Am. J. Hum. Genet. 59:423–430, 1996

Allele-Specific Replication of 15q11-q13 Loci: A Diagnostic Test for Detection of Uniparental Disomy

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Summary

Allele-specific replication differences have been observed in imprinted chromosomal regions. We have exploited this characteristic of an imprinted region by using FISH at D15S9 and SNRPN (small nuclear ribonucleo protein N) on interphase nuclei to distinguish between Angelman and Prader-Willi syndrome patient samples with uniparental disomy of chromosome 15q11-q13 (n = 11) from those with biparental inheritance (n = 13). The familial recurrence risks are low when the child has de novo uniparental disomy and may be as high as 50% when the child has biparental inheritance. The frequency of interphase cells with asynchronous replication was significantly lower in patients with uniparental disomy than in patients with biparental inheritance. Within the sample population of patients with biparental inheritance, those with altered methylation and presumably imprinting center mutations could not be distinguished from those with no currently detectable mutation. This test is cost effective because it is performed on interphase cells from the same hybridized cytological preparation in which a deletion is excluded, and additional specimens are not required to determine the parental origin of chromosome 15.

Introduction

The Prader-Willi and Angelman syndromes (PWS and AS, respectively) are clinically distinct genetic disorders that map to chromosome 15q11-q13, an imprinted region (Ledbetter et al. 1981; Butler et al. 1986; Kaplan et al. 1987; Magenis et al. 1989). Several genetic etiologies exist for each syndrome. They include de novo deletions that span ~4 Mb (Butler and Palmer 1983; Donlon et al. 1986; Knoll et al. 1989; Magenis et al. 1989; Nicholls et al. 1989*b*; Williams et al. 1990), uniparental disomy (UPD) of 15q11-q13, and biparental inheritance and disomy (BPD) with or without a detectable mutation in the imprinting process (Nicholls et al. 1989*a*; Knoll et al. 1991; Malcolm et al. 1991; Mascari et al. 1992; Glenn et al. 1993*a*; Reis et al. 1994; Buiting et al. 1995). De novo deletions are paternally derived in PWS and maternally derived in AS and occur in ~70% of each of the patient populations. Maternal UPD is observed in >25% of the PWS population (Mascari et al. 1992) and paternal UPD in <5% of the AS population (Knoll et al. 1991; Malcolm et al. 1991). The remaining patients (<5% PWS and ~25% AS) have BPD. Patients with BPD either have alterations in the regulation of imprinting characterized by aberrations in DNA methylation and mutations of DNA sequences upstream of the SNRPN (small nuclear ribonucleo protein N) gene or, as in the case of AS, normal DNA methylation without a detectable mutation in the imprinting process (Nicholls et al. 1989*a*; Knoll et al. 1991; Malcolm et al. 1991; Mascari et al. 1992; Meijers-Heijboer et al. 1992; Wagstaff et al. 1992; Clayton-Smith et al. 1993; Glenn et al. 1993*a*; Reis et al. 1994; Buiting et al. 1994, 1995; Sutcliffe et al. 1994). Patients within this class of mutations are rare in both syndromes (<5% of AS and PWS).

Familial recurrence risks may be as great as 50% when the proband exhibits BPD with or without a mutation in the imprinting process. By contrast, the risk is generally no greater than population incidence when either a large deletion or de novo UPD is observed (Clayton-Smith et al. 1993; Woodage et al. 1994; Williams et al. 1995).

Imprinting has been shown, to date, to display characteristics of parent-of-origin allele specificity in gene expression, gene methylation, and asynchronous replication timing. Within 15q11-q13, several imprinted loci—SNRPN (Glenn et al. 1993*b*, 1996; Nakao et al. 1994; Reed and Leff 1994), D15S63 (Dittrich et al. 1992), IPW (Wevrick et al. 1994), D15S9/ZNF127/DN34 (Driscoll et al. 1992), and the expressed sequence tags

Received April 14, 1996; accepted for publication May 28, 1996.

