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Rapid detection of chromosome 18 aneuploidies in amniocytes by using primed in situ labeling (PRINS) technique*

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Abstract: This paper presents a feasible method for rapid detection of the interphase nuclei of uncultured amniocytes for chromosomes 18 by using our modified primed in situ labeling (PRINS) technique. A total of 262 independent, uncultured amniotic fluid samples were analysed in a blind fashion before the karyotype was available. In addition, 62 samples were examined by fluorescence in situ hybridization (FISH) for comparison. In more than 95% of the samples PRINS reactions with primer 18cen were successfully induced. Two samples were properly identified and correctly scored as trisomic 18. PRINS reaction could be performed automatically in less than one hour with a programmable thermocycler. Our studies showed that the PRINS technique is simple, rapid and cost-effective. It is as sensitive and specific as FISH; can enhance the accuracy of standard cytogenetic analysis; and allows identification of chromosomes 18 aneuploidies in uncultured amniocytes in significantly less time.

Key words: Primed in situ labeling (PRINS), Prenatal diagnosis, Chromosome 18
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INTRODUCTION

Prenatal diagnosis of fetal genetic disease is routinely accomplished by conventional microscopic analysis of banded metaphase spreads prepared by in vitro culture of cells. But there are some disadvantages in the banding analysis method. The amniocytes must be cultured for several days prior to analysis even under the best circumstances. It is labour-intensive, time-consuming and more than two weeks are commonly needed. Under the above circumstances, there is a perceived need for methods for rapid detection of the major aneuploidies and for rapid and accurate detection of the major chromosomes 21, 18, 13, *X* and *Y* fetal aneuploidies. They could be useful adjunctive diagnostic aids to traditional cytogenetics.

Fluorescence in situ hybridization (FISH) to interphase nuclei with chromosome-specific DNA probes can now rapidly and accurately detect the most common autosomal trisomies and aneuploidies of the sex chromosomes (Eiben et al., 1998). However, the production of chromosome-specific probes by FISH still remains difficult, expensive. Most cytogenetic laboratories are not qualified to synthesize DNA probes or libraries. Primed in situ labeling (PRINS) presents an alternative technique, being faster and approximately 1/10 as expensive as FISH (Velagaleti et al., 1999; Coullin et al., 2002).

Since the introduction of PRINS by Koch et al. in 1989, the technique has been greatly improved and now provides a rapid alternative to FISH for many investigations, particularly the identification of chromosome aneuploidy in tumour cell lines and pre- and postnatal diagnosis (Gosden and Lawson, 1995). The method consists of annealing oligonucleotides (or denatured double-stranded DNA fragments) to complementary sequences on fixed chromosomes, followed by a DNA polymerase-driven extension in the presence of labeled deoxynucleotides. Newly synthesized DNA can be visualized by fluores-

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cence detection. As oligonucleotides (or denatured double-stranded DNA fragments) are unlabeled, high concentrations can be used without giving rise to background signals; if a probe is bound non-specifically to structures in the cell, this will not cause labeling since only correctly hybridized probe can function as primer for chain elongation. The use of high concentrations accelerates hybridization and minimizes damage to tissue structures. At the same time, sensitivity is higher than in conventional FISH since a multitude of reporter molecules can be incorporated during chain elongation.

The aneuploidies of chromosomes 21, 18, 13, X and Y can account for up to 95% of live-born chromosome abnormalities which are accompanied by birth defects (Whiteman and Klinger, 1991). The trisomy 18 comprise the second most common chromosomal aneuploidies in newborns. Rapid detection of chromosome 18 aneuploidies in amniocytes is of special significance.

In the present paper, we present the first major prospective study directly comparing aneuploidy detection of PRINS for chromosome 18 in uncultured amniocytes with the results obtained by FISH and cytogenetic analysis.

