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Martin Werner · Ludwig Wilkens · Anette Nasarek
Joëlle Tchinda · Paul Komminoth

Detection of karyotype changes in interphase cells: oligonucleotide-primed in situ labelling versus fluorescence in situ hybridization

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Abstract Interphase cytogenetics is a rapidly developing technique which is usually performed by fluorescence in situ hybridization (FISH). Recently, oligonucleotide-primed in situ synthesis (PRINS) has become established as a method of labelling centromeric regions of chromosomes in metaphase spreads. We tested the suitability of PRINS in detecting the exact copy number of chromosomes 1, 3, 7 and 8 in intact interphase cells of 17 cytological preparations derived from normal and neoplastic tissues. Control procedures consisted in preparation of metaphase spreads of lymphocytes of healthy donors, conventional cytogenetics in some of the specimens, and omission of the primers or *Taq* polymerase from the reaction mixture. All specimens were additionally examined by FISH and analysed blind by two experienced observers. Both PRINS and FISH revealed a corresponding distribution of hybridization signals for all chromosomes examined in specimens of normal bone marrow (n = 5), normal liver cells (n = 5), three samples of acute nonlymphocytic leukaemia in which conventional chromosome analyses had shown monosomy 7 or trisomy 8, and in four hepatocellular carcinomas that displayed trisomy 1. Overall, statistical analysis revealed no significant difference in the signal distribution between the two techniques. Our results demonstrate that PRINS is as reliable as FISH for detecting chromosome copy numbers in interphase nuclei of intact cells. The PRINS method, however, is easier to perform, faster and less expensive, holding great potential for future applications in diagnostic pathology.

Key words In situ hybridization · Molecular cytogenetics · Chromosomes · Leukaemia · Hepatocellular carcinoma

Dedicated to Professor Dr. Axel Georgii, Director of the Institute of Pathology, Medical University of Hannover, Germany, on the occasion of his 70th birthday.

M. Werner (✉)
Institute of Pathology, Technical University of Munich,
Ismaninger Strasse 22, D-81675 Munich, Germany
Tel.: (49) 89-4140 4170, Fax (49) 89-4140 4865

L. Wilkens · A.Nasarek · J. Tchinda
Institute of Pathology, Medical University of Hannover,
Hannover, Germany

P. Komminoth
Department of Pathology, University of Zurich,
Division of Cell and Molecular Pathology, Zurich, Switzerland

Introduction

Interphase cytogenetics is a rapidly developing technique that allows the assessment of karyotype changes in single nondividing cells derived from tissue specimens [9]. Apart from the visualization of chromosome gains or losses, this technique has also been applied for detecting chromosomal translocations [16, 20, 24]. The method of choice for determining karyotype changes in interphase nuclei is fluorescence in situ hybridization (FISH). FISH requires DNA probes several hundred or thousand base pairs (bp) in length that are produced by molecular cloning techniques. The dependence on these probes, is the major disadvantage of FISH, however, since they are difficult to generate, and rather expensive if obtained commercially.

Oligonucleotide primed in situ synthesis (PRINS) has recently been established for the characterization of metaphase chromosomes [11]. The PRINS method is based on the *Taq* polymerase mediated incorporation of labelled nucleotides into newly synthesized DNA, which is primed by the hybridization of oligonucleotides to unique chromosome specific sequences in centromeric tandem repeats [5, 12]. So far, PRINS has only been applied to metaphase speads prepared from peripheral blood, bone marrow or amnionic fluid cultures that also contain interphase nuclei of lysed cells [2, 3, 7, 8, 14, 17]. Its use for detecting chromosome changes in intact cells from either cytological or surgical pathology specimens, however, has not been reported, to the best of our knowledge.