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0002-9297/96/5902-0019$02.00

PAR-1 and PAR-5 (Sutcliffe et al. 1994)—have been identified and shown to display differential DNA methylation. Each of these is expressed from the paternal chromosome only. Imprinted chromosome regions also display allele-specific asynchronous replication timing over domains as large as 4 Mb in 15q11-q13 (Kitsberg et al. 1993; Knoll et al. 1994; LaSalle and Lalande 1995). While most regions in 15q11-q13 show paternal-early and maternal-late replication timing, a small region of 15q11-q13 shows the opposite pattern (Knoll et al. 1994; LaSalle and Lalande 1995). Differential DNA methylation (Dittrich et al. 1992; Glenn et al. 1993*a*; Reis et al. 1994; Buiting et al. 1995) has been employed as a diagnostic tool for PWS and AS, but gene expression and replication timing have not.

In many diagnostic centers, the initial laboratory testing regime for a patient suspected of having AS or PWS often includes routine metaphase chromosome analysis along with FISH, to exclude the presence of a deletion. In the absence of a deletion, DNA analyses of the patient and his or her parents are performed to determine whether the proband displays UPD or BPD of chromosome 15q11-q13. If neither a deletion or UPD are detected, the patient is referred to a research laboratory for assessment of potential imprinting mutations. We have extended the use of FISH to include analysis of allele-specific replication on interphase nuclei to determine whether both deletion exclusion and discrimination of UPD from BPD is possible with the same hybridized cytological preparation.

Material and Methods

Patient Samples

Lymphocytes or lymphoblastoid cells were available from 24 nondeletion AS or PWS patients whose genotypes had been established by studies of genetic polymorphism (table 1). DNA methylation status at D15S63 (Dittrich et al. 1992) and/or SNRPN (Sutcliffe et al. 1994) was also determined on lymphocytic DNA for most of these patients. Cells from three normal control individuals who are parents of affected children were included to demonstrate asynchronous replication at the loci studied. Lymphocytes were either cultured directly from whole blood or were transformed before routine harvest and fixation. Cell pellets, which contained both metaphase and interphase cells, were stored in Carnoy's fixative at 4°C from overnight to 5 years before being dropped onto microscope slides for FISH. Previous findings have demonstrated no differences in asynchronous replication between phytohemagglutinin-stimulated lymphocytes or Epstein-Barr virus–transformed lymphoblastoid cells (Kitsberg et al. 1993; Knoll et al. 1994).

DNA Probes

Chromosome 15q11-q13–specific DNA probes for loci D15S9 and SNRPN were used for FISH. These probes were selected because they reside within the AS/PWS chromosomal region and are imprinted (Driscoll et al. 1992; Sutcliffe et al. 1994). Phage clone 34-10 (D15S9) (Knoll et al. 1993) was labeled with digoxigenin-11-dUTP or biotin-16-dUTP via nick-translation under standard conditions (Knoll and Lichter 1994). SNRPN, a cosmid clone from the 3′ end of the SNRPN gene, was labeled with digoxigenin-11-dUTP via nick-translation and is commercially available (Oncor; Ozcelik et al. 1992). Each new batch of labeled probe was tested on normal lymphocytes, and only those batches with $\geq$90% hybridization efficiency were used.

FISH

All cytological preparations were coded. Cells were dropped onto wet microscope slides, air dried, and aged at room temperature for 1–14 d prior to FISH. The cells were denatured, hybridized with SNRPN and 34-10 individually and washed at stringencies as described by Knoll and Lichter (1994). Digoxigenin-labeled probes were detected with rhodamine-conjugated antibody and biotin labeled probes were detected with avidin-fluorescein. Total nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; 100 ng/ml).

For analysis, hybridization signals were viewed with an epifluorescence microscope equipped with a dual band (fluorescein isothiocyanate [FITC]/Texas red; Omega Optical) or a triple-band pass filter set (FITC/Rhodamine/DAPI; Chroma Technology). The nuclear counterstain was viewed through a standard single-band pass filter (Zeiss). Representative cells were imaged with a Photometrics CCD camera (fig. 1). For imaging, each fluorochrome was viewed with the appropriate single-band filter, individually captured in gray scale by using IP Lab software (Signal Analytics), pseudocolored and the three images merged.