MATERIALS AND METHODS

1. Samples

A total of 262 samples at 14－33 weeks gestation (average 16 weeks), containing 15 to 17 ml of amniotic fluid were obtained. For PRINS and FISH analysis, 3－5 ml of the fluid were removed and 12 ml were used for standard karyotype analysis. After conventional amniotic cell culture, metaphase spreads were used for PRINS to control signal localization.

2. Slide preparations for FISH and PRINS in uncultured amniocytes

Approximately 3－5 ml of fresh amniotic fluid samples were centrifuged for 6 min at 1000 r/min. The pellet was resuspended and incubated in 2－3 ml of fresh 0.28% KCl at 37℃ for 40 min. After 0.8－2 ml fresh ice-cold methanol/glacial acetic acid (3:1) was added to it slowly for 5 min at room temperature, the suspension was centrifuged for 5 min at 1000 r/min. The pellet was resuspended in 0.5－1 ml of fresh ice-cold methanol/glacial acetic acid (3:1). Immediately before the PRINS or FISH process, 10－25 μl fixed suspension was dropped on a slide and air-dried. Metaphase spreads were established according to standard cytogenetic procedures.

Prior to reaction, the freshly prepared slides were incubated in formaldehyde-PBS/$MgCl_2$ solution (100 ml PBS/50 mmol/L $MgCl_2$ + 2.7 ml 37% formaldehyde solution) for 1 min at room temperature, then rinsed in 1x PBD buffer for short time, dehydrated through an ethanol series 70%, 80%, 100% at room temperature, and air-dried.

3. Primer

Primer 18cen (Koch et al., 1995): 5′-CGT TTC AAA ACT TCT CTA TGA AAA GAA AGG TTC TAC TCC TTT A-3′, is specific for the centromere of chromosome 18. The oligonucleotide primer was synthesized by MWG Biotechnology (MWG, Ebersbach, Germany). The optimal concentration of the oligonucleotide was 100 pmol/50 μl final reaction volume.

4. FISH and PRINS reactions

For FISH analysis we used the Quint-essential™ 18q-specific DNA probe (Oncor Inc., Gathersburg, MD, USA). Hybridization, posthybridization washes and detection were as the manufacturer's recommendations.

PRINS reaction was performed on the same day of slide preparation. For each slide, the reaction mixture was prepared in a final volume of 50 μl containing 100 pmol of the oligonucleotide, 5 μl of 10 × dNTP (0.5 mmol/L each of dATP, dCTP, dGTP, 0.4 mmol/L of dTTP, 0.1 mmol/L of digoxigenin-11-dUTP), 10 μl of 5 × polymerase buffer (10 x polymerase buffer in 50% of 87% Glycerol) and 2.5 U Tth Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The reaction mixture was applied on the slide and covered with a 24 mm × 60 mm coverslip. Slides were transferred to a programmable thermal cycler (Hybaid, Ashford, UK), incubated at 94 ℃ for 3.5 min to denature chromosomal DNA, at temperature lowered to 67℃ under which annealing of primer and chain elongation were performed for 30 min. The reaction was stopped by washing in pre-

warmed stop buffer (50 mmol/L NaCl, 50 mmol/L EDTA, pH 8.0) for 1 min at annealing temperature. The slides were then transferred to 4 × SSC, 0.05% Tween 20 at room temperature.

5. Detection

Detection of the labeled sites was performed by immunocytochemistry and conventional fluorescence microscopy. Detection of digoxigenin incorporated into synthesized products was done with anti-digoxigenin fluorescein Fab fragments (Boehringer Mannheim, Mannheim, Germany). The preparations were counterstained by 4′,6-diamidino-2-phenylidole (DAPI. 0.1 μg/ml) or propidium iodide in antifade solution (Oncor, Gaithersburg, USA). Documentation was achieved with a ZEISS Axioplan2 microscope equipped with corresponding fluorescence filters and a linked computer with software in situ image system version 1.90 (MetaSystem, Altlussheim, Germany).

