Development of an Alu-PCR Amplified YAC Probe Suitable for Enumeration of Chromosome 13 on Uncultured Lymphocytes and Amniocytes by Fluorescence in situ Hybridization

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Abstract

The main objective of the present study was to develop an efficient and reliable probe to be routinely used for detection of chromosome 13 copy numbers by interphase FISH. To achieve this, a Yeast Artificial Chromosome (YAC) containing sequences specific for human 13q12 (744D11), was cultured and the whole yeast genomic DNA was extracted. The human insert within the isolated DNA was amplified by inter-Alu PCR reaction and the PCR product was used as probe to detect the copy number of chromosome 13 using FISH experiments on 150 uncultured amniotic fluid samples after labelling with biotin or digoxigenin. Using this method 97 percent or more of the randomly evaluated cells from uncultured blood samples and 93 percent of the uncultured amniocytes showed two distinct signals on each cell. The intensity of signals was comparable to those of chromosome-specific alphoid DNA probes. The results suggest that this approach can be reliably used for prenatal detection of chromosome 13 aneuploidy.

Keywords: Trisomy 13; YAC clone; FISH; Alu-PCR

Introduction

Unique targets in the genome can be detected by complementary single copy probes in metaphase and interphase cells [1]. This type of probes has been important for a broad spectrum of cytogenetic and molecular genetic experiments. Moore et al have used the single copy probes to detect the numerical alterations in metastatic melanoma by a DNA fluorescence in situ hybridization probe panel and array comparative genomic hybridization [2]. Laureys et al have employed these type of probes to identify the breakpoint-flanking markers on chromosomes 1 and 17 of a constitutional translocation T (1;17) (p36;q12-21) in a patient with neuroblastoma [3]. In clinical genetic applications, they are valuable tools to detect numerical and structural abnormalities such as deletions, inversions and translocations. In a prospective study of

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4000 amniotic fluid samples, postal et al used the single copy probes and the multiplex ligation-dependent probe amplification method for aneuploidy detection [4]. A similar study was carried out to detect the chromosomal abnormalities in neonates by Lin et al[5].

There is a fairly good correlation between hybridization efficiency and the size of the target DNA. When the size of the target DNA detected by a given probe decreases (e. g. using probe fragments cloned in phage or plasmid vectors), the percentage of successfully delineated target sites also decreases [6]. With probes containing approximately 2 kb single-copy sequences, a maximum of 40% to 50% of all target sites can presently be detected [7]. Although single-copy DNA fragments of about 1 kb have been successfully mapped by fluorescence *in situ* hybridization [8], the discrimination of weak specific signals against background dots is likely to require statistical analysis [9]. Since the size of DNA segments that can be carried in various types of vectors is different [10], the detection efficiency of probes hybridizing to the single copy targets depends on the vector type used for cloning [11]. The two main types of vectors which have been widely used to produce single copy probes are cosmids and yeast artificial chromosomes (YACs) [12].

Cosmids are hybrid vectors, derived from plasmids and λ phages facilitate genomic cloning which is able to carry up to 45 kb of foreign DNA and are efficient probes which yield highly specific signals on metaphase chromosomes. But they result in weak signals when hybridised to uncultured amniocytes [13,14].

Cosmid contigs which are overlapping cosmid clones specific for a single target DNA can overcome the limitations of single cosmids to some extend since they have acceptable spatial resolution of the fluorescence signal [15]. However, we found them unsatisfactory for prenatal enumeration of chromosome 13, as the detection efficiency was relatively low and mainly dependent on quality of uncultured amniocyte preparations (under publication).

Yeast artificial chromosomes (YACs) with the ability to carry several kilo bases of exogenous DNA have vastly improved the strategies of isolation and cloning of genes important for human disease [16,17], as well as the construction of physical maps of regions of complex genomes [18,19,20]. These probes have been successfully used for prenatal detection of aneuploidies on uncultured lymphocytes and amniocytes [21, 22]. In the present study, we used the Alu-PCR product of a YAC clone to specifically detect the copy number of chromosome 13 on uncultured lymphocytes and amniocytes. Our results indicate that the fluorescence signals produced by the probe are comparable to those of centromeric probes with high hybridization and detection efficiency.