Replication Analysis

The probes selected for hybridization are within the 15q11-q13–imprinted domain (Kuwano et al. 1992; Knoll et al. 1993; Mutirangura et al. 1993). Three distinct hybridization patterns on interphase nuclei were scored. They are G1 (two single hybridization signals corresponding to unreplicated chromosomes), G2 (two distinct pairs of signals corresponding to cells in which both homologues had replicated), and G1/G2 (one single and one double signal corresponding to cells in S phase in which only one homologue has replicated) (see fig. 1). The replication pattern was scored if nuclei were intact and nonoverlapping and both homologues were hybridized. Homologues were scored as replicated if a doublet or signal pair was observed and the distance between the doublet signals was $\leq$2 signal widths apart. For each probe, the numbers of cells in G1, G1/G2, and G2 from usu-

Table 1

Summary of Molecular Findings

Individuals	Diagnosis	Genotype[a]	DNA Methylation[b]	Reference
Patients:				
Patient 4	PWS	UPD	MAT	PWS 9, Mascari et al. 1992; Present study
Patient 7	PWS	UPD	MAT	PWS 12, Mascari et al. 1992; Present study
Patient 8	PWS	UPD	MAT	PWS 13, Mascari et al. 1992; Present study
Patient 9	PWS	UPD	MAT	PWS 14, Mascari et al. 1992; Present study
Patient 12	PWS	UPD	MAT	PWS 17, Mascari et al. 1992; Present study
Patient 13	PWS	UPD	MAT	PWS 18, Mascari et al. 1992; Present study
Patient 21	PWS	UPD	MAT	PWS 25 , Mascari et al. 1992; Present study
Patient 22	PWS	UPD	MAT	PWS 26, Mascari et al. 1992; Present study
Patient 24	PWS	UPD	MAT	PWS 28, Mascari et al. 1992; Present study
WJK64[c]	PWS	UPD	...	Nicholls et al. 1989*a*
WJK75[c]	PWS	UPD	...	Present study
WJK1[c]	AS, sib of WJK4	BPD	...	Knoll et al. 1991
WJK4[c]	AS, sib of WJK1	BPD	...	Knoll et al. 1991
WJK14[c]	AS	BPD	...	Knoll et al. 1991
Patient 10	PWS—atypical	BPD	MAT/PAT	PWS 15, Mascari et al. 1992; P. K. Rogan, unpublished data
CHB94-268	AS	...	MAT/PAT	Present study
CHB94-324	PWS—atypical	BPD	MAT/PAT	Present study
CHB94-486	AS	...	MAT/PAT	Present study
CHB94-515	PWS—atypical	...	MAT/PAT	Present study
JKB342	AS	BPD	MAT/PAT	Present study
DBH[c]	PWS, sib of DKH	BPD	MAT[d]	PWS-U, Buiting et al. 1995; Saitoh et al., in press
DKH[c]	PWS, sib of DBH	BPD	MAT[d]	PWS-U, Buiting et al. 1995; Saitoh et al., in press
JK391[c]	AS	BPD	PAT[d]	AS-C, Buiting et al. 1995
Patient 14	PWS	BPD	MAT[d]	PWS 19, Mascari et al. 1992; Present study
Controls:				
JKB341	Parent	...	MAT/PAT	Present study
GRH[c]	Parent	...	MAT/PAT	PWS-U, Buiting et al. 1995
DLH[c]	Parent	...	MAT/PAT	PWS-U, Buiting et al. 1995

[a] Genotype analysis by RFLP and/or microsatellite analyses of parents. Ellipses (...) = not determined.
[b] Methylation status at D15S63 or SNRPN with maternal (MAT) and/or paternal (PAT) alleles present.
[c] Epstein-Barr–transformed lymphoblastoid cells; otherwise peripheral lymphocytes.
[d] Methylation pattern is not concordant with genotype.

ally ≥100 interphase cells were scored by two individuals. The presence of G2 cells (in the absence of metaphase cells) established that the cultures were actively growing at the time of fixation.

Statistical Analysis

The G1, G1/G2, and G2 data were fit to general linear models (GLM) specifying either genotype, probe, or methylation status, or scorer as independent variables. Repeated measures analysis of variance was used (procedure GLM, SAS Institute). Methylation data were not available for some patients. The cell counts for G1, G1/G2, and G2 were specified as repeated measures of the same subject. Between-subject effects for all replication data on G1, G1/G2, and G2 were detected by multivariate analysis of variance.

