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Detection of Chromosome Aberrations in Interphase Nuclei using Fluorescene In Situ Hybridization Technique

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We report here several experiences of interphase cytogenetics, using fluorescence in situ hybridization (FISH) technique, for the detection of chromosome aberrations. FISH, using alpha satellite specific probes of 18, X, Y chromosomes, was done in interphase nuclei from peripheral blood of patients with Edwards' syndrome, Klinefelter's syndrome and Turner's syndrome with healthy male and female controls, respectively. The distributions of fluorescent signals in 100 interphase nuclei were well correlated with metaphase findings. Nowadays FISH plays an increasingly important role in a variety of research areas, including cytogenetics, prenatal diagnosis, tumor biology, gene amplification and gene mapping.

Key Words: *Fluorescence in situ hybridization, Interphase cytogenetics, Chromosome aberration.*

INTRODUCTION

Since the introduction of in situ hybridization, the technique has been used for the localization of DNA sequences in interphase and metaphase nuclei from many different cell types. In the seventies, the first methods were developed to overcome the disadvantages of the original autoradiographic method, notably, the lower resolution and the long exposure times needed. This resulted in the use of biotinylated DNA probes and fluorescence visualization of the DNA-DNA hybrids. Advantages of the latter method are the use of nonisotopically labeled probes and a signal that can be generated in a few hours. Thereafter, an important technological improvement of the biotin-avidin detection system was the introduction of signal amplification by successive treatments with fluorescently labeled avidin and biotinylated antiavidin antibody (Pinkel et al., 1986), achieving virtually the same sensitivity as radioactive in situ hybridization.

The recent availability of satellite DNA probes specific for individual chromosomes has provided a new class of molecular markers for the cytogenetic and genetic analysis of the human genome (Moyzis et al., 1987; Willard, 1989). Chromosome specificity is a general, although not the sole, mode of satellite DNA evolution and has been described for a number of classical satellites (Cooks and Hindley, 1979), α satellite (Mitchell et al., 1985) and β satellite (Waye and Williard, 1989) DNA probes. Such DNA probes are increasingly used in for molecular cytogenetic analysis of both metaphase chromosomes and interphase nuclei (Cremer et al., 1986). Several studies have shown that the results of in situ hybridization to interphase nuclei ("interphase cytogenetics") correlate well with metaphase findings (Cremer et al., 1988).

In this paper, we would like to introduce several experiences, showing the clinical usefulness of interphase cytogenetics using the FISH technique.

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MATERIALS AND METHODS

Patients

Suitble materials were available from two adult patients with sex-linked disease and one autopsy case with multiple congenital anomalies (Table 1).

As a control for ISH, a suspension of cultured lymphocytes obtained from two healthy individuals (a women and a man; Table 1) was used.

Karyotype Analysis

Metaphase cells of healthy individuals, and patients 1, 2 and 3 were obtained following culturing in vitro

Table 1. Clinical and cytogenetic data

Patient No.	Age (years)/ Sex	Diagnosis	Karyotype
1.	Newborn/F	Edwards' syndrome	47, XX, +18=10[a]
2.	32/M	Klinefelter's syndrome	47, XXY=8
3.	13/F	Turner's syndrome	45, XO=9:46, XX=10
4.	9/F	Normal female	46, XX=11
5.	Newborn/M	Normal male	46, XY=10

[a]: Number of counted metaphase

for 72 hours with phytohemagglutinin (PHA), in RPMI 1640 containing 10% BCS.

Metaphase cells were harvested and Giemsa trypsin G-banding (GTG-banding) was performed according to standard protocol (Seabright, 1971).

In Situ Hybridization (ISH)

For ISH studies, the following probes were used: alphoid repetitive DNA probes specific for centromeric regions of chromosomes X, Y, 18 further designated as αpX, αpY, and αp18 respectively.

Preparations for ISH were made by centrifuging cells onto slides. Standard noncompetitive ISH protocols were used for ISH with the repetitive probes (Raap et al., 1989; Kibbelaar et al., 1991), and CISS hybridization was performed for ISH with chromosome-specific libraries (Cremer et al., 1990).

Slides were treated with RNase A for 60min at 37°C (10μg in 100μl of 2 X SSC under a $24 \times 50mm^2$ coverslip), followed by a pepsin digestion (50μg/ml) in 0.01 M HCl for 10min at 37°C and a postfixation step in 1% acid-free formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature.