The aim of our study was to investigate the suitability of PRINS for demonstrating numerical chromosome

changes in intact interphase cells, and to compare this method with the conventional FISH analysis. Once the reliability of PRINS reactions had been demonstrated in bone marrow cells in which the exact chromosome pattern was known from conventional chromosome banding, the PRINS technique was applied to cytological preparations of solid tissues (normal liver and hepatocellular carcinoma [HCC]). We investigated chromosomes 1, 3, 7 and 8, which are known to be affected by numerical changes in acute leukaemias or HCC [16, 22]. Our results demonstrate that PRINS is a reliable and efficient method, which has shorter turnaround times and is less expensive than FISH in detecting numerical chromosome aberrations in interphase nuclei of intact cells.

Materials and methods

Heparinized bone marrow aspirates and formaldehyde/methanol fixed bone marrow biopsies were obtained from eight patients. Bone marrow biopsies were embedded in methyl methacrylate as previously described [1]. Five patients displayed nonspecific findings in bone marrow histopathology, and three patients had acute nonlymphocytic leukaemia (ANLL).

Conventional cytogenetics from bone marrow aspirates was performed according to standard protocols [27] and revealed normal diploid karyotypes in the five patients without evidence of neoplastic disease. Two patients with ANLL had trisomy 8 in 74% and 73%, respectively, and one patient with ANLL had monosomy 7 in 48% of the metaphases examined. For interphase cytogenetics of bone marrow aspirates, cells were fixed with methanol:acetic acid (3:1) for 20 min, smeared onto glass slides and air dried at room temperature.

Cytological imprint specimens from hepatocellular carcinomas (n = 5) and the corresponding normal liver tissue were prepared directly after surgical removal (hemihepatectomy) and fixed with paraformaldehyde (4%) for 10 min, followed by proteinase K digestion (0.1% in phosphate buffered saline, PBS) for 10 min at 37°C, and air drying at room temperature.

Probes

For PRINS reactions, oligonucleotides specific for tandem repeats in the centromers of chromosomes 1, 3, 7 and 8 were obtained from Boehringer, Mannheim, Germany. For FISH analyses, digoxigenin-labelled alpha satellite probes specific for the centromeric region of chromosomes 1, 3, 7 and 8 (D1Z5, D3Z1, D7Z1, D8Z1) were purchased from Oncor/Amersham-Buchler (Braunschweig, Germany).

Oligonucleotide-primed in situ labelling

The air-dried slides were heated for 1 min at 96°C using a flat thermocycler (Omnigene TRC3, Hybaid, UK) followed by lowering of the temperature to 94°C for 3 min. Then, 30 µl of a solution containing 5 µl of the respective PRINS primer (250 pM), 50 mM Tris/HCl pH 8.3, 250 mM KCl, 0.05% gelatin, 7.5 mM $MgCl_2$, 0.05% Tween 20, 0.05% Triton X 100, 0.05% Nonidet p40, 500 µM dATP, dCTP and dGTP, respectively, and 50 µM DIG-11-dUTP, 450 µM dTTP, and 2.5 units *Taq* polymerase (all nucleotides and enzymes were purchased from Boehringer, Mannheim, Germany) was added. The specimens were incubated under a coverslip sealed with rubber cement for 30 min at 60°C. Then, the rubber cement was carefully peeled off and the coverslip removed by immersing the slides in 500 mM NaCl/50 mM EDTA for 1 min at 60°C. Slides were then washed three times in 0.2% Tween 20/PBS at 37°C, incubated with FITC labelled anti-DIG Fab fragments (Boehringer, Mannheim, Germany) diluted 1:1000 in PBS/1% bovine serum albumin for 30 min at 37°C, and washed again as described above. For counterstaining, 15 µl propidium iodide/antifade was added to the samples, which were then coverslipped (Oncor, Amersham/Buchler, Braunschweig, Germany).

