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Cytogenetical diagnosis in paraffin-embedded fetoplacental tissue using comparative genomic hybridization

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Comparative genomic hybridization (CGH) is a FISH-related technique used to assess global chromosomal aberrations in a variety of human tumours. Recently CGH has been applied to cytogenetic analysis of fresh frozen fetoplacental tissues. Here we report the application of CGH to paraffin-embedded placental samples. Ten samples from paraffin-embedded blocks of 6 control placentas and fetoplacental tissue from 10 aneuploidies, and 2 unbalanced aberrations were evaluated. Balanced karyotype profiles were obtained from samples of healthy placentas and all samples from the same placenta appeared to have similar confidence intervals. CGH analysis of four cases of trisomy 21, three cases of trisomy 18, one case of trisomy 13, one case of trisomy 15 and one case of trisomy 7 all showed overrepresentation of the respective trisomic chromosome. The CGH profile was also in accordance with the karyotyping of a case with isochromosome 21. The CGH profile of a case with der (2)t(2;6)(q37.3;q22.2) revealed partial trisomy for chromosome 6 between q21 and q27. CGH may be a useful adjunct in prenatal genetic diagnosis when retrospective diagnosis is needed from archival samples. Copyright © 2000 John Wiley & Sons, Ltd.

KEY WORDS: comparative genomic hybridization; aneuploidy; fetal karyotype

INTRODUCTION

In prenatal diagnosis, conventional Giemsa banding of metaphase chromosomes and fluorescent *in situ* hybridization (FISH) are the most widely used techniques for detecting genetic aberrations. While FISH can be applied in situations where only interphase cells are available, with interphase FISH, only a few regions of the genome can be explored at a time necessitating a prediction of expected cytogenetic abnormalities based on phenotype.

Comparative genomic hybridization (CGH) is a molecular cytogenetic approach using ratio-imaging FISH for detection of unbalanced chromosomal aberrations (Kallioniemi *et al*., 1992). It has mostly been used as a research tool for evaluation of human solid tumours where access to metaphase spreads is often limited. The technique uses DNA extracted directly from the tissue to be analysed and therefore circumvents the need for metaphase spreads and cell culture. A mixed probe of labelled test genomic DNA and an equimolar amount of differently labelled reference DNA prepared from cells with normal chromosome complements is used for chromosomal *in situ* suppression hybridization to normal metaphase spreads. Hybridized test and control DNA sequences are detected with different fluorochromes. The ratios of fluorescence intensities reflect the relative copy number in the test genome compared with the control genome. Thus, CGH provides the possibility for searching the whole genome for unbalanced genetic material in a single hybridization reaction.

The use of CGH for fresh frozen fetoplacental samples has been reported in a few studies (Bryndorf *et al.*, 1995; Daniely *et al.*, 1998;1999; Yu *et al.*, 1997). However, the applicability of this technique in paraffin-embedded fetoplacental tissue has not been evaluated. A policy of genetic analysis is recommended for recurrent abortions and all stillbirths to avoid parental anxiety, unnecessary diagnostic tests and to provide appropriate counselling of the parents for future pregnancies (Wolf and Horger 1995; ACOG Committee Opinion No. 178, 1996). Work-up for recurrent pregnancy losses is usually started after two or three losses from which fresh fetoplacental tissue is no longer available for culture. CGH may be a potentially powerful method for detection of prenatal cytogenetic aberrations when retrospective karyotyping is needed, i.e. in cases of recurrent abortion and fetal demise.

The purpose of this study was to evaluate the applicability of CGH in paraffin-embedded fetoplacental samples for genetic analysis.

