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Development of a probe consist of three cosmids to enumerate the chromosome 13 on uncultured lymphocytes or amniocytes using interphase FISH

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Abstract

Background: To produce a reliable probe suitable for aneuploidy detection of chromosome 13 on uncultured lymphocytes and amniocytes by fluorescence in situ hybridization (FISH), we used a contig of three overlapping cosmids mapped to 13q12.3.

Methods: The cosmid DNA carrying the expected sequences of human chromosome 13 was isolated from host cells and labelled with biotin-11-dUTP. The hybridization and detection conditions with FITC-Avidin were optimised using a series of cultured and uncultured lymphocytes and amniocytes.

Results: Intensive signals were detected when a combination of three overlapping cosmids was used to enumerate the chromosome 13 on interphase nuclei. An average of 87 and 85.5 percent of interphase cells prepared from lymphocytes and amniocytes showed accurate number of specific signals for chromosome 13.

Conclusion: The results obtained in present study indicate that the probe was capable of detecting the copy number of chromosome 13 on interphase cells prepared from peripheral blood or amniotic fluid cells providing that the uncultured amniotic fluid cells are free of cytoplasmic residues, RNA and protein debris.

Keywords: Chromosome 13, interphase FISH, cosmid-contig

Introduction

Trisomy of chromosomes 13, 18, 21 and aneuploidy of chromosomes X and Y account for 95 percent of chromosomal abnormalities in human populations [1]. Classical cytogenetic techniques can detect the numerical and structural abnormalities of chromosomes [2]. However for prenatal diagnostic applications, diagnosis is labour-intensive and time consuming as it depends on the culture of foetal cells and analysis of metaphase chromosomes, which can be available within 15 days at best [3,4].

Fluorescence in situ hybridization (FISH) with chromosome specific probes can detect the number of copies of a particular chromosome present in interphase nuclei [5-8]. The major advantage of this technique is that there is no requirement for cell culture and hence the results can be available in two days [9]. At present FISH is used as adjunct for classical cytogenetic analysis, as the technique does not allow a diagnosis of certain structural abnormalities [10]. However using the right probes and techniques it is now possible to detect a major chromosome aneuploidies by uni-colour FISH.

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Centromeric probes which typically hybridize to alphoid DNA repeats on centromeric regions of chromosomes are well suited for analysis of aneupoidies in interphase nuclei, as they produce strong and distinct signals [11]. However there is no access to such probes for chromosomes 13 and 21, as they share the same repeat sequences [12]. A number of single copy probes specific for chromosome 13 are commercially available; however we found them ineffective for interphase FISH analysis. For this reason we optimized the use of a cosmid contig composed of three overlapping cosmids to enumerate the chromosome 13 on interphase cells. Cosmid contigs are overlapping cosmid clones specific for a single target DNA, which are stable in use, have high signal-to-noise ratios, acceptable spatial resolution of the fluorescence signals, and exhibit high hybridization and detection efficiencies [13,14]. Using these DNA probes it is possible to detect the major chromosomal aneuploidies present in uncultured amniotic fluid samples in a routine prenatal diagnosis [15]. Quantitative analysis for hybridization of an unselected series of uncultured lymphocytes and amniocytes with the probe showed that the probe can be reliably used for prenatal diagnosis of trisomy 13.

Methods

Sample preparation

Uncultured lymphocytes, from approximately 1 ml of heparinised peripheral blood sample was prepared by hypotonic solution and fixed according to the standard cytogenetics methods. The fixed cells were dropped on to clean microscope slides and air dried. Uncultured amniocytes were prepared according to Liehr et al [16], with minor modifications. 2-3 ml of amniotic fluid sample was centrifuged and suspended in 3 ml trypsin/EDTA and incubated for 15 min at 37°C. After centrifugation, the pellet was resuspended in 5ml of 37.5 mM KCl and incubated at 37°C for 15 minutes. The hypotonic solution was substituted with Carnoy fixative. After spanning, resuspension and incubation at -20°C for 5 min in 3 ml fixative, the cell suspension was separated by a last centrifugation. The supernatant was discarded and the cells were diluted in 200 µl of the remaining supernatant and placed on slide. The target DNA was denatured in 70% formamide/2×SSC for 2-3 minutes. The denatured DNA was immediately transferred into ice cold 70% ethanol, dehydrated through ethanol series (70%, 90% and 100%), air dried and stored at -20ºC until used.