After dehydration, the metaphase preparations were denatured in 70% formamide at 70°C for 2min, and the slides were transfered immediately to 70% ethanol at −20°C. After further dehydration in 90% and 100% ethanol at room temperature, the slides were kept at 42°C.

Repetitive probes were dissolved in a 50% deionized formamide. Probe DNA was denatured for 10min at 75°C, quenched on ice, allowed to preanneal at 37°C, and mixed with a hybridization mixture containing 20% dextran sulphate, 2 X SSC, and 0.1% BSA. For single target repetitive DNA probes, 5μl of hybridization mixture was applied with a probe concentration of 2ng/μl. Hybridization took place at 37°C for 15 to 18 hours.

Immunological Detection

Slides were washed three times in 50% formamide, 2 X SSC at 45°C, followed by three washes in 0.1 X SSC at 60°C. Slides were preincubated for 30min at 37°C in 4 X SSC, 3% BSA, followed by three layers of immunological detection at 37°C for 30min each. The excess of antibodies was removed by three washing steps (37°C, 5min each) in the buffer used for the immunological detection, adding 1% Tween 20, but without blocking reagent. Three subsequent steps were performed: the first layer contained avidine-FITC (VECTOR Laboratories); the second layer, goat antiavidine biotinylated (VECTOR); the third layer, avidine-FITC (VECTOR). The buffer used for the three steps contained 4 X SSC, 0.1% Tween 20, and 1% BSA. Finally, the slides were embedded in a dextran sulphate mixture with PI (for FITC), for counterstaining and the antifade reagent DABCO and PPD (Sigma).

RESULTS

Banding analysis

Cytogenetical and clinical data are summarized in Table 1. An autopsy case having multiple congenital anomalies revealed typical trisomy 18 on karyotypic analysis. All karyotypes of Klinefelter's syndrome were 47, XXY. Karyotype of Turner's syndrome was 50% 45, XO and 50% 46, XX (mosaicism). Karyotypes of a normal female and normal were 46, XX and 46, XY, respectively. All results of banding analysis are shown in table 1.

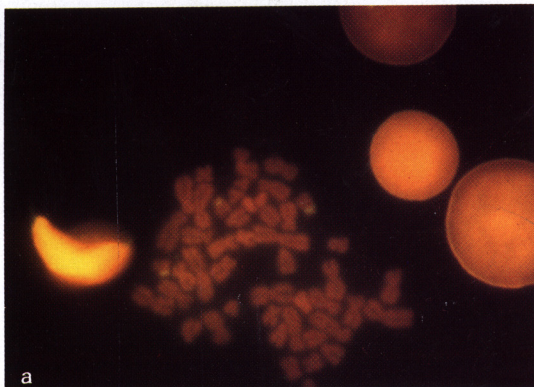
In situ hybridization

Results of FISH are shown in Fig. 1, 2 and 3 (Fig. 1 for Edwards' syndrome, Fig. 2 for Klinefelter's syndrome, Fig. 3 for normal female).

For Edward's syndrome, most of the metaphase cells revealed three clear intense signals at the centromeric lesions of chromosome 18 and interphase cells also revealed three signals in the nuclei (Fig. 1). In the case of Klinefelter's syndrome, two clear strong signals and one weak small signal were noted at the centromeres of two X chromosomes and one Y chromosome,

Table 2. Distribution of spots per cell

Patient No. & Diagnosis	Probe	Spots Per Nucleus (% positive nuclei) by FISH			
		Interphase cells			
		0	1	2	3
1. Edwards' syndrome (47, XY, +18)	αp18	0	20	10	70
2. Klinefelter's syndrome (47, XXY)	αpX & αpY	0	9	17	74
3. Turner's syndrome	αpX	0	80	20	0
(45, X/46, XX)	αpY	97	3	0	0
4. Normal female	αpX	0	16.7	83.3	0
(46, XX)	αpY	0	0	0	0
5. Normal male	αpX	2	80	18	0
(46, XY)	αpY	0	91	9	0