Fluorescence in situ hybridization

The air-dried slides were rinsed in 2×SSC, the DNA denatured in 70% formamide 2×SSC for 2 min at 70°C, and the samples dehydrated in graded ethanols at –20°C. Then 15 ng of digoxigenin-labelled alpha satellite probes (previously denatured for 5 min at 70°C) in 30 µl hybridization buffer containing 65% formamide 2×SSC and blocking DNA (Hybrisol VI, Oncor, Amersham/Buchler, Braunschweig, Germany) were applied to each sample under a coverslip followed by incubation overnight at 37°C. After stringency washes in 2×SSC for 5 min at 72°C, slides were rinsed once in 0.1% Tween 20 in PBS for 1 min at room temperature, and detection of hybridized probes was performed by incubating FITC-labelled anti-digoxigenin (30 µl) for 5 min at 37°C. The reaction was amplified by incubation with a rabbit anti-sheep antibody (30 µl) for 15 min at 37°C followed by FITC-labelled anti-rabbit antibody (30 µl) for 15 min at 37°C (all incubations were performed under a coverslip). After washing in 0.1% Tween 20/PBS, nuclei were counterstained with 15 µl propidium iodide/antifade (all reagents were purchased from Oncor, Amersham/Buchler, Braunschweig, Germany).

Metaphase preparations of blood lymphocytes obtained from healthy donors served as controls for both PRINS and FISH.

PRINS and FISH reactions were analysed blind by two experienced investigators using a Zeiss Axiophot fluorescence microscope (Oberkochen, Germany) with oil immersion (final magnification ×1000). Only signals with bright and approximately equal staining in a corresponding nucleus were counted, and paired spots were considered as one signal, at least 100 intact, nonoverlapping nuclei being evaluated. The number of hybridization spots for each chromosome was calculated by determining the mean value of the numbers counted by both investigators. The Chi-square test was used for statistical analyses. The 3× standard deviation was determined to define pathologic loss or gain of chromosomes [16, 25]. Documentation of PRINS and FISH analyses was performed with a CCD camera (CH250/A, Photometrics, Tucson, Arizona, USA) and a capture image analysis system (Vysis, Stuttgart, Germany).

Results

In normal control metaphases of healthy donors, PRINS and FISH reactions revealed strong signals in the centromeric regions of the chromosomes examined – chromosomes 1, 3, 7 and 8 (Fig. 1a, b). Dispersed interphase nuclei in those preparations also displayed two distinct and easily scorable signals for all chromosomes examined. Controls for PRINS (omission of primers or *Taq* polymerase) revealed no signals in metaphases or interphase nuclei.

To investigate the reliability of PRINS in detecting chromosomes in interphase cells, this technique was compared with FISH in cytological preparations of various normal and tumour tissues. We first examined five samples of bone marrow with a normal chromosome pattern shown by conventional chromosome banding in metaphase preparations. Both the PRINS and the FISH methods revealed a similar distribution of hybridization spots in the interphase nuclei of the cytological preparations. The majority of nuclei in each sample exhibited

