OUTSTANDING CONTRIBUTION

Detection of chromosomal aberration in fetuses arising from recurrent spontaneous abortion by comparative genomic hybridization

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Chromosomal abnormalities (mostly aneuploidy) account for ~50% of fetal losses in the first 8–15 weeks of gestation. Cytogenetic analysis of aborted fetal material depends on conventional tissue culturing and karyotyping. This technique is laborious and is subject to problems including external contamination, culture failure and selective growth of maternal cells. Comparative genomic hybridization (CGH) was used to determine the chromosomal constitution of 27 fetuses arising from recurrent spontaneous abortion. In 12 samples, the CGH results were compared to the results obtained by conventional cytogenetic techniques. Correlation was found in 75% of samples. Overall, CGH detected chromosomal abnormalities in 48% of the samples, including trisomies, monosomies, and partial chromosome gains and losses. The preliminary data in this study show that CGH can be added, at least as a complementary method, to the traditional cytogenetic techniques used in the investigation of recurrent spontaneous abortions.

Key words: chromosomal abnormalities/comparative genomic hybridization/conventional cytogenetics/recurrent spontaneous abortions

Introduction

Chromosomal abnormalities (mostly aneuploidy) account for ~50% of fetal losses between the first 8–15 weeks of gestation (Byrne and Ward, 1994; Abruzzo and Hassold, 1995). The cytogenetic findings in different spontaneous abortions occurring in the same couple were found to be non-random in several studies (Hassold, 1980; Drugan et al., 1990; Eiben et al., 1990). This is particularly valid for trisomies, and for other aneuploidies as well. This phenomenon cannot be explained by gonadal mosaicism in one of the parents, as the risk for having another trisomic conception is not restricted to a particular chromosome (Hassold, 1980).

Chromosomal abnormality in one of the parents can be found in up to 5–7% of couples who experience multiple spontaneous abortions. These couples are at high risk for having chromosomally unbalanced offspring and they are advised to have a prenatal diagnosis for future pregnancies (Abuelo and Barsel-Bowers, 1983; Drugan et al., 1990). However, when normal karyotype is found in such couples, the possibility of prenatal diagnosis relies almost exclusively on cytogenetic analysis of the previously aborted fetus. Therefore, karyotyping of the abortuses is extremely important for prognostic reasons (Eiben et al., 1990).

Cytogenetic analysis of aborted fetal material depends on conventional tissue culturing and karyotyping. This technique is laborious and subject to problems such as external contamination, culture failure and selective growth of maternal cells (Eiben et al., 1990). Comparative genomic hybridization (CGH) is a new molecular–cytogenetic assay capable of detecting chromosomal gains and losses by fluorescence in situ hybridization (Kallioniemi et al., 1992). In this assay, two differentially labelled genomic DNAs (test and reference) are hybridized to normal human metaphase spreads with the presence of unlabelled repetitive DNA (Cot-1 DNA). Differential fluorescent hybridization signals represent gains and losses of the test DNA relative to the reference DNA. The ratio between test and reference DNAs is quantitated and analysed by using a digital image analysis system (du Manoir et al., 1993; Holdsworth and Chaganti, 1994; Bryndorf et al., 1995). This technique bypasses the technical problems associated with tissue culturing, since it depends on DNA isolation from samples to be analysed rather than on preparation of metaphase spreads. It provides a whole genome screen for unbalanced aberration, and can detect the origin of extra or missing chromosomal material (Bryndorf et al., 1995).

CGH has been applied mostly in the field of cancer genetics, but can also be used in clinical cytogenetic laboratories (Bryndorf et al., 1995).

In the present study, the CGH technique was applied to determine the chromosomal constitution of fetuses arising from recurrent spontaneous missed abortions.

Materials and methods

Study population

Twenty-seven abortions were evaluated. The parents of all these abortuses had previously experienced at least two spontaneous abortions. The aetiology accounting for these abortions was undetermined. Of these 27 abortions, 12 were successfully cultured and analysed both by CGH and conventional cytogenetic techniques. In the other 15 abortions, the determination of the chromosomal constitution relied on CGH analysis alone.

Tissue samples

Chorionic villus and placental samples of spontaneously aborted fetal material were obtained from the Department of Obstetrics and...
Conventional cytogenetic processing

Tissue samples were cultured on coverslips in Petri dishes containing 5 ml RPMI medium supplemented with 20% fetal calf serum (FCS), 1% streptomycin/penicillin and 1% glutamine. Colchicine was added 4 h before the cytological preparation and samples were processed using standard techniques. All specimens were G-banded using trypsin–Giemsa. Samples were visualized under a light microscope (Zeiss, Axioscope; Jena, Germany). A minimum of 20 cells was scored and five cells were analysed per sample.