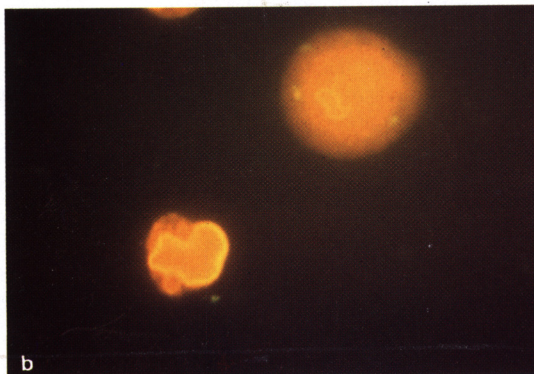
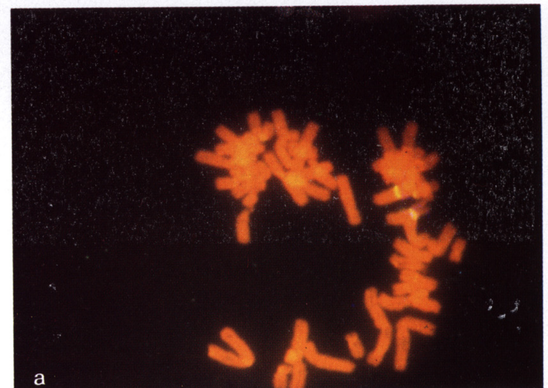


Fig. 1. a. FISH with αp18 showing three spots in one metaphase of Edwards' syndrome (patient 1).
b. FISH with αp18 showing three sports in one interphase of a Edwards' syndrome (patient 1). PI counterstaining.



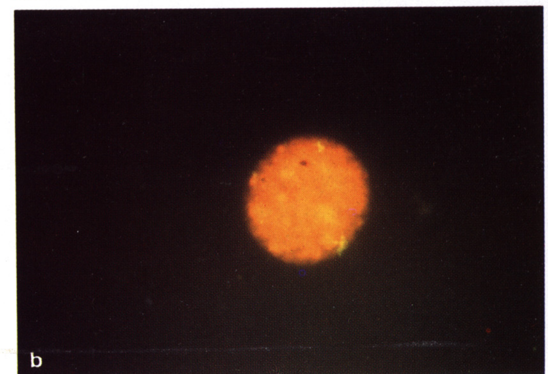


Fig. 2. Double-target FISH with the αpX and αpY probes. Colocalization of one of the weak spots on Y chromosome and the two spots on two X chromosomes of Klinefelter's syndrome (patient 2); in a metaphase cell (a), in an interphase cell (b). PI counterstaining.

respectively, and interphase cells revealed three signals (two intense signals and one weak signal) (Fig. 2). In Turner's syndrome, there were variable signals from zero to two. In the normal female and male, metaphase and interphase nuclei revealed two X in the female (Fig. 3) and one X and one Y in the male (data not shown).

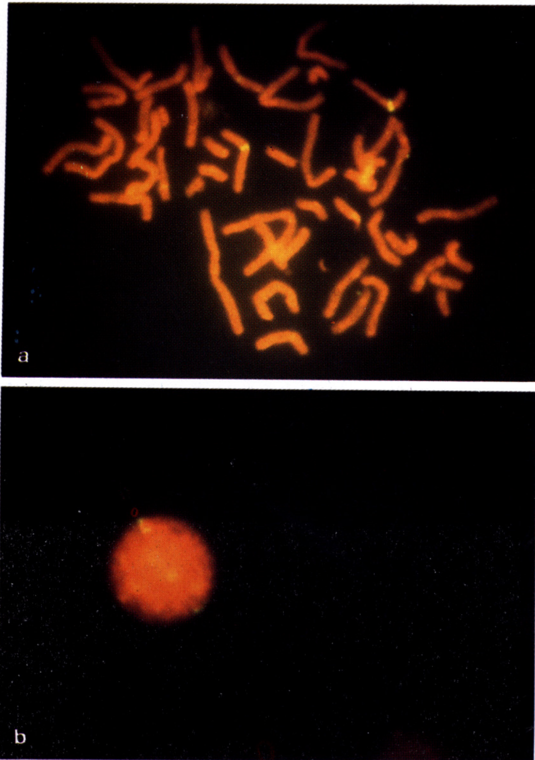


Fig. 3. a. FISH with αpX showing two spots in one metaphase of a normal female (patient 4).
b. FISH with αpX showing two spots in one interphase of a normal female (patient 4). PI counterstaining.

DISCUSSION

We present here several experiences of interphase cytogenetics, using fluorescence in situ hybridization (FISH) technique and discuss its clinical usefulness.