Materials and Methods

Sample Preparation

Metaphase and interphase cells of peripheral blood samples were prepared according to the standard cytogenetic methods. Uncultured amniocytes were prepared according to Liehr et al [23], with minor modifications. At first, 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 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 preparation was finalized with 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 in ice cold 70% ethanol, dehydrated through ethanol series (70%, 90% and 100%), air dried and stored at -20ºC until used.

Probe Preparation

Yeast genomic DNA was extracted from cultured yeast cells in selective AHC medium according to the procedure described by Sherman et al [24]. Two Alu primers: BK-33 (5'-CTGGGATTACAGGCGTGAGC-3') priming to the 5' end of the Alu consensus sequences (nt positions 15-34) and SR1 (5'-CCACTGCAC TCCAGCCTGGGG-3') close to the 3' end (nt position 241-261) [25] were then used to selectively amplify the chromosome 13 specific DNA sequence inside of the YAC 744D11. The PCR assay was performed as described by Lengauer et al [26] with minor modifications. 100 ng of the primer was each at a concentration of 0.25 μM in a total volume of 50 μl PCR buffer containing of 250 μM of each of the four dNTPs, and 2.5 units of Taq DNA polymerase (perkin-Elmer/cetus). After an initial denaturation at 96°C for 5 min, 30 cycles of PCR were carried out with denaturation at 96°C for 1 min, annealing at 37°C for 30s and extension at 72°C for 6 min. A 10 min extension was performed at the end of the last cycle.

Ten microlitre aliquots of amplified DNA sequences were fractionated by electrophoresis in a 1.3% agarose gel in1x T.B.E (0.9 M Tris-HCL, 0.9 M boric acid and

20 mM EDTA). PCR products were ethanol precipitated, dissolved in TE (10 mM Tris-HCL, 1mM EDTA, PH 8), and used for nick translation with biotin-11-dUTP. Prior to hybridization, the labelled probe DNA was denatured in hybridization buffer at 70-75°C for 10 minutes.

Chromosome 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. [27] with the following modifications: For hybridization 100-150 ng of Alu-PCR amplified YAC DNA was used as probe after pre-annealing with 100 ng of human placental DNA. The hybridisation areas were detected by FITC-Avidin and the signals were amplified once using biotinilated anti-avidin D and FITC-Avidin as a second layer. The hybridised cells were counter stained 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 signal counting was carried out in a blind fashion and the results were compared to the cytogenetic findings. A minimum of 50 interphase cells were analysed for each sample. The cut-off scheme employed by Lier et al [28] was used to differentiate between normal and trisomic samples for chromosome 13. A sample was considered as normal if 93% or more of cells showed two specific signals. A trisomy 13 result was reported where a minimum of 87% of cells demonstrated three signals on each cell. Samples simultaneously showing one, two or three signals on a minimum of 40%, 49% and 21% of cells respectively, were defined as mosaics. Data were analyzed using chi-square test and SPSS v.15 as statistical software. A P-value of less than 5% was defined as the significant level.

Results

The labelled probe was initially hybridised to normal metaphase preparations from peripheral blood using CISS hybridisation protocols. Specific and intensive signals with minimum of background fluorescence were produced on the long arm of chromosome 13 (13q12) in all of the metaphase cells hybridised with the labelled probe. Figure 1-a show a metaphase cell from cultured peripheral blood sample hybridised to this probe. Similar results were achieved when the probe was hybridised to a number of normal uncultured lymphocytes (Fig. 1-b). A minimum of 50 nuclei was analysed for each hybridisation area. More than 96 percent of the hybridized cells displayed two signals specific for chromosome 13.

The probe was then used to enumerate the copy number of chromosome 13 on uncultured amniocytes prepared from 150 amniotic fluid samples and studied under a fluorescence microscope. A minimum of 50 interphase cells were analysed for each sample and the results were compared to those obtained from standard cytogenetic technique. The signals observed on hybridised cells were intense and could be readily detected by direct microscopy observation (Fig. 1-c). An average of 93 per cent of the cells displayed two signals specific for chromosome 13 on 149 samples. A single sample demonstrated 3 signals in 47 cells out of 50. No false positive or false negative results were detected.

