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Prader Willi/Angelman and DiGeorge/ Velocardiofacial Syndrome Deletions: Diagnosis by Primed In Situ Labeling (PRINS)

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A recently developed methodology—primed in situ labeling (PRINS)—can be used in place of fluorescence in situ hybridization (FISH) to diagnose microdeletions. To demonstrate the efficiency, sensitivity, and specificity of PRINS in the diagnosis of microdeletions, we studied groups of patients with Prader Willi/Angelman (PWS/AS) syndrome and DiGeorge/velocardiofacial syndrome (DGS/VCFS). Results obtained by PRINS were then confirmed with the results obtained with FISH. Oligonucleotide primers specific for *SNRPN* and *GABRB3* were used for PWS/AS syndromes. For DGS/VCFS, the primers used were *DGCR2*/*TUPLE1* loci. Labeling patterns obtained by PRINS and FISH were analyzed and scored under a fluorescence microscope. Five normal subjects served as controls and were used for standardization of the PRINS protocol. In all, 20 study patients were involved: 10 PWS/AS and 10 DGS/VCFS. Five of the 10 patients referred with the clinical diagnosis of PWS/AS showed absence of labeling for *SNRPN* and *GABRB3* on one chromosome 15, confirming deletion of the two loci. Similarly, 6 of the 10 patients referred for DGS/VCFS showed deletion for the *DGCR2*/*TUPLE1* loci on one chromosome 22. The remaining patients and controls had normal patterns for all the loci as indicated by FISH and PRINS. Concordant FISH and PRINS results were obtained in all patients and controls studied. © 2001 Wiley-Liss, Inc.

KEY WORDS: FISH; PRINS; microdeletion; Prader Willi/Angelman; DiGeorge/velocardiofacial syndrome

INTRODUCTION

Microdeletion syndromes are caused by loss of DNA sequences encompassing a single gene or contiguous genes. Most microdeletions arise de novo during gametogenesis. This is often because of uneven crossovers and may involve repeat sequences in the genome [Smeets et al., 1992; Horsthemke et al., 1996; Lupski, 1998; Tharapel et al., 1999; Shaffer and Lupski, 2000]. Some microdeletions occur at the terminal regions of chromosome, whereas others are interstitial.

Two interstitial regions predisposed to microdeletions are on chromosome 15, bands q11.2q13, and chromosome 22, bands q11.2q23. Deletion at 15q11.2q13 can produce two clinically distinct syndromes, depending on the maternal or paternal origin of the deleted chromosome. When chromosome 15 deletion is paternally derived, the result is Prader-Willi syndrome (PWS). The incidence of PWS is generally quoted as 1/16,000 live births (MIM 176270), although recent reports suggest a higher prevalence. If the same deletion is maternally derived, the result is Angelman syndrome (AS). Imprinting phenomena associated with PWS/AS are well known [Knoll et al., 1989; Driscoll et al., 1992; Nicholls, 1993].

Deletion at band 22q11.2 can result in DiGeorge syndrome (DGS), which has a prevalence rate of 1/4000 live births (MIM 188400). Patients with velocardiofacial syndrome (VCFS) share a deletion segment (22q11.2) common with DGS. Occasionally, the deletion in VCFS may extend further to band 22q23 with more severe clinical manifestations.

In general, microdeletions are beyond the resolution of conventional cytogenetics. Fluorescence in situ hybridization (FISH) has contributed substantially to the identification and understanding of microdeletions or contiguous gene syndromes. Now primed in situ

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labeling (PRINS) can be used as a cost-effective alternative to FISH. The new methodology combines the principles of the polymerase chain reaction and FISH [Koch et al., 1989], allowing one or more unique primer sequences to anneal directly onto chromosomes or nuclei fixed on slides. Initially, the PRINS technique was limited to identification of centromeres, telomeres, and *Alu* repeats [Koch et al., 1991; Gosden et al., 1991; Gosden and Lawson, 1994]. Later, PRINS was used to determine the chromosomal origin of marker chromosomes and for aneuploidy detection [Pellestor et al., 1995; Velagaleti et al., 1997]. Cinti et al. [1993] used PRINS to localize the *FACTOR IX* gene, a single copy gene on the X chromosome.

We have modified PRINS for localization of several unique sequences and for identification of subtle rearrangements such as the translocation of *SRY* from Yp11.3 to Xp22 in XX males [Kadandale et al., 2000a,b]. By using PRINS, we have also confirmed deletion of *RBM* and *DAZ*, two candidate genes for the azoospermia factor in an azoospermic man [Kadandale et al., 2002].