Probe preparation

Three cosmids 29G3, 121F8 and 167H1 kindly provided by Dr. I. S. Edelman, (Colombia Biochemistry University) were cultured in liquid terrific broth medium containing kanamycin. DNA probe was isolated from the amplified host cells using Circle-prep kit. Approximately 1µg of isolated DNA was used for nick translation with biotin-11-dUTP. The labelled DNA was used as probe for FISH after denaturation at 70°C for 10 minutes.

Chromosomal in situ suppression hybridization

Chromosomal in situ suppression (CISS) hybridization and probe detection with fluorescein isothiocyanate (FITC) conjugated to avidin were carried out according to carter et al. [17] with the following modifications: For hybridization 80 ng of each labelled cosmid DNA was used individually or in combination after pre-annealing with 60 ng of human placental DNA. The signals were amplified once by biotinylated antiavidin-D and FITC avidin. Cells were counterstained with 0.4 µg/ml 4,6 diamino-2-phenylindol-dihydrochloride (DAPI) and 0.2 µg/ml propidium iodide in mounting medium AF1 (Citiflour Ltd) and were evaluated with conventional fluorescence microscope. The pictures were prepared using a digital Imaging microscopy (Zeiss Axioscope equipped with a cooled charged device and related software).

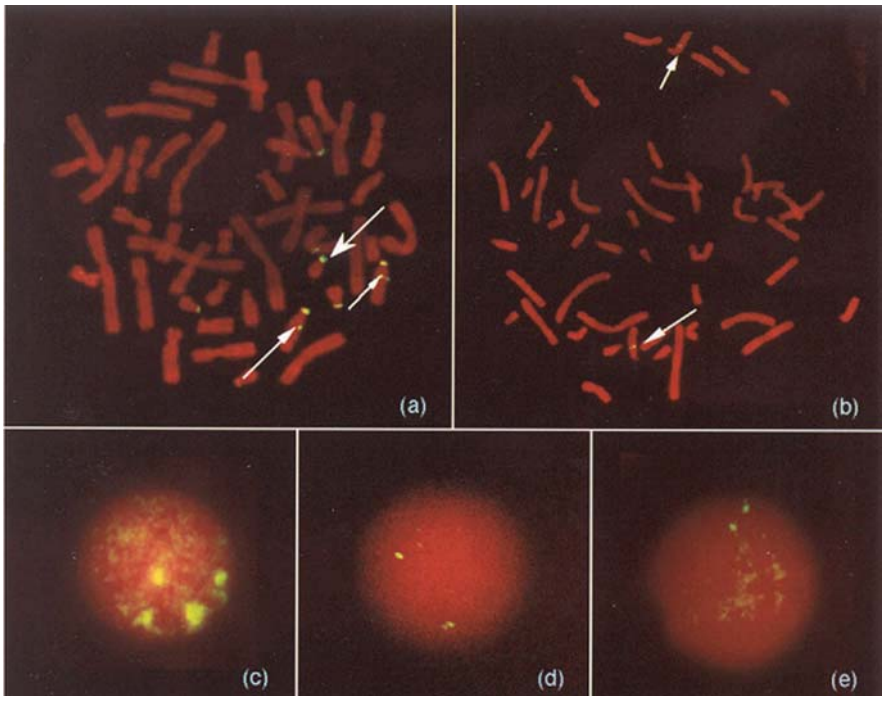

Fig. 1. Illustration of the cross-hybridization of one of the chromosome 13-specific cosmids with other chromosomes. (a) A metaphase spread hybridized with cosmid 29G3, resulted incross-reactivity with centromeric regions ofother acrocentric chromosomes. Small arrows show the specific signals and the large arrows show the cross-hybridization; (b) a metaphase cell hybridized with both 121F8 and 167H1 cosmids. Specific signals with no cross-hybridization (small arrows) on; (c) an interphase nucleus hybridized with 29G3 cosmid showing cross-hybridization with other chromosomes; (d) an interphase nucleus hybridized with cosmid 121F8 and (e) an interphase nucleus hybridized with cosmid 167H1.