Discussion

Prevention of Trisomy 13 with an incidence of 1 in 5000 live births [29] by prenatal diagnosis is currently part of the health care programs in most countries. Conventional cytogenetic techniques are the most commonly used procedures for detection of chromosome abnormalities, however for prenatal diagnosis it is time consuming, as it needs 12-15 days to report a result.

Using interphase fluorescence in situ hybridisation methods it is possible to achieve the results within 24 hours, as there is no need for cell culture. However the accuracy of the results depends on the detection efficiency of the probes.

The centromeric probes are suitable for detection of aneuploidies by interphase FISH, but there is no access for a chromosome 13 specific centromeric probe, as it share the same nucleotide sequence as chromosome 21 and hence double the signal numbers on interphase cells. Unique-sequence probes can be an alternative, providing that the insert size within the vector is long enough to produce intense signals with high detection efficiency. There are a number of commercially available probes specific for chromosome 13 which can be used for interphase FISH, however they are extremely expensive and the efficiency of probes may decrease in transfer. The major objective of the present study was to develop a specific probe for chromosomes 13 with high hybridisation and detection efficiency and reproducible in lab to avoid waiting for probes for long times and to ensure that the efficiency of the probes does not decrease due to inappropriate transfer in to the lab.

At the time that the present study was started it had

been shown that DNA fragments several kb long can be successfully cloned in yeast artificial chromosomes (YACs). Several studies had also successfully used the YAC clones for gene mapping [30] and detection of aneuploidies on uncultured lymphocytes and amniocytes [31,32] by *in situ* hybridisation methods. According to these knowledge we used the 744D11 YAC clone to produce our own probe. Through the present study the whole process of the YAC clone culture, DNA isolation, Alu-PCR amplification and DNA labelling were done in our lab. The labelled DNA was then used as probe to detect the copy number of chromosome 13 on uncultured lymphocytes and amniocytes in a prospective study. The probe that was produced in this study revealed to be highly specific with a detection efficiency of 96 percent in uncultured lymphocytes and 93 perceent in uncultured amniocytes which are comparable to the similar commercial probes. The cost of the probes per reaction was also significantly reduced. These results indicate that the 744D11 YAC can be reliably used for enumeration of chromosome 13 by interphase FISH, however more studies is required to reveal its suitability for detection of mosaic form of trisomy 13.
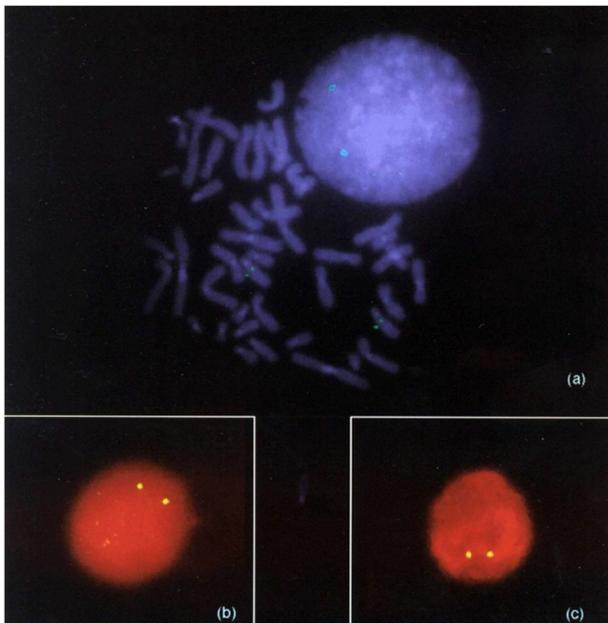

Figure 1. (a) A metaphase spread prepared from cultured lymphocytes hybridized with the biotin labeled 744D11 YAC, (b) An uncultured lymphocytes hybridized with the biotin labeled probe, (c) An uncultured amniocytes hybridized with the biotin labeled probe.

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