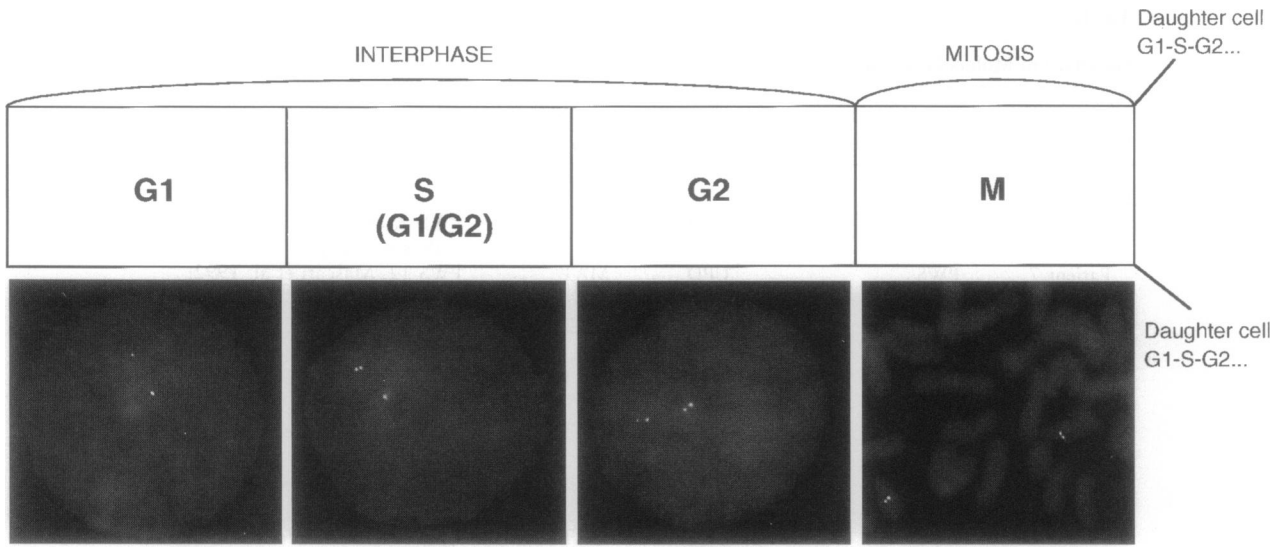

Figure 1 Schematic of different phases of the cell cycle (G1, or gap 1; G2, or gap 2; S, or DNA synthesis; and M, or mitotic) and corresponding computerized images of DAPI-stained cells hybridized with SNRPN. G1, S, and G2 comprise interphase of the cell cycle, and M is the mitotic part in which metaphase chromosomes are recognizable. In G1 the locus has not yet replicated, and two single hybridizations are observed. In S (also referred to as "G1/G2"), one of the chromosomes has replicated (double signal) and the other has not (single hybridization signal), while in G2, both chromosomes have replicated (two pairs of double hybridizations). In metaphase of mitosis, both chromosomes show hybridization at 15q11-q13 in the absence of a deletion.

Principal component analysis was performed with the procedure FACTOR (SAS Institute) for each of the dependent (G1, G1/G2, G2) and significant independent variables (genotype, scorer, methylation) found in the GLM. Components with eigenvalues >1 were analyzed. The loading or influence of each variable for each principal component represented its contribution to the total variance of all the variables. In order to verify that loadings of the variables were independent for different components, data were also transformed orthogonally prior to performing the analysis to determine whether the results differed from the nontransformed data.

Results

The probes selected for hybridization are within the imprinted domain in AS and PWS (Kuwano et al. 1992; Knoll et al. 1993; Mutirangura et al. 1993) and show three distinct hybridization patterns on interphase nuclei (fig. 1). These three patterns correspond to the G1 (gap 1), S (synthesis), and G2 (gap 2) phases of the cell cycle. In G1 cells, DNA synthesis has not yet occurred, and two single and separated hybridizations are observed. In S-phase cells, DNA replication has occurred on only one homologue and are referred to as "G1/G2 cells." This pattern appears as a single distinct hybridization and a pair of closely spaced hybridization signals. In G2 cells, both homologues have replicated, and each homologue appears as a pair of hybridization signals. These three patterns are present in all individuals.