Results

To map the probes on their specific targets the cosmids were individually or in combination were hybridized to metaphase spreads prepared from peripheral blood samples. The CISS and sonicated human placental DNA were employed to prevent the cross reactivity of probes with non specific DNA sequences. Hybridization of 29G3 cosmid to cultured and uncultured peripheral blood cells resulted in cross reactivity of the labelled probes with other chromosomes. Figure 1a shows a metaphase cell hybridized with cosmid 29G3. Strong nonspecific signals are observed on the short arm of all acrocentric chromosomes. Four to six signals were observed on most of the uncultured lymphocytes hybridized to the probe as illustrated by Fig. 1c. The signals observed on nonspecific sites were stronger than those on specific targets. No cross hybridization was observed when the cosmids 121F8 and 167H1 were hybridized either individually or in combination on metaphase cells (Fig. 1b). Hybridization of uncultured lymphocytes with the cosmids 121F8 and 167H1 resulted in two signals on each nucleus (Figs. 1d and e). However the signals were not strong enough to be easily distinguished from background fluorescence.

To overcome this problem the cosmid probes were individually cultured in agar plates containing terrific broth. A single colony from each probe was then transferred to a liquid medium and amplified overnight. The DNA probe was then isolated from the amplified cells which had been generated from a single bacterial host cell and labelled with biotin-11-dUTP. Specific signals with no cross hybridization were obtained when each of the three cosmids or the contig composed of the three cosmids were hybridized to normal cultured or uncultured lymphocytes (Fig. 2a-d). The hybridization condi-

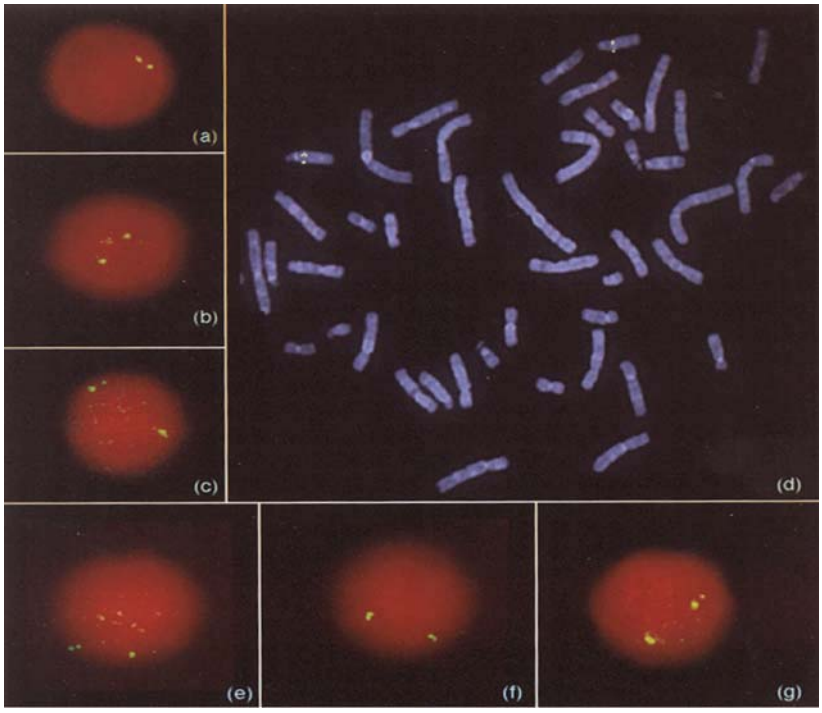

Fig. 2. Metaphase and interphase cells are hybridized with the cosmids and the cosmid contig specific for chromosome 13. (a-c) Uncultured lymphocytes hybridized with cosmids 29G3, 121F8 and 167H1 respectively; (d) a metaphase spread hybridized with the cosmid contig composed of all three cosmids; (e) an uncultured lymphocyte simultaneously hybridized with cosmids 121F8 and 167H1; (f) an uncultured lymphocyte hybridized with the contig; and (g) an uncultured amniocyte hybridized with the contig.

tions were optimised using various concentrations of probe and competitor. Specific signals with the least background fluorescence were produced using 80 ng of each probe and 5μg of competitor DNA.