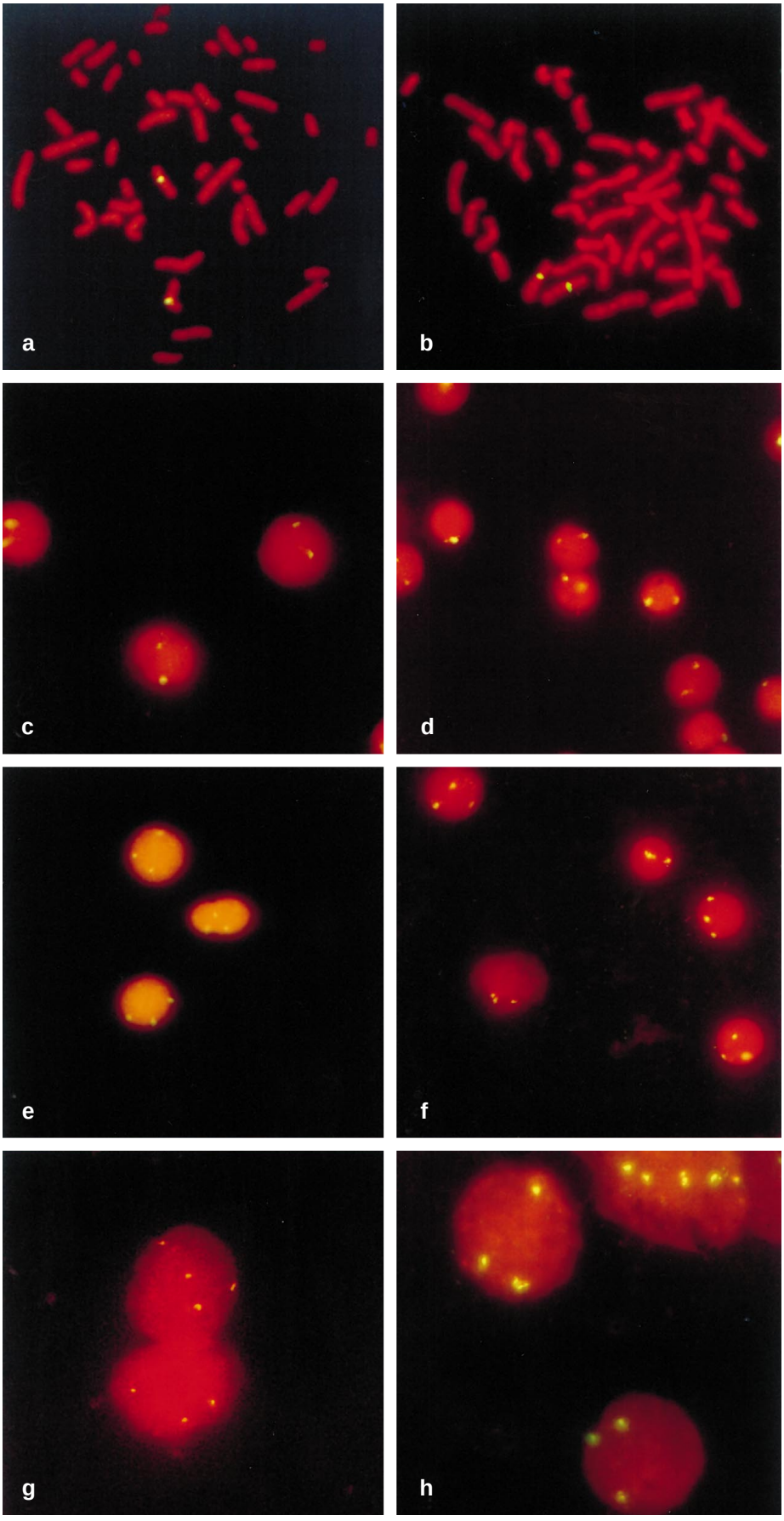

Fig. 1 Comparison of fluorescence in situ hybridization (FISH; **a**, **c**, **e**, **g**) and oligonucleotide-primed in situ labelling (PRINS; **b**, **d**, **f**, **h**). **a**, **b** Metaphase spreads of a healthy donor displaying strong staining in the centromers of both chromosomes 7 by FISH (**a**) with the digoxigenin-labelled D7Z1 probe and PRINS (**b**) with specific oligonucleotides. **c**, **d** Bone marrow smear of a patient without evidence of neoplastic bone marrow disease and with normal diploid karyotype according to conventional cytogenetics: **c** FISH with the centromeric D7Z1 probe specific for chromosome 7 revealing two distinct signals per nucleus; **d** PRINS with oligonucleotides specific for the centromeric region of chromosome 7 displaying two signals in most nuclei. **e**, **f** Bone marrow cells of a patient with acute nonlymphocytic leukaemia (ANLL) exhibiting trisomy 8 as clonal karyotype change in conventional chromosome analysis; both FISH (**e**) with the digoxingenin-labelled D8Z1 probe and PRINS (**f**) with oligonucleotides specific for chromosome 8 exhibited three hybridization spots in most nuclei. **g**, **h** Imprint specimens of a hepatocellular carcinoma displaying trisomy 1 on FISH (**g**) with the D1Z1 probe and PRINS (**h**) with oligonucleotides specific for chromosome 1. Detection of FISH by FITC-labelled anti-digoxigenin followed by an amplification step with a bridging antibody and FITC-labelled anti-rabbit antibody. PRINS reactions have been visualized by FITC-labelled anti-digoxigenin. Note that there are no significant differences between the two methods in signal intensity and distribution. Counterstaining with propidium iodide/antifade, ×1000 (**a–f**), ×1600 (**g, h**)