Detection of UPD by Interphase Analysis

Normally, the paternal allele replicates prior to the maternal allele at D15S9 and SNRPN in a subset of cells (table 2; Kitsberg et al. 1993; Knoll et al. 1994; Malcolm and Donlon 1994). If both alleles are inherited from one parent, as in the case of UPD, the alleles are predicted to replicate synchronously. The present study validates this prediction at both D15S9 and SNRPN in UPD patients with PWS. The results from each patient were fit to a GLM that was dependent on the numbers of cells in G1, G1/G2, and G2. Significantly lower numbers of G1/G2 cells ($F = 139.67$; $P = .0001$) and significantly higher numbers of G1 cells ($F = 19.25$; $P = .0001$) were observed in the patients with UPD ($n = 11$). The percentages of G1 and G1/G2 cells for patients with UPD ranged between 78% and 90% and 3% and 11%, respectively, compared with 56%–76% and 21%–36% for patients with BPD at these loci (fig. 2). These probes did not show significant differences in replication timing at either G1 ($F = 0.19$; $P = .66$) or G1/G2 ($F = 0.08$; $P = .77$).

BPD of Chromosome 15q11-q13

Asynchronous replication in patients with BPD at the probe loci was similar to normal control individuals (fig. 2; table 2; Kitsberg et al. 1993; Knoll et al. 1994). The BPD patient population included six with normal methylation patterns at D15S63 and/or SNRPN, four with altered methylation patterns due to mutations in the

Table 2

Summary of Replication Data

Class and Probe	Mean Number of Cells ± SD		
	G1 (%)	G1/G2 (%)	G2 (%)
UPD:			
SNRPN	92.3 ± 13.5 (86.4)	7.9 ± 2.7 (7.4)	6.6 ± 3.1 (6.2)
34-10	95.3 ± 13.6 (83.7)	9.2 ± 2.9 (8.1)	9.3 ± 3.9 (8.2)
BPD with normal methylation:			
SNRPN	89.8 ± 25.2 (64.5)	38.2 ± 12.6 (27.5)	11.1 ± 7.4 (8)
34-10	76.1 ± 8.7 (68.3)	27.4 ± 3.0 (24.6)	7.9 ± 2.9 (7.1)
BPD with abnormal methylation:			
SNRPN	77.3 ± 10.7 (66.9)	30.3 ± 3.6 (26.2)	7.9 ± 4.3 (6.8)
34-10	74.4 ± 13.6 (64.9)	32.8 ± 10.2 (28.6)	7.4 ± 3.8 (6.5)
Control:			
SNRPN	68.5 ± 6.0 (66.1)	30.3 ± 1.7 (29.2)	4.8 ± 2.2 (4.6)
34-10	69.3 ± 5.8 (64)	31.3 ± 4.5 (28.9)	7.7 ± 5.1 (7.1)

imprinting process, and three in whom methylation patterns were not examined. This allele-specific replication assay did not discriminate between BPD patients with altered methylation patterns from those with no detectable mutation. This is because some patients with mutations that result in altered methylation show asynchronous replication, methylation status for all individuals was not correlated with replication asynchrony to the same extent as the individual's genotype, though both associations were significant (G1, $P = .02$; G1/G2, $P = .01$).

Distinguishing UPD from BPD

Principal component factor analysis revealed that the proportion of cells in the G1/G2 phase were associated with methylation status and with the individuals' genotype (UPD or BPD) (table 3). Genotype, however, was the most significant factor contributing to the variance in frequency of asynchronously replicating cells between UPD and BPD patients. Two different observers scored the data. Observer differences were detected when all replication data (G1, G1/G2, and G2) were analyzed together ($F = 5.75$; $P = .02$), but these differences contributed to a different principal component than either genotype or methylation status (table 3, factor 3). Therefore, the interpretation of genotype on the basis of the degree of asynchronous replication was not influenced by which individual performed the analysis. Since both of the probes analyzed gave similar results, their effects on the variance of each principal component were also negligible. Orthogonal transformation of these data confirmed that the observed variance due to genotype was related to the frequency of G1 or G1/G2 cells rather than to which observer carried out the analysis. Furthermore, the variance in allele-specific replication asynchronicity due to genotypic differences was 30-fold greater than the observer associated variance (eigenvalue = 5.02 vs. 0.17).