MATERIAL AND METHODS

Tissue from paraffin-embedded blocks of six term placentas and 12 samples of abortion material diagnosed with aneuploidies or unbalanced aberrations by conventional karyotyping were evaluated by CGH. A total of 10 biopsies from chorionic villi of six healthy term pregnancies collected prospectively were classified as the normal group. Though conventional karyotyping of these six cases was not done, they were

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assumed to be normal since the pregnancy history and the newborns were normal. Fetoplacental samples with chromosomal aberrations were provided from second trimester therapeutical terminations carried out over the past six years. There were 10 cases with aneuploidies, four with trisomy 21 and three with trisomy 18, one each with trisomy 7, trisomy 15 and trisomy 13; and two cases with unbalanced aberrations. The latter included one case with isochromosome 21 in addition to a normal chromosome 21 and one case with translocation der(2)t(2;6)(q37.3;q22.2).

The biopsy materials were fixed in buffered formalin and embedded in paraffin. Hematoxylin eosin stains were evaluated for each sample to assess the presence of decidual tissue microscopically. Samples with minimal or no decidual contamination were selected and the decidua was removed from those with decidual contamination by a scalpel after marking the area under a microscope in order to have as high a fetal/maternal tissue origin ratio as possible.

Placental DNA samples were prepared from a total of 5–10 tissue slices each 30 μm thick, that were deparaffinized with xylene 3×10 min and ethanol 100% 2×10 min. The tissue was incubated overnight in 1 ml of 1 mol/l sodium thiocyanate, and then digested with 100–200 μl proteinase K from 10 μg/μl stock for 3–5 days at 55°C prior to DNA extraction with phenol/chloroform/isoamylalcohol and ethanol precipitation.

Reference genomic DNA was prepared from healthy male peripheral blood according to standard protocols. The amounts of placental and reference DNAs were measured by means of a dsDNA fluorescein dye (PicoGreen™, Molecular Probes Incorporation, Oregon) on a fluorescence plate reader. Normal metaphase spreads were prepared according to standard protocols from phytohaemagglutinin-stimulated peripheral blood lymphocytes of a karyotypically normal male using standard procedures of hypotonic treatment and methanol/acetic acid fixation. Test or reference DNA samples were indirectly labelled by nick-translation with biotin-11-dUTP or digoxigenin-11-dUTP, respectively. The length of the placental or reference DNA was checked by gel electrophoresis. For optimum hybridization conditions, DNA molecules should be 500–1500 bp in length.

A total of 500 ng of test and 500 ng of reference DNA were combined with 50 μg human cot-1 DNA and 20 μg of salmon sperm DNA and were precipitated with ethanol. The DNA was lyophilized and resuspended in 70% deionized formamide, $4 \times$ SSC, 20% dextran sulphate, denatured by incubation at 75°C for 5 min and the DNA then was reannealed at 37°C for 20–30 min. Normal human chromosome spreads on pretreated slides were denatured in a solution of 70% deionized formamide, $2 \times$ SSC at 72°C for exactly 1 min 40 sec. The slides were dehydrated with graded ethanol series and air-dried. The hybridization mixture (12 μl) was applied to the slides, the edges were sealed with rubber cement, and the slides were incubated for 72 h in a moist chamber at 37°C.

After washing off the coverslips, specimens were agitated in prewarmed 50% formamide, $2 \times$ SSC at 42°C three times, 5 min each. A second wash was done with $0.1 \times$ SSC at 60°C, agitating for 5 min (three times) and the slides were incubated with blocking solution (3% BSA, $4 \times$ SSC, 0.2 Tween 20) for 30 min at 37°C. The detectors, avidin FITC (providing green fluorescence for test DNA) and anti-digoxigenin rhodamine (providing red fluorescence for reference DNA) diluted in detection buffer were applied and the slides were incubated at 37°C for 45 min before counterstaining with DAPI for 3 min. After air drying, antifade was applied.