6. Statistical analysis

A minimum of 50 hybridized nuclei were scored for each sample. Two independent investigators analyzed each sample in a blind fashion. Cases in which ≥80 percent of nuclei displayed two signals, normal or disomic cells were assured; whenever three signals were found in ≥ 60 percent of cells in a case, it was suspected as trisomic and was categorized as normal or abnormal, according to the karyotype analysis.

The data were statistically analyzed using Fisher's exact probability test method. A P value less than 0.01 was considered significant; more than 0.05 was defined as no significant difference.

RESULTS AND DISCUSSION

A total of 262 independently uncultured and cultured amniotic fluid samples were analyzed by PRINS. The cultured amniotic fluid samples were used for signal localization analysis. All amniotic fluid samples were simultaneously processed for routine cytogenetic analysis. For comparison with the results of PRINS, 62 of the 262 samples were additionally analyzed by FISH. Specific chromosomal labeling were obtained in the interphase nuclei of uncultured amniocytes as well as in the metaphase preparations and interphase nuclei obtained from cultured amniocytes with primer 18cen by PRINS (Fig. 1).

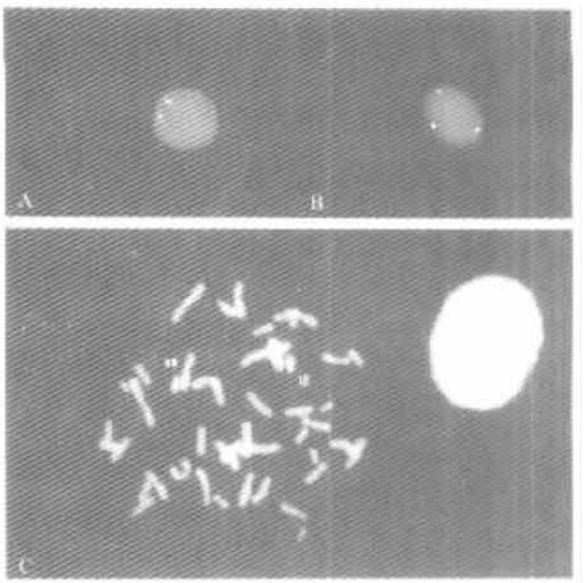

Fig. 1 Photomicrographs of in situ labeling by PRINS for chromosome 18

A: interphase nucleus showed two fluorescent signals in uncultured amniocyte of normal sample; B: three signals were observed in trisomy 18 (counterstained by DAPI); C: the primer 18cen specifically labeled chromosome 18 in cultured amniocyte of normal sample (counterstained by propidium iodide).

The PRINS assay was considered successfully in 242 of the 262 samples tested since at least 50 nuclei were counted in each probe. In nine samples, only 15 – 49 nuclei were counted; the result was classified as "problematic". Eleven samples displayed no signal. Among the technically successful hybridizations of 249 normal disomic samples, 248 samples showed ≥80% hybridized nuclei with two signals; one sample displayed 76% hybridized nuclei with two signals. Two different samples of trisomy 18 were recognized and over 85% of hybridized nuclei displayed three signals labeling (Table 1). The results of all samples were correlated further by conventional karyotype analysis and the false-positive and false-negative was 0%.

Conventional FISH was applied independently to 60 normal samples and two samples of trisomy 18 successfully assayed by PRINS. All FISH reactions were successful and all normal

samples displayed > 80% of 50 hybridization nuclei with two signals. In trisomy 18, the two samples showed > 90% hybridization nuclei with three signals. The comparison results on normal samples obtained from PRINS and FISH techniques are summarized in Table 2. The statistical results of Fisher's exact probability test using the observed signal distribution of cells showing one, two, and three signals indicated that there were no significant difference in detecting results between FISH and our modified PRINS methods. Because of its usability in clinical diagnosis, the 18q-specific DNA probe was chosen and used in FISH experiments here for comparison.