Table 1 Mean distribution of hybridization signals in PRINS and FISH reactions obtained in five samples of cytogenetically normal bone marrow and five samples of normal liver. There were no significant differences between the two methods

Tissue	Chromosome	PRINS/FISH Signals per nucleus (%)				
		0	1	2	3	>3
Bone marrow	1	2/3.6	9/9.2	86.4/85.4	2/1.4	0.6/0.4
	3	2/2	8.8/9.8	87.4/86.2	1.4/1	0.4/1
	7	2.8/3	8.4/9.4	86.6/86	1.8/1.6	0.4/0
	8	2.8/2.8	9.4/10.2	85.8/85.6	1.8/0.8	0.2/0.6
Liver	1	1.7/1	9.7/7.1	83.6/83.6	3.5/5.8	1.5/2.5
	8	2/2.8	10.3/8.7	83.5/84.8	3.5/3.2	0.7/1.5

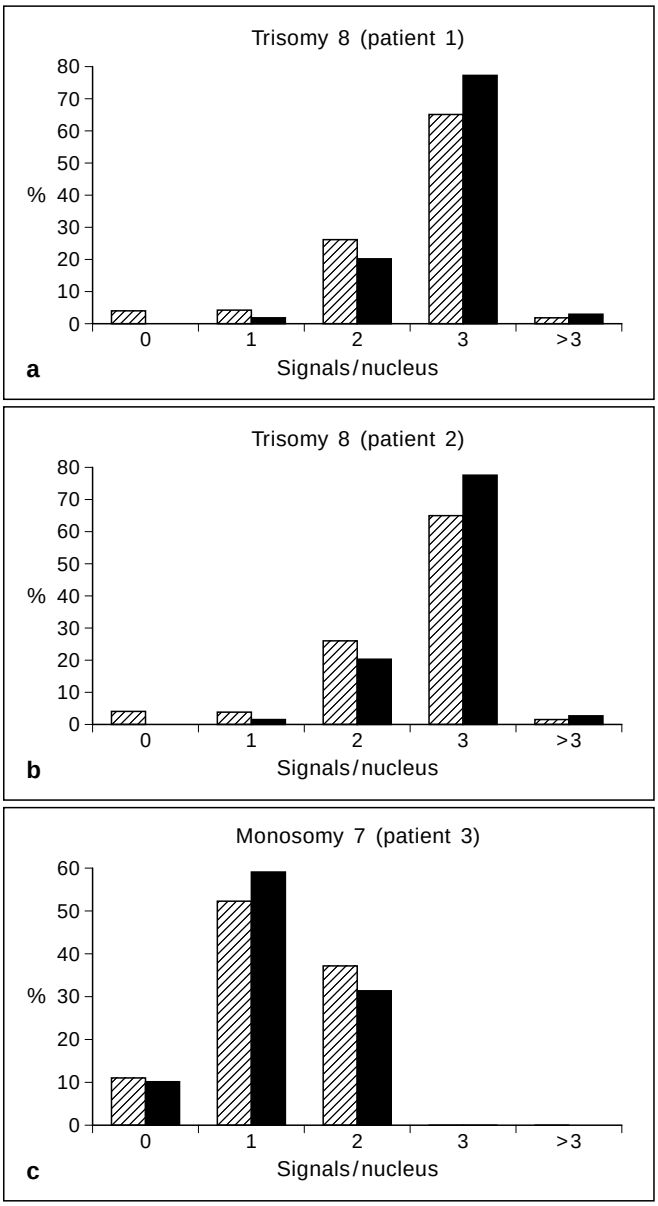

Fig. 2a–c Comparative distributions of hybridization signals obtained by PRINS (*lighter bars*) and FISH (*darker bars*) in bone marrow cells of patients with ANLL, and **a, b** trisomy 8 or **c** monosomy 7