Discussion

This study extends the use of interphase FISH from the detection of aneuploidy (Cremer et al. 1988; Tkachuk et al. 1990; Ried et al. 1992) to the discrimination of UPD from BPD on chromosome 15 (by using probes from an imprinted region). UPD patients had a significantly lower population of asynchronously replicating cells (3%–11%) than those with BPD at the tested loci (21%–36%). The G1/G2 cells in the UPD specimens may be the result of a low level of stochastic asynchronous replication and/or a <10% inefficiency of probe hybridization or visualization (see Material and Methods). There was no overlap between the UPD and BPD patient populations in the mean percentage and range of cells undergoing asynchronous replication. Therefore, we suggest scoring the frequency of G1/G2 cells for distinguishing UPD from BPD. While the mean frequency of G1 cells was also significantly different between UPD and BPD, a smaller difference between the ranges were observed. Asynchronous replication on interphase nuclei, however, was not useful in discriminating among the different BPD classes, i.e., those with altered methylation (and presumably imprinting mutations) from those with no detectable genetic abnormality. This finding implies that methylation does not significantly affect the overall frequency of asynchronously replicating cells. This suggests that the frequency of asynchronous replication is related to parent of origin of the chromosome rather than gene expression or methylation status.

The phenomenon of asynchronous replication as detected by FISH is not understood. It may reflect true differences in replication timing or chromatin conforma-