Hybridization with the individual cosmids 29G3, 121F8 and 167H1 produced two signals on an average of 75%, 69% and 71% among the hybridized normal interphase cells respectively (Fig.2a-c). Hybridization with the combination of cosmids 121F8 and 167H1 resulted in two signals on an average of 79 percent of hybridized cells (Fig. 2e). An average of 87 percent of the hybridized cells displayed two signals when the contig composed of the three cosmids was hybridized (Figure 2f). The signals produced by a combination of all three cosmids were intense and could be clearly detected on the long arm of chromosome 13 (13q12) or on uncultured lymphocytes using a conventional fluorescence microscope. These experiments revealed the suitability of the cosmid contig for analysis of chromosome 13 copy number on uncultured lymphocytes.

For prenatal detection of chromosome 13 copy number, an unselected series of 50 uncultured amniocytes was hybridized with the contig. The analysis was carried out by scoring a minimum of 50 nuclei for each sample. Cells covered by cytoplasm, and degenerate and clumped cells were not counted. Four of the 50 samples could not be analysed as most of the cells were degenerate or were covered by cytoplasm. All the remaining 46 samples were detected since normal and this was subsequently confirmed by the cytogenetic results. Figure 2g shows an uncultured amniocyte displaying two signals specific for chromosome 13. An average of 85.5 percent of the hybridized cells produced two signals, 2.2 percent of the cells showed no signal, 8.5 per cent showed one signal and 3.8 percent showed three signals.

Discussion

The accuracy, reproducibility and reliability of analysis by FISH depend on various factors. However specificity, size and complexity of the probes used are among the factors which can most significantly affect the hybridization and efficiency of detection [18]. Centromeric repeat probes are suitable for detection of aneuploidies in interphase nuclei, as they generate strong and distinct signals [19,20]. However, there are difficulties with use of these probes for some chromosomes. In general, the significant polymorphisms that characterize most of the repetitive sequences influence the signal size and can lead to misdiagnosis [21]. A centromeric repeat probe (L1.26) which hybridizes to both chromosomes 13 and 21 is available [22], but it is difficult to use for interphase analysis since it cannot distinguish between the chromosomes 13 and 21 and thus twice the number of signals on the interphase cells. Furthermore the centromeric position of the probe does not allow identification of Robertsonian translocations [23].

Cosmid probes commonly contain 25-45 kb of cloned insert DNA and are efficient probes which yield highly specific signals on metaphase chromosomes. However they result in weak signals when hybridized to uncultured amniocytes [24]. Using a contig of these DNA probes it is possible to detect major chromosomal aneuploidies presented in uncultured amniotic fluid samples in a routine prenatal diagnosis [25]. We developed a contig of three overlapping cosmids hybridizing to 13q12.3 and used as a probe to enumerate the chromosome 13 on interphase cells. One of the cosmids (29G3) showed cross-hybridization with short arms of the other acrocentric chromosomes. This problem was thought to be originated in the presence of bacterial host cells carrying other DNA sequences in the same glycerol stock. Isolation of a single colony of bacterial cells originating from a single cell and amplification of the isolated cells in a liquid medium was a possible solution for this difficulty. However picking a colony from the other cell type was another possible error in experiment. Using this method, specific fluorescent signals were produced on the long arm of chromosome 13. The signals generated by cosmid 29G3 were stronger than those produced by the two other cosmids. A greater intensity of signals was obtained when a combination of the three overlapping cosmids was used to enumerate the chromosome 13 on interphase nuclei. Using a combination of the three cosmids, a range of 87-91 percent of uncultured lymphocytes resulted in two specific signals for chromosome 13. The detection efficiency was in the range of 82-88 percent when the uncultured amniocytes hybridized with the contig.

Conclusion

Our results indicated that the probe developed in this study was capable of detecting the copy number of chromosome 13 on interphase cells prepared from peripheral blood or amniotic fluid cells providing that the uncultured amniotic fluid cells were in high quality.

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