Genomic DNA probes and labelling procedure

High molecular weight genomic DNA was prepared from each sample by proteinase K and RNase digestion followed by phenol–chloroform–isoamylalcohol extractions according to standard protocols (Sambrook et al., 1989).

Reference DNA was extracted from peripheral blood of karyotypically normal males, using standard protocols (Sambrook et al., 1989). A 1 µg sample from each test and reference DNA was directly labelled with Spectrum Green dUTP and Spectrum Red dUTP (Vysis) respectively by nick translation. The labelled DNA was denatured in 70% formamide and 2 M NaOH for 2 min, followed by 0.1% Nonidet P-40 in 2×SSC for 2 min and dehydrated in a series of ice-cold ethanol.

Equal amounts of Spectrum Green (Vysis) labelled test DNA and Spectrum Red (Vysis) labelled reference DNA (1 µg each) and 40 µg of unlabelled Cot-1 DNA (GIBCO BRL Life Technologies, Inc., Gaithersburg, MD, USA) were mixed and ethanol precipitated and then resuspended in 14 µl hybridization buffer (50% formamide and 10% dextran sulphate in 2×SSC). This probe mixture was denatured for 5 min in 75°C, pre-annealed for 30 min in 37°C and then placed on slides with the denatured chromosomes. A glass coverslip was added and sealed with rubber cement. Hybridization took place in a moist chamber at 37°C for 72 h. Slides were washed in 0.4×SSC at 75°C for 2 min, followed by 0.1% Nonidet P-40 in 2×SSC for 2 min at room temperature. Target chromosomes were counterstained with 4,6-diamino-2-phenylindole (DAPI) (Sigma, St Louis, MO, USA) and mounted with Vectasheild (Vector Laboratories, Burlingame, CA, USA).

Digital image analysis

CGH results were analysed using an epifluorescence microscope (Zeiss Axioscope, Germany) equipped with 100 W mercury lamp and a cooled charge-coupled device camera (Photometrics, Tucson, AZ, USA) controlled by a Cytovision image analysis system (Applied Imaging). Analysis was performed as described previously (Lundsteen et al., 1995). Briefly, 10–15 metaphases were chosen for image analysis with high fluorescence intensity and uniform hybridization. Gray-level images were captured separately for each fluorochrome (DAPI, Spectrum Green and Spectrum Red). Karyotyping was performed based on DAPI banding by inverting the DAPI image. Green and red fluorescence intensities were determined along each chromosome medial axis at 1-pixel intervals. Green-to-red fluorescence intensity ratio profiles were calculated after background correction and normalization of the green-to-red ratio for each metaphase to 1.0. Mean ratio profiles for each chromosome were determined after data from all analysed metaphases were combined. Trisomies or partial chromosome gains were defined as having a green-to-red ratio >1.2. Monosomies or partial chromosome losses were defined as having a green-to-red ratio <0.80 (Bryndorf et al., 1995).

Results

Of the 27 samples evaluated, 12 were assessed both by conventional cytogenetic analysis and CGH. In 15 additional samples, chromosomal constitution was determined solely by CGH because of tissue culture failures.

Results of the conventional cytogenetics and CGH analysis performed in 12 samples are summarized in Table I. In nine cases, cytogenetic analysis and CGH analysis were in agreement and consistent. Five samples (M-13, M-19, M-20, M-66, 97-M-6) were found to be chromosomally balanced, both by conventional cytogenetics and CGH. The other four samples (M-21, M-89, M-10, M-16) showed chromosomal aberrations that were detected by both methods. In three samples there was a discrepancy between the two analyses: in two samples (M-14, M-17), conventional cytogenetics revealed a balanced karyotype, whereas CGH showed partial amplification of two different chromosomes (chromosomes 17 and 6). In an additional case (M-90), cytogenetic analysis exhibited a mosaic in which 20% of the cells were trisomic (for chromosome 16), while CGH did not detect this aberration. Overall, CGH revealed an abnormality in 50% (6/12) of the samples.
Investigation of recurrent spontaneous abortions using CGH