Routine karyotyping using G-banding procedure is well suited to detection of numerical aberrations and structural aberrations involving approximately 5 or more megabases (Mb) of DNA. In addition, it provides information about population heterogeneity and about the occurrence of multiple aberrations per cell. However, karyotyping can be applied only to cells that can be stimulated into mitosis and reliably banded. This is a significant limitation, especially in prenatal diagnosis and solid tumors. In breast cancer, for example, many of the cells that proliferate in culture are near diploid, even when the tumor appears highly aneuploid by DNA content analysis. Karyotypic information is also difficult to correlate with phenotype since many phenotypic markers are lost during mitosis. Due to such problems many cytogeneticists have been looking for better methods of analysing in situ state and have shown much interest in interphase nuclei.

Along with the development of molecular genetic technique many centromere specific and whole chromosome specific probes have been produced and their applications to cytogenetic analysis have been increasing. In situ hybridization with chromosome specific probes can be applied to interphase nuclei, thereby increasing the number of cells that may be studied without culturing. It is of particular importance in cases when analyzable metaphase cells cannot be obtained, especially in prenatal diagnosis and solid tumors. At first centromere specific, highly repetitive satellite DNA sequences were used as probes, mainly because of their high signal to noise ratios. But now chromosome specific probes are also used. In interphase nuclei, hybridization with the chromosome specific libraries resulted in a large, rather fuzzy signals that could not be interpreted very well. In contrast hybridization with centromere specific probes yielded small, but distinct signals in interphase nuclei.

Fluorescence in situ hybridization (FISH) has now been developed to the point where it can be considered as a significant adjunct to the more established methods for detection and characterization of numerical and structural aberrations in human cancer (Trask et al., 1990; Rodriguez et al., 1992). Some of the advantages of FISH for single cell genetic analysis include (1) it is a relatively easy and fast technique (2) it gives a more reliable quantitative analysis of in vivo occurrance of chromosomal aberration (3) many cells particularly interphase cells, can be analyzed (4) changes in gene copy number and gene structure can be detected (Bar-Am et al., 1992; Bajalica et al., 1992) (5) multiple target sequences simultaneously with different fluorescent colors can be detected (Nederlof et al., 1989a).

Also there are some limitations of FISH including (1) only one or, at best, four chromosome segments can be studied in one experiment to establish numerical abnormalities (2) the signals are easily quenched out, so it is difficult to handle.

In our studies, all five cases revealed expected signals in interphase nuclei, which were well correlated with metaphase findings. But in small percentages lower signal numbers than expected were observed, especially in case 1 and 2. In the case of Turner's syndrome (case 3), showing mosaicism (45X/46XX), one signal with X specific probe was predominently noted, even though routine karyotyping revealed roughly a ratio of 1:1 (one X:two X). So in the cas of mosaicism, there are many problems in reaching an appropriate diagnosis using only interphase

cytogenetics.

The correspondence between the number of hybridization signals and the number of target chromosomes is not perfect. Uncoupling between signal number and chromosome number can occur for several reasons. One is that the hybridization signals extend over a significant fraction of the nucleus and, at best, are more or less randomly distributed. Thus, there is a significant probability of this occurring increasingly as the number f target chromosomes increases (a common occurrence for highly aneuploid solid tumors). Hybridization signal coalescence limits detection of cell populations carrying monosomies to those in which the monosomic population is present at frequencies greater than 10%. In some tissues (e.g., in the brain), the situation is even worse. There, the nonrandom organization of the nucleus reproducibly juxtaposes the repeated sequences from two homologous chromosomes so a single hybridization signal is generated.

Another important reason for uncoupling between hybridization signal number and chromosome copy number is unsuspected variation in nuclear organization so that one hybridization signal splits into two parts. Determination of chromosome copy number using repeat sequence probes also is complicated by heteromorphic variability between individuals in the size of the tandem repeat targeted by the repeat sequence probes. In some cases, the size of the repeat on one chromosome may be almost undetectably small. In these cases, a normal cell population may appear as monosomic for the heteromorphic chromosome. In other situations, karyotypic instability may lead to duplication of the tandem vice versa. Chromosome number determination based on hybridization signal number in these cases will be incorrect (Gray et al., 1992).

Now a multicolor in situ hybridization provides a versatile tool for the detection of specific chromosome aberrations; not only in metaphases but also in interphases nuclei. This method will be a useful supplement to modern cytogenetic approaches.

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