The sensitivity and specificity of PRINS allow localization of DNA segments that are too small to be detected by conventional FISH. For the present study, we modified the PRINS method for diagnosis of gene deletions in PWS/AS and DGS/VCFS. The PRINS results were compared with results obtained by FISH with standard commercial probes.

MATERIALS AND METHODS

Subjects

Three normal males and two normal females served as controls. The Prader Willi/Angelman patient group consisted of five males and five females. Of these 10 patients, 4 males and 4 females had clinical features of PWS. Symptoms included hypotonia, obesity, small hands and feet, small penis, excessive appetite, speech problems, and mild to moderate mental retardation. Two of the 10 patients, a male and a female, had the clinical diagnosis of AS. Both presented with jerky arm movements, frequent inappropriate laughter, pale deep-set eyes, maxillary hypoplasia, prognathia, absence of speech, and severe mental retardation.

The DiGeorge/velocardiofacial group also consisted of 10 patients in all. Of these, four males and three females referred with clinical diagnosis of DGS showed mild dysmorphic features, hypoplastic thymus and parathyroid, and various aortic arch abnormalities. Three other patients had clinical diagnosis of VCFS (one male and two females). They exhibited short stature, long face, submucosal cleft palate, thin long hands and feet, tetralogy of Fallot, and mild to moderate mental retardation.

Cytogenetics

Venous blood was collected from patients and controls in sodium heparin. Phytohemagglutinin-stimulated lymphocyte cultures were initiated and harvested, and slides were prepared according to conventional protocols. For G-banding, the slides were exposed to Wright stain after treatment with pancreatin. A 20-cell chromosome analysis was performed on each patient. Chromosome analysis was not repeated on the controls, all of whom had normal karyotypes.

FISH

Probes for FISH were obtained from Vysis Inc. (Downers Grove, IL). Only the *SNRPN* probe was tested because the *SNRPN* locus is consistently known to be deleted in patients with Prader-Willi/Angelman. For FISH analysis of the patients with DGS/VCFS, the *N25* locus FISH probe was used (Vysis Inc.). Hybridization and detection were performed according to the manufacturer's instructions. For each probe, 20 metaphase spreads and 80 interphase nuclei were analyzed on each patient.

PRINS

For the Prader-Willi/Angelman group, in addition to *SNRPN* we also used sequences specific for *GABRB3*. For DG/VCFS group, we used sequences unique for *DGCR2/TUPLE1*. Aliquots of mitogen-stimulated lymphocyte preparations used for cytogenetic analysis and FISH were also used for PRINS studies. Slides were immersed in 0.02 N HCl for 20 min, washed, and air dried through an ethanol series. Chromosomal DNA was denatured in 70% formamide/2x standard sodium citrate (SSC) (pH 7.0) for 2 min at 72°C, dehydrated again through a cold ethanol series, and air dried. PRINS reactions were carried out separately for each locus, namely, *SNRPN* and *GABRB3* for PWS, and *DGCR2/TUPLE1* for DGS/VCFS syndromes. Multiple primers for the same locus were used and are listed in Table I. The reaction mixture, prepared in a final volume of 40 μl, contained 50 pmol of the oligonucleotides (for the specific locus), 0.2 mM each dATP, dCTP, dGTP, 0.02 mM dTTP, 0.02 mM biotin-16-dUTP (Roche Molecular Biochemicals, Indianapolis, IN), 50 mM KCl,

TABLE I. Primer Sequences Used in the Investigation*

Locus	Primer sequence	Annealing temp (°C)
SNRPN	5′-ACGTACCAGAGGTGGAAGTC-3′	
	5′-GTCTTCAGAAGCATCAAGTTT-3′	
	5′-ATGACTGTGGAGGGGCCAC-3′	
	5′-AGGCATTATGGCTCCTCCAC-3′	63
GABRB3	5′-TTCTGCAGGCACCGTCGGG-3′	
	5′-AGAGGTCCGAGTCCCGTG-3′	
	5′-AGGAAGGCTTTTCGGCATCT-3′	
	5′-TAAGTGGGCTGCGCGCGGC-3′	68
DGCR2/TUPLE1	5′-GCGGTGCAACCCTGGGCA-3′	
	5′-CAGTATGTTATCACTGGCCGG-3′	
	5′-CGCCTGGTCGTCAGCTGC-3′	
	5′-CAACCACAATGGCAAGCCGA-3′	
	5′-AAGTTCCCAGAAATTCTAGCTA-3′	
	5′-GTCATCCATGAACTGTTTGAC-3′	65.5

*Oligonucleotide primer sequences for PRINS were derived by searching the published sequences for the respective genes.