Table II. Samples analysed using CGH alone

<table>
<thead>
<tr>
<th>Findings</th>
<th>Sample</th>
<th>CGH analysis</th>
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<tbody>
<tr>
<td>Balanced</td>
<td>M-4</td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td>M-7</td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td>M-12</td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td>M-24</td>
<td>Balanced</td>
</tr>
<tr>
<td>Balanced chromosomal constitution</td>
<td>M-86</td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td>M-87</td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td>M-104</td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td>M-117</td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td>97M-19</td>
<td>Balanced</td>
</tr>
<tr>
<td></td>
<td>M-15</td>
<td>Loss of Y ch.</td>
</tr>
<tr>
<td></td>
<td>M-22</td>
<td>Loss of ch. 19</td>
</tr>
<tr>
<td>Numerical aberrations</td>
<td>M-26</td>
<td>Gain of ch. 22</td>
</tr>
<tr>
<td></td>
<td>M-28</td>
<td>Gain of ch. 8</td>
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<tr>
<td></td>
<td>M-31</td>
<td>Gain of ch. 15</td>
</tr>
<tr>
<td></td>
<td>M-46</td>
<td>Gain of ch. 16</td>
</tr>
<tr>
<td>Structural rearrangements</td>
<td>M-18</td>
<td>Loss of 7q32→qter</td>
</tr>
<tr>
<td></td>
<td>M-23</td>
<td>Loss of 8q23→qter</td>
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<tr>
<td></td>
<td>M-25</td>
<td>Gain of 8q21.3→qter</td>
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<tr>
<td>Partial gains and losses</td>
<td>M-11</td>
<td>Gain of 18cen→pter</td>
</tr>
<tr>
<td></td>
<td>97M-21</td>
<td>Gain of 10p13→q24</td>
</tr>
</tbody>
</table>

as compared with 41.6% (5/12) abnormality displayed by conventional cytogenetics.

Table II presents the results of CGH analysis in the remaining 15 samples, for which no cytogenetic evaluation was possible. In eight samples (53.3%), chromosomal constitution was balanced, and in seven samples (46.6%), amplifications and deletions of various chromosomes were detected.

Analysis of numerical aberrations by conventional cytogenetics and CGH

Numerical aberrations were displayed successfully by CGH in four samples (Figure 1). An agreement between conventional cytogenetics findings and CGH analysis was found in two cases. In one case (M-21), cytogenetic analysis revealed mosaic karyotype 46,XX/47,XX + 18. The percentage of the trisomic cells was 50%. CGH analysis found a complete gain in chromosome 18. The other case (M-89) had a trisomic karyotype 45,X0 (Turner syndrome) as the test DNA indicated one copy of the X chromosome (as in the reference male), and no Y chromosome. In the second case (M-26), a gain was detected along the entire chromosome 22, while all the other regions were within the normal green/red ratio range.

Analysis of unbalanced structural rearrangements and extra chromosomal material by CGH

Four samples with unbalanced structural rearrangements were detected by CGH (Figure 2). One sample (M-16) had a female karyotype with extra chromosomal material on the p-ter arm of chromosome 18. This extra material was suspected to be from the Y chromosome heterochromatic region. A FISH experiment with the Y heterochromatic region probe confirmed this assumption. CGH analysis provided confirmation and a gain in the Y chromosome was found (in this experiment, we used a female DNA as a reference).

In three cases, CGH analysis revealed partial chromosomal gains and losses. In all of these samples, the analyses depended solely on CGH as metaphase spreads could not be obtained. Cytogenetic analyses performed on the parents of these abortuses revealed a balanced structural rearrangement in each couple that could account for the CGH findings. In sample M-18, CGH analysis detected a gain in chromosome 4q26→qter and a loss in chromosome 7q32→qter. Conventional cytogenetic analysis of the parents revealed that the father was a carrier of a balanced translocation 46,XY t(4;7)(q26;q32). We concluded that the CGH analysed abortus inherited from the father the translocated chromosome 7 and a normal chromosome 4. In another sample (M-23), a loss of chromosome 8q23→qter was detected. Cytogenetic analysis of the parents revealed that the father was a carrier of a pericentric inversion of chromosome 8. The abortus was an unbalanced recombinant of this inversion caused by crossing over within the inversion loop during meiosis I. Sample M-25 represented another spontaneously aborted fetus of the same couple. CGH analysis revealed in this case a gain in chromosome 8q21.3→qter as a consequence of the same inversion.

High level of sporadic aneuploid cells revealed by CGH and cytogenetic analysis

Sample (M-10) revealed a high level (35%) of sporadic aneuploid cells by cytogenetic analysis. CGH analysis revealed a disturbed hybridization pattern: many amplifications and deletions in many chromosomes. The same finding was demonstrated even after a second CGH experiment. We assume that these results reflect the unstable chromosome constitution of the tissue.

Partial chromosomal gains revealed by CGH

In four samples, CGH analysis revealed partial chromosomal gains (Figure 2). In two samples (M-14, M-17), these gains (in chromosomes 17 and 6 respectively) were not detected by conventional cytogenetic analysis. In the other two cases (M-11, 97M-21), a gain was found in chromosomes 18 and 10 respectively. These last two samples were evaluated solely by CGH. In all these cases, the CGH experiment was repeated twice in order to verify the results of the first analysis.