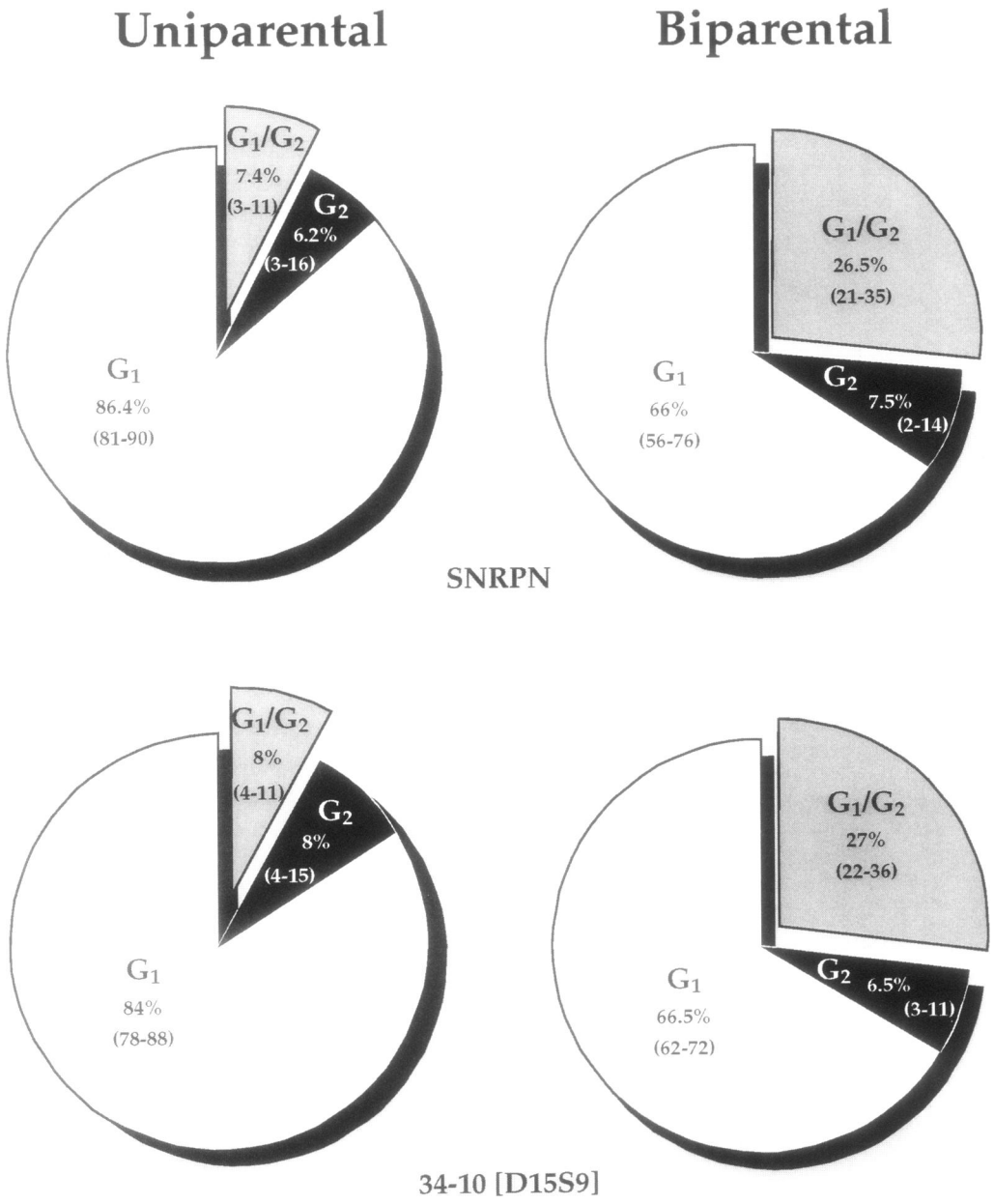

Figure 2 Mean percentage and range of cells in each part of interphase for patients with uniparental disomy (*left*) and biparental inheritance (*right*). At each locus (SNRPN and D15S9), the proportion of cells in G1/G2 is significantly lower in cases with UPD than in cases with BPD ($P = .0001$).

tional changes that affect hybridization efficiency (Hansen et al. 1995). Regardless of the underlying cause of the phenomenology, this test provides a reliable way of distinguishing patients with different recurrence risks. Most deletions and UPDs are sporadic, and parents of these patients have recurrence risks comparable to the general population, while the recurrence risk may be $\leq 50\%$ in families in which the BPD genotype is detected (Clayton-Smith et al. 1993; Woodage et al. 1994; Williams et al. 1995; Webb et al. 1995).

This simple test can provide additional information to the proband's family in a single physician visit. This test, like DNA methylation testing (Dittrich et al. 1992), does not require analysis of parental specimens for UPD detection. It is advantageous, however, in that it can be used simultaneously with routine cytogenetics and FISH

Table 3

Principal Component Analysis

Variables	Factor 1	Factor 2	Factor 3
G1/G2	.82056	.40174	−.15514
Methylation	.90009	−.04363	.03747
Genotype	.96918	.05335	−.01376
Scorer	−.03788	.48256	.40579
Probe	.05207	−.12100	.87355
Eigenvalue	2.75	2.44	1.05

NOTE.—The Procedure Factor (SAS Institute) was used to determine eigenvalues for each factor and loadings for the above variables. These variables were selected because they showed significant contributions to the GLM. Factor 1 has large positive loadings (or influence) for genotype and methylation. Factor 2 has small loadings on genotype, methylation, and probe. Factor 3 has small loadings on genotype, methylation, and scorer.

to distinguish between chromosomal deletions, familial chromosomal rearrangements, UPD, and BPD with altered methylation. This test does, however, rely on clinical features to distinguish between AS and PWS.

From the practical point of view, cytogenetic testing is currently the most widely available test for AS and PWS, and therefore the application of this test would benefit the maximum number of patients. Thus, (1) patient samples are already being examined for deletions by FISH; (2) interphase FISH replication analysis utilizes the same hybridized preparation as for metaphase analysis; (3) it requires little additional effort to discriminate between UPD and BPD (15–30 min/probe/score 100 nuclei); and (4) it can be performed on archived fixed cell pellets. By combining the results of FISH analysis from both metaphase and interphase analyses, the genetic etiology in >95% of PWS patients and ~75% of AS patients can be determined.

Acknowledgments

The authors gratefully acknowledge the statistical expertise of Dr. Thomas TenHave, Division of Biostatistics at Pennsylvania State University College of Medicine. This work was supported by the Beth Israel Pathology Foundation, Inc. (to J.H.M.K.), and by NIH grants HD18658 (to J.H.M.K.) and HD29098-01 (to P.K.R.).

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