TABLE II. Results of FISH Analysis

No. of subjects	No. of cells[a]	Probe	Hybridization pattern
Controls (5)	100	*SNRPN*	Normal pattern $\geq$ 95%
	100	*N25*	Normal pattern $\geq$ 95%
PWS/AS (5 patients)	100	*SNRPN*	Normal pattern $\geq$ 95%
PWS/AS (5 patients)[b]	100	*SNRPN*	Deleted $\geq$ 90%
DGS/VCFS (4 patients)	100	*N25*	Normal pattern $\geq$ 95%
DGS/VCFS (6 patients)[c]	100	*N25*	Deleted $\geq$ 90%

[a]Twenty metaphases and 80 interphase nuclei were assessed per patient per probe.
[b]Four patients with PWS and one with AS.
[c]Five patients with DGS and one with VCFS.

10 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, 0.01% bovine serum albumin, and 2 U of Taq DNA polymerase with TaqStart antibody (Clontech, Inc., Palo Alto, CA). The PRINS reactions were carried out on a programmable thermal cycler (MISHA, Shandon Lipshaw, Pittsburgh, PA), and the program consisted of a single cycle of 10 min at the respective annealing temperatures (Table I) and 30-min extension at 72°C. Slides were washed in 0.4x SSC/0.3% NP40 at 72°C for 2 min. PRINS label was amplified with the Tyramide Signal Amplification system (TSA-Indirect, NEN Life Science Products, Boston, MA). Signals were detected with Texas-red-conjugated avidin and counterstained with diamino-phenylindol, the preparations were analyzed under the fluorescence microscope, and representative images were obtained (Applied Imaging, Santa Clara, CA). As in the FISH experiments, 20 metaphase spreads and 80 interphase nuclei were analyzed for each locus in each patient.

RESULTS

For each control and patient sample, 20 metaphases and 80 interphase nuclei were analyzed per probe/locus. FISH hybridization pattern for *SNRPN* and *N25* are summarized in Table II. PRINS pattern for *SNRPN*, *GABRB3*, and *DGCR2/TUPLE1* are summarized in Table III. Of the 10 patients with PWS/AS, 5 showed deletion for *SNRPN* by both FISH and PRINS (Fig. 1A–1D). Among the 10 patients with DGS/VCFS, 6 had deletion for *N25* by FISH. The same patients also showed deletion for *DGCR2/TUPLE1* by PRINS (Fig. 1E–H).

DISCUSSION

Our ability to localize single copy genes via PRINS methodology has opened various applications in diagnosis and research. This validation study documents an important clinical application of PRINS. Presently, cytogenetic diagnoses of PWS/AS and DGS/VCFS are made exclusively by FISH probes. However, the accuracy and reliability of FISH depends on the specificity of the probes themselves, and FISH probes can contain overlapping DNA segments outside the targeted loci and can cross-hybridize to unintended targets. The target-specific PRINS approach can overcome this limitation. As previously stated by others [Bottema and Sommer, 1993; Pellestor et al., 1999], the complementation process of the primer and its target is specific, and any mismatch between the primer and the intended gene sequence can prevent annealing and extension.

These observations underscore the potential efficiency of the PRINS method. Moreover, PRINS results can be available within 2–4 hr, whereas a typical FISH procedure takes at least 12 hr. As a final point, analysis of PWS/AS and DGS/VCFS accounts for more than 60% of all FISH studies requested by clinicians. The reliability of PRINS demonstrated in the present study, its relative speed, and the comparative cost of the procedure point to PRINS as a significant tool in clinical