two signals for chromosomes 1, 3, 7 and 8 (for all chromosomes examined: PRINS, mean 86.6%, standard deviation: 2.8%; FISH: mean, 85.8%, standard deviation: 2.9%; Table 1, Fig. 1c, d).

Cytological imprints prepared from normal liver also showed two spots in most nuclei (for all chromosomes examined: PRINS: mean 83.3%, standard deviation: 1.8%; FISH: mean: 83.8%, standard deviation: 4.1%), with no difference in signal distribution between PRINS and FISH (Table 1). Both methods revealed a small percentage of nuclei with a higher (PRINS: ≤7%, FISH: ≤15%) or lower number (PRINS: ≤14%; FISH: ≤11%) of signals expected in normal cells. No significant differences between the PRINS and FISH methods were found when the cut-off levels for pathological trisomy or monosomy of chromosomes 1, 3, 7 and 8 were compared (Table 2).

The bone marrow samples of three patients with ANLL, in which conventional cytogenetics had demonstrated either monosomy 7 or trisomy 8 as a clonal karyotype change, exhibited the appropriate numerical chromosome changes in the interphase cell preparations with both the PRINS and the FISH methods (Fig. 1e, f). There was no significant difference in the percentage of cells with abnormal chromosome numbers between the PRINS and the FISH methods (Fig. 2a–c).

In touch preparations of four HCC, FISH revealed trisomy 1, which was confirmed by PRINS with primers for chromosome 1 (Figs. 1g, h, 3a, b). Chromosome 8 was trisomic according to both methods in only one of four tumour specimens (Fig. 3c, d). One HCC was not informative because of extensive necrosis of the specimen yielding to small numbers (<100) of intact cells.

Statistical evaluation of the results of all 17 preparations examined revealed no significant difference in the signal distribution between PRINS and FISH experiments. There was no intraobserver difference in the signal distribution counted blindly by the two investigators.

Discussion

By applying the PRINS and the FISH methods to the detection of aneusomic chromosome counts in intact interphase cells of pathology specimens, we have demonstrated that the two techniques yield comparable results. In

Table 2 Cut-off levels (%) for monosomy and trisomy for the PRINS and FISH method, respectively

Bone marrow	PRINS/FISH			
Chromosomes	1	3	7	8
monosomy	>14.0/>15.2	>11.7/>16.4	>11.5/>21.2	>14.6/>17.2
trisomy	>3.9/>2.9	>2.9/>3.4	>4.7/5.2	>6.6/3.1
Liver	PRINS/FISH			
chromosomes	1	8		
monosomy	>14.8/18.6	>19.2/13.8		
trisomy	>5.1/>13.0	>12.9/12.6		