Fluorescence signals were visualized by conventional epifluorescence microscopy (Olympus AX70 Provis). The whole hybridization field was scanned to select the best metaphase spreads on the slide to which extracted control and test DNA are hybridized, and an average of 10–15 images from each slide were digitized using a CytoVision™ computerized imaging system equipped with a COHU monochrome cooled charged-coupled device camera. DAPI, FITC and rhodamine images were visualized using filter sets selective for each of the fluorochromes. Exposure times for each fluorochrome were chosen in such a way that results in maximum pixel values equalling half of the dynamic range of the camera. All optical settings, as well as exposure times, were kept constant for images obtained in a single case.

The image representing the DAPI stain was inverted and used for semi-automated karyotyping. Green and red fluorescence intensities were determined from each chromosome from *p*-telomere to *q*-telomere by integrating intensities at 1-pixel intervals along the chromosome medial axis. After background correction and normalization of the green–red ratio for each entire metaphase to 1.0, green–red intensity profiles were calculated for all chromosomes.

Data from all images were combined, and an average ratio profile for each chromosome was calculated. Profiles at the centromeres and close to the telomeric regions are excluded from analysis since *in situ* suppression prevents reliable hybridization in these regions. Trisomies and monosomies were defined as a green–red intensity ratio of 1.25 and 0.75, respectively. These thresholds were used also for partial under-representation or over-representation of chromosomes.

RESULTS

The results of the CGH studies are summarized in Table 1. Balanced karyotype profiles were obtained from samples of six normal placentas. The width of the confidence intervals appeared similar in all cases. In all 10 cases with a trisomic chromosome identified by fetal chromosome analysis, CGH was able to correctly show the overrepresentation in the trisomic chromosome. A typical example of a fetoplacental trisomy 21 is shown in Figure 1. In each of the additional cases exhibiting chromosomal anomalies, the ratio for the

Table 1—Results of the CGH profiles and conventional cytogenetics

Number	Karyotype	CGH result
1	Normal female karyotype	Balanced
2	Normal female karyotype	Balanced
3	Normal female karyotype	Balanced
4	Normal female karyotype	Balanced
5	Normal male karyotype	Balanced
6	Normal male karyotype	Balanced
7	Normal male karyotype	Balanced
8	Normal female karyotype	Balanced
9	Normal female karyotype	Balanced
10	Normal male karyotype	Balanced
11	47, XY, +21	47, XX, +21
12	47, XX, +21	47, XX, +21
13	47, XX, +21	47, XX, +21
14	47, XX, +21	47, XX, +21
15	47, XX, +13	47, XX, +13
16	47, XX, +18	47, XX, +18
17	47, XY, +7	47, XY, +7
18	47, XY, +18	47, XY, +18
19	47, XX, +18	47, XX, +18
20	47, XY, +15	47, XY, +15
21	46, XY, i (21)(q10)	46, XY, i (21)(q10)
22	46, XX, der(2)t(2;6)(q37.3;q22.2)	46, XX, partial trisomy at 6q16.2–23.2 and 6q25.1–27

trisomic chromosome was above the cut-off ratio and typical examples of the CGH profile of the respective chromosomes are presented in Figure 2. The CGH profile showed a gain of chromosome 21 DNA similar to the conventional karyotype for case 21 with 46, XY, 21 i(21)(q10). The CGH profile revealed a trisomic area from 6q16 to 6q27 with a short segment at 6q24-25 that fell just below the 1.25 intensity ratio chosen to define trisomies. The deletion at 2q37.7 was not revealed; however, the telomeric regions were not included in the analysis for the reason mentioned above.

DISCUSSION

In this study, we evaluated 22 fetoplacental tissue samples from paraffin-embedded blocks of which 10 were from healthy term pregnancies and 12 were from abortion material with an abnormal karyotype. In all cases from healthy pregnancies, balanced profiles were obtained. In all 10 trisomies, the CGH profile revealed the presence of the anticipated extra chromosome.