Discussion

In the present study, we used the CGH technique for detecting chromosomal aberrations in aborted fetal material from 27 cases of recurrent spontaneous abortions. In a subset of these
cases \((n = 12)\), we compared CGH to conventional cytogenetic analysis. From our preliminary results, it seems that all major anomalies detected by conventional cytogenetics can be detected by CGH. In only one case did conventional cytogenetics show a mosaicism where 20% of cells displayed a trisomy for chromosome 16, whereas CGH diagnosed a balanced chromosomal constitution. The reported sensitivity of CGH in detecting a mosaic pattern is \(~40\%\) abnormal cells (Bryndorf et al., 1995). Indeed, in another case in our series, 50% mosaicism for trisomy 18 was detected both by CGH and by conventional cytogenetics. Thus, there seems to be a good agreement between the two techniques and no major abnormality was misdiagnosed by CGH. Moreover, two cases of chromosomal aberrations (gain of 17q23→qter; gain of 6p21.3→q25), were detected solely by CGH and were missed by conventional methods.

CGH alone, unlike conventional cytogenetics, revealed crucial information regarding the chromosomal constitution of the abortus: one trisomy, one monosomy, and three cases of gains and losses as a result of a structural rearrangement carried by one of the parents. This information is important for genetic counselling and has immediate clinical application and implication. Since conventional cytogenetics could not be applied to these cases (due to culture failure), CGH seems to provide a suitable alternative.

In four samples, gains and losses of several chromosomal regions were detected by CGH alone. In two of these samples, conventional cytogenetics failed to detect these chromosomal aberrations and in the other two, cytogenetic analysis was not performed due to culture failure. Since these experiments were duplicated and confirmed, it seems to reflect a true abnormality rather than technical artefact. Conventional cytogenetic analysis depends on the quality of the metaphase spreads. Missed fetal material grows poorly in tissue cultures. Therefore, the
metaphase spreads obtained from these tissue cultures are often of poor quality. This can account for the misdiagnosis of these chromosomal aberrations by the conventional method, especially when the gained or deleted chromosomal region is very small. Another possibility is that these aberrations were not present in all of the cells and were missed by banding analysis because of insufficient number of scored cells. The true significance of the findings by CGH only, unsubstantiated by conventional cytogenetics, is still unclear. It may well be that some of the chromosome abnormalities detected by CGH have no clinical significance or implication. Yet, it could represent a genuine finding that was missed by cytogenetic analysis alone.

The current published estimates on the occurrence of chromosomal abnormalities in spontaneous fetal losses are ~50% (Byrne and Ward, 1994; Abruzzo and Hassold, 1995). In the present series, cytogenetics revealed chromosomal abnormalities in ~40% of the tested cases, compared with 48% for CGH. The relative low number of samples that were cytogenetically analysed in this study compared with others, can account for the discrepancy in chromosomal abnormality revealed. The higher incidence of abnormal chromosomal constitution revealed by CGH compared with conventional cytogenetics can be explained by the higher resolution of CGH in detecting chromosomal abnormalities, over conventional cytogenetics. This advantage, and the analysis of DNA instead of metaphase spreads, makes the CGH an important complementary technique in clinical cytogenetics.

Conventional cytogenetic analysis of fetal material depends on tissue culturing and karyotyping. This technique has some problems, including external contamination, culture failure and selective growth of maternal cells (Eiben et al., 1990). The application of DNA-based methods such as fluorescent polymerase chain reaction (Findlay and Quirke, 1996; Findlay et al., 1996), placental mRNA expression (Brizot et al., 1996), analysis of interphase nuclei by FISH (Horiuchi et al., 1997) and CGH, can overcome the limitations associated with conventional cytogenetic analysis and increase the sensitivity of detection to the level of a single copy gene within a single cell.

Despite these promising results, the limitations of widespread use must be emphasized. CGH analysis is suboptimal for both telomeric and pericentricromic regions, because of low fluorescence intensities and highly polymorphic regions that are blocked by Cot-1 DNA respectively. Thus, chromosomal abnormalities of these regions need to be evaluated by an alternative technique. CGH cannot detect balanced chromosomal rearrangements, and it gives no information on the chromosome architecture. Finally, contamination of normal cells prevents reliable detection of chromosomal aberrations (Kallioniemi et al., 1994).

More studies comparing larger numbers of cases, evaluating CGH compared with conventional cytogenetic analysis for detecting chromosomal abnormalities, need to be carried out. At the present time, the preliminary data in this study show quite an optimistic picture as to the applicability of CGH to the investigation of missed abortions and to the clinical genetics setting, at least as a complementary method to the traditional cytogenetic techniques.

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Reference


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