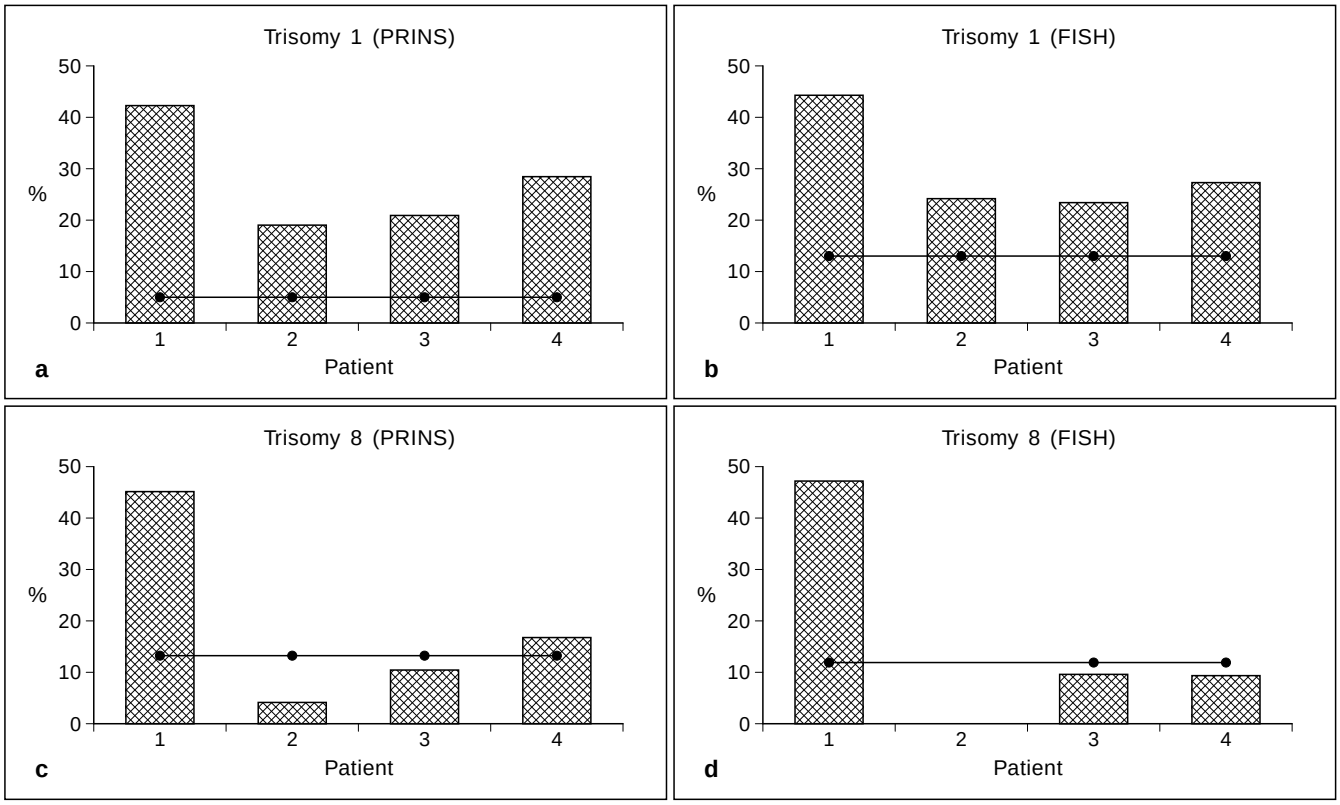

Fig. 3a–d Results of PRINS and FISH in four cases of hepatocellular carcinoma (HCC) (patients 1–4). Percentage of cells with trisomy counts for chromosome 1 by **a** PRINS and **b** FISH, and percentage of cells with trisomy for chromosome 8 by **c** PRINS and **d** FISH. All HCC patients examined had trisomy 1 according to both PRINS and FISH (**a, b**). Only case 1 displayed trisomy 8 (**c, d**)

imprint preparations of normal liver cells and in normal bone marrow cells, the percentages of disomic cells obtained here for chromosomes 1, 3, 7 and 8 did not differ significantly between PRINS and FISH, and there was good agreement with earlier FISH studies [10, 16]. Moreover, the cut-off levels for defining gains or losses of chromosomes were also not significantly different with the two methods, suggesting that PRINS is as sensitive as FISH for detecting low numbers of aneusomic cells. Applying FISH with chromosome 4-specific probes and PRINS with chromosome 9- and chromosome 18-specific primers in haploid human sperm, Pellestor et al. [19] suggested that both techniques can be used in a complementary fashion.