There are certain limitations of CGH. First, the technique is valid only for unbalanced aberrations. The sensitivity for partial gains and losses are around the range of 10–15 mb, thus smaller aberrations are not detectable by CGH (Kalousek, 1998). Mosaicism is also difficult to detect by CGH since DNA extraction pools the genetic material from the normal and abnormal cells. Finally, the procedure is

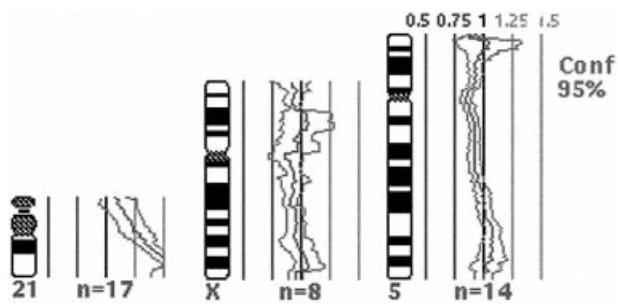

Figure 1—Chromosomes 21, X and 5 from a trisomic karyotype. (Case no. 11: 47 XY, +21)

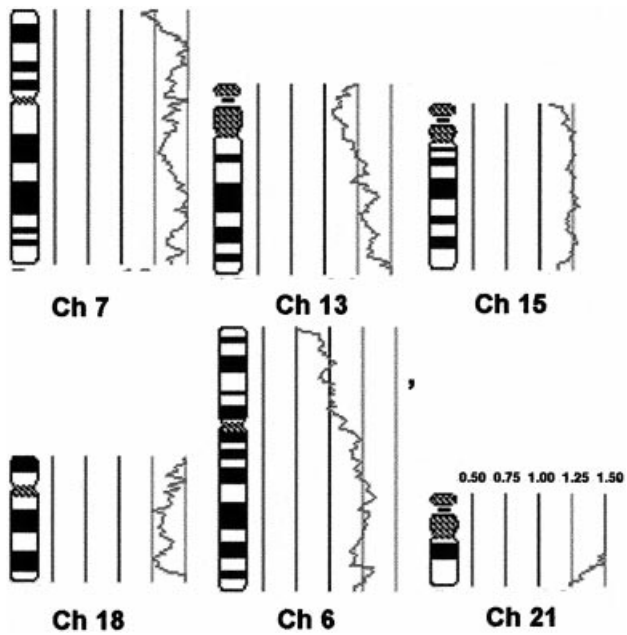

Figure 2—CGH profile of the trisomic chromosomes 7, 13, 15, 18 and chromosome 6 in 46,XX,der(2)t(2;6)(q37.7 : q22.2)

extremely dependent on the test DNA quality and the quality of target metaphase spreads.

The efficacy of CGH in revealing aneuploidies as shown in this study is promising in prenatal diagnosis. Recent reports show that for first trimester losses the percentage of genetically abnormal embryos may be as high as 69–83% and the majority of these are aneuploidies (Strom *et al.*, 1992; Ohno *et al.*, 1991). For recurrent abortions, the data indicate that certain couples are at risk for repeated trisomic conceptions (Hassold *et al.*, 1980; Warburton *et al.*, 1987). There is growing evidence that karyotype analysis of the conceptus is beneficial and is even a better strategy than karyotypic analysis of parental blood in this group of patients (Wolf and Horger, 1995; ACOG Committee Opinion No. 178, 1996; Kalouset 1998; Ford *et al.*, 1996). The frequency of cytogenetic abnormalities is reported as 5–10% among stillbirths with the majority being trisomies and monosomy X. Furthermore, the cost of CGH is within a reasonable range compared with other cytogenetical methods using *in situ* hybridization. We estimated an approximate cost of US $150 for one assay including the chemicals and the bench work material.

Regardless of the important contribution of chromosomal anomalies to fetal losses and the recommendation for genetic diagnosis, the clinical reality is that most specimens are not sent for chromosomal analysis. CGH analysis of archival material in those cases might be a useful adjunct to guide the management of future pregnancies.

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