Table 1 Results of PRINS for chromosome 18 assays

	Cases (n)	%
PRINS performed	262	100
PRINS not successful (no hybridization)	11	4.2
PRINS successful		
Trisomy 18 (≥50 nuclei and >80% three signals)	2	0.8
Disomy 18 (≥50 nuclei and ≥80% two signals)	239	91.2
Result problematic (≥50 nuclei but 76% two signals)	1	0.4
Result problematic (15 – 49 nuclei and ≥80% two signals)	9	3.4

Table 2 The results of PRINS and FISH scoring in normal uncultured amniocytes for chromosome 18 (60 samples; 3000 nuclei)

Chromosome 18	1 signal		2 signals		3 signals	
	PRINS	FISH	PRINS	FISH	PRINS	FISH
Number of nuclei	111	87	2790	2886	99	27
Percentage (%)	3.7	2.9	93	96.2	3.3	0.9
P value	0.472		0.894		0.275	

Scoring results were based on all technically successful hybridizations.

Chromosomal abnormalities are currently routinely diagnosed by cytogenetic analysis of metaphase chromosomes. However, cytogenetic diagnosis could be enhanced by techniques that could allow more rapid detection of common abnormalities. PRINS combines the sensitivity of polymerase chain reaction (PCR) and the specificity of FISH. In this study, we present a modified PRINS technique for rapid localization of sequence-specific fluorescent tagging. The PRINS reaction time in our protocol was much shorter and pretreatment was simpler. The results of our study clearly validated the specificity of the generated primers and we had demonstrated that PRINS provides efficient prenatal detection of chromosomal aneuploidies in uncultured cells from amniotic fluid.

Cell quality and proper slide preparation are important factors for successful PRINS reaction. As amniocytes are fewer than lymphocytes in the suspension, especially in the early pregnancy, proper sample-handling, use of the fixogum to mark the areas of interest, and pretreatment with formaldehyde and alcohol can improve slide quality.

In order to assess the efficiency of the method under various practical conditions, various combinations of primer or enzyme concentration and reaction times were tested. The best results, strong labeling and low background, were obtained with 100 pmol of oligonucleotide and *Tth* DNA polymerase, and 30 minutes annealing and elongation time. We applied a thermal cycler equipped with a flat plate allowing the simultaneous treatment of four slides. The metallic plate block was equipped with a plastic lid to ensure good heat transfer and uniformity of temperature on the plate.

We observed slides of 11 cases with areas containing no signals at all. These slides or parts

of the slide were considered technically unsuccessful hybridizations or inadequately labeled. This could be due to variations in slide preparation or subtle events occurring during the reaction. Subtle variations in sample fixation also influenced hybridization efficiency. Undetected maternal cell contamination, increased background fluorescence due to excessive cellular debris, weak hybridization signals, or unknown causes contributed to the observed false negatives. The specificity of the reaction also greatly depended on the setting of proper annealing temperature, so each annealing temperature must be exactly tested.

CONCLUSIONS

PRINS technique seems to have great potential for prenatal diagnosis and constitute an efficient complement to existing technologies, such as FISH and PCR. These preliminary results show that PRINS combined with a simple enrichment procedure provides a rapid and simple method for rapid detection of the interphase nuclei of uncultured amniocytes. The results of the present study support use of PRINS to enhance the effectiveness of standard cytogenetics, allowing accurate identification of trisomic 18 constituents in uncultured amniocytes in significantly less time. Here we only obtained two different samples of trisomy 18. Our experience showed that PRINS is easy to perform, cost-effective and an interesting alternative to in situ hybridization. As the sample-handling protocols are developed, prenatal diagnostic applications of PRINS undoubtedly will expand. Some modifications of the original PRINS reaction have also been published (Wilkens et al., 1997). The multi-color PRINS (Hindkjær et al., 1994) allows for the detection of more than one target sequence within one cell by repeating the PRINS reaction using differently labeled nucleotides. With multi-color PRINS, subsequent PRINS reactions with different primers and different reporter molecules bound to the incorporated nucleotides are possible.

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