The fact that small percentages of the normal bone marrow or liver cells examined displayed more or less than the two signals expected with FISH or PRINS is a well-known phenomenon in interphase cytogenetics [10]. Trisomy 1, which was revealed in four specimens of HCC by both methods, is a frequent finding in some of the malignant cells in those tumours [16]. In ANLL, trisomy 8 or monosomy 7 occurs as the clonal karyotype changes, affecting variable numbers of tumour cells [22].

Compared with FISH, the PRINS technique has proven to be equally accurate, easier to perform and quicker, allowing results within 2 h. By incorporating directly fluorochrome-labelled nucleotides into newly synthesized DNA, the turnaround time of the PRINS reaction can be reduced to no more than a few minutes, as has been shown in metaphase preparations, but signals are

Table 3 Comparison of PRINS and FISH methods

	PRINS	FISH
Probes	Oligonucleotides (custom made)	cosmid probes (commercial)
Length of probes/primers	20–30 nt[a]	Several hundred or thousend bp [b]
Method	Synthesis of labelled DNA by *Taq* polymerase	In situ hybridization with labelled probe
Detection	Direct or indirect	Direct or indirect
Materials	Methaphases, cytological preparations, tissue sections (?)	Metaphases, cytological preparations, sections from fresh frozen and paraffin embedded tissues
Turnaround time	≤2 h	≤2 days
Advantage	Easy, quick, inexpensive	Established protocols
Disadvantage	Emerging technique	Generation of probes difficult and expensive

[a] Nucleotides
[b] Base pairs

weaker than with the indirect method [6, 7, 13]. FISH, in contrast, usually takes 2 working days [16, 20, 24], and faster protocols with shorter hybridization times result in significantly weaker hybridization signals.

The intensity of the signals produced by PRINS depends on the size of the products synthesized by the *Taq* polymerase. By targetting tandem repeats that are present in the centromers as well as in the telomers of all chromosomes [15, 26], specific oligonucleotides can bind repeatedly to these regions, presenting multiple sites for incorporation of labelled nucleotides. The signal intensity can be further increased by performing several cycles of the PRINS reaction (so-called cycling PRINS), which accumulates labelled copies of the target sequence at the site of synthesis [23].

Another advantage of PRINS is the use of oligonucleotide primers which are easy to generate and rather inexpensive compared with the cosmid probes required for the FISH method. Overall, we estimate that the PRINS reaction costs approximately one-tenth as much as FISH. Nonspecific binding, which can be a problem in oligonucleotide in situ hybridization, did not occur in PRINS if the protocols (reaction times and incubation temperatures) were followed exactly. Probe penetration is better with small oligonucleotides than with the long DNA probes applied in FISH, resulting in faster hybridization conditions. However, in PRINS a short proteinase K predigestion of liver epithelial and HCC cells was necessary to allow access of the *Taq* enzyme. We did not observe a loss of reactivity of the *Taq* polymerase during primer extension, which may be caused by adherence of the molecule to the glass slide. With the exception of a flat heating block thermal cycler, no special equipment is required to perform the PRINS reaction. Two ordinary heating blocks, which are available in most laboratories, can also be used for PRINS. Table 3 summarizes the main differences between the PRINS and the FISH methods.

It appears that the full potential of the PRINS method has not yet been exhausted. For example, recent reports indicate that PRINS is also suitable for semiautomation [18], which opens up a wide variety of clinical applications not only for the study of genetic diseases but also for haematological and solid neoplasms. Furthermore, combinations of PRINS and FISH may soon allow for the efficient diagnosis of structural chromosome changes, including balanced translocations. These applications will be further facilitated by the recently described signal amplification methods [21, 28], which may – with or without cycling PRINS procedures – enable the detection of single copy genes. So far, this has only been successfully achieved in the case of the factor IX gene [4].

The PRINS reaction is a rapid and reliable method for visualization of numerical chromosome aberrations in interphase nuclei of pathology specimens. It has the potential to further increase the diagnostic possibilities of molecular cytogenetics and to complement FISH methods in detecting numerical and structural chromosome changes which are found in many neoplastic cells and in precursor lesions.

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