TABLE III. Results of PRINS Analysis

No. of subjects	No. of cells[a]	Loci	Labeling pattern
Controls (5)	100	*SNRPN*	Normal pattern $\geq$ 90%
	100	*GABRB3*	Normal pattern $\geq$ 90%
	100	*DGCR2/TUPLE1*	Normal pattern $\geq$ 90%
PWS/AS (5 patients)	100	*SNRPN*	Normal pattern $\geq$ 90%
	100	*GABRB3*	Normal pattern $\geq$ 90%
PWS/AS (5 patients)[b]	100	*SNRPN*	Deleted $\geq$ 80%
	100	*GABRB3*	Deleted $\geq$ 80%
DGS/VCFS (4 patients)	100	*DGCR2/TUPLE1*	Normal pattern $\geq$ 90%
DGS/VCFS (6 patients)[c]	100	*DGCR2/TUPLE1*	Deleted $\geq$ 80%

[a]Twenty metaphases and 80 interphase nuclei were assessed per patient per probe.
[b]Four patients with PWS and one with AS.
[c]Five patients with DGS and one with VCFS.

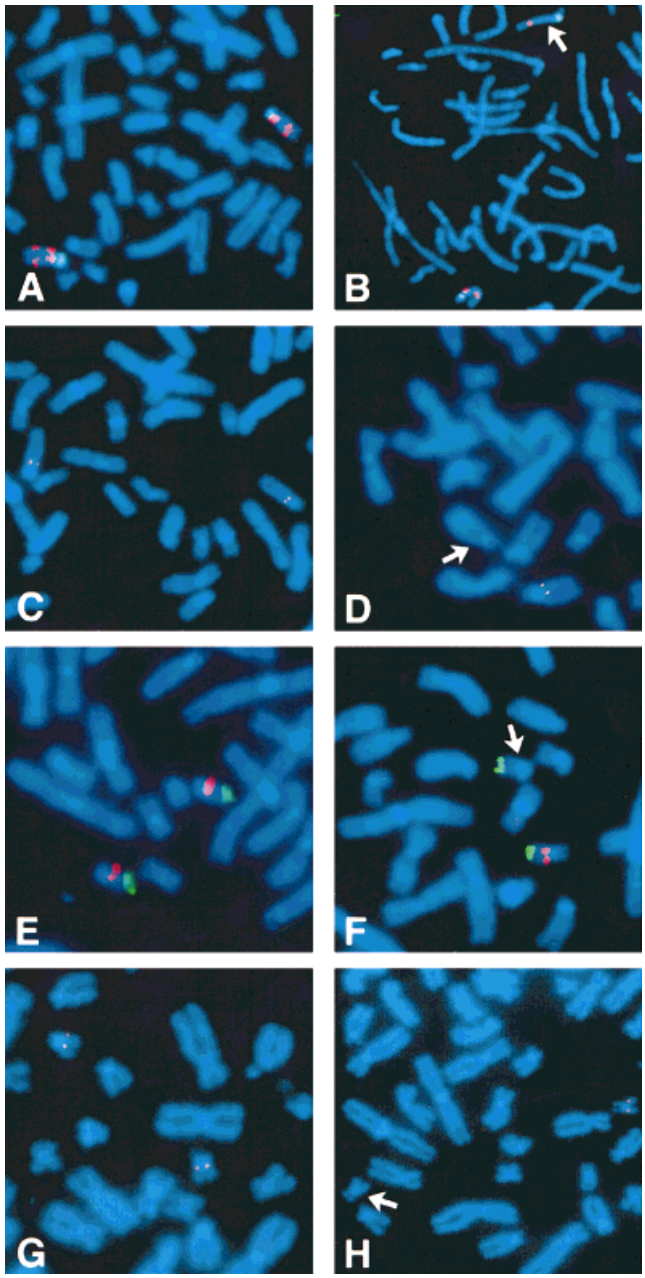

Fig. 1. **A:** Chromosome 15 centromeres appear in green. Adjacent to the centromeres in red is the normal pattern for *SNRPN* by FISH. Control probe PML appear on the distal long arm (15q22) in red. **B:** Deletion of *SNRPN* by FISH (arrow). **C:** Normal pattern for *SNRPN* by PRINS. **D:** Deletion of *SNRPN* by PRINS (arrow). **E:** Normal pattern for *N25* (DGS) by FISH in red. Control probe *ARSA* (22q13) in green. **F:** Deletion of *N25* by FISH (arrow). **G:** Normal pattern for *DGCR2/TUPLE1* by PRINS. **H:** Deletion of *DGCR2/TUPLE1* by PRINS (arrow).

cytogenetic laboratories. Thus, it seems reasonable to assume that PRINS will soon be a competitive alternative to FISH in the evaluation of PWS/AS and DGS/VCFS.

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