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Comparative genomic hybridization analysis of fetal samples arising from recurrent spontaneous abortion

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Abstract

Objective: This study determined the chromosomal constitution of recurrent spontaneous abortions by Comparative genomic hybridization (CGH) analysis of fetal samples.

Material and methods: 40 pregnant women with early recurrent pregnancy losses aged 21 to 42 years old from obstetric departments of Tehran University of Medical Sciences affiliated hospitals entered the descriptive study. Hibridization analysis of fetal samples whose standard karyotyping were not possible due to poor sample quality was performed. Number of successful chromosome analysis using CGH comparing with all samples analyzed was determined.

Results: CGH was able to determine the chromosomal constitution of all samples. Overall, CGH detected chromosomal abnormalities including trisomies and monosomies in 18 samples (46%).

Conclusion: CGH can be used to reveal the chromosomal constitution of fetal samples when the sample quality does not permit an accurate chromosome analysis. CGH can also play a role as a complementary method, to the traditional cytogenetic techniques used in the investigation of recurrent spontaneous abortions.

***Key words**: Comparative Genomic Hybridization, Recurrent Abortion, Chromosomall abnormalities, Iran*

Introduction

Spontaneous abortion is a common clinical problem. It is estimated that 10%-15% of all clinically recognized human pregnancies end as a result of early pregnancy losses (1). Unbalanced chromosomal abnormalities account for about 50% of fetal losses in the first trimester of gestational life (2).Therefore, the development of an efficient and accurate karyotyping analysis of an aborted conceptus is of great importance. Chromosome study not only can help families understand the possible causes of the problem but it can also help clinicians to consider possible genetic causes and refer the family for genetic counseling as well as warrant further investigations. However, it is common that information on conventional cytogenetic analysis is

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frequently unavailable due to the various technical pitfalls including, culture failures of old and non-sterile tissues or the selective overgrowth of maternal cells during tissue culturing, which inhibits the karyotype from being representative of the conceptus (3-5). These problems, and as a consequent, lack of such information greatly affect families with repeated pregnancy losses. Comparative genomic hybridization (CGH) is a recently developed molecular cytogenetic technique that is capable of detecting chromosomal gains and losses by fluorescence in situ hybridization (6). In this assay, two differentially labeled genomic DNAs (test and reference) are co-hybridized to normal 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 analyzed by using a digital image analysis system (7). Since this technique depends on DNA isolation from samples to be analyzed rather than on preparation of metaphase spreads, it therefore bypasses the technical problems associated with tissue culturing. CGH can provide a whole genome screen for unbalanced aberration, and detects the origin of extra or missing chromosomal material (6).

Since its development, CGH has been applied mostly on samples from solid tumors in the field of cancer genetics, but it has also been used in clinical cytogenetic laboratories for the diagnosis of unbalanced chromosomal abnormalities (8).

In the present study, the CGH technique was applied to determine the chromosomal constitution of fetuses arising from recurrent spontaneous missed abortions whose standard karyotyping where not possible due to poor sample quality.

Materials and methods

Patients and sampling

This descriptive study included 40 pregnant Iranian women with early pregnancy losses. Maternal ages ranged from 21 to 42 years old, and the gestational periods at the times of embryos' demise were estimated by ultrasound scan and established to be 50—95 days. All cases experienced repeated pregnancy losses and recurrent abortions. All of the 40 specimens, had been previously sent for routine cytogenetic analysis and except in 8 cases the culture had been refused due to old samples or unsterility and therefore, possible culture failures. In 8 samples both CGH and routine culture were set up and both were successful.

Tissue preparation and DNA extraction

Chorionic villi and placental samples of spontaneously aborted fetal material were obtained from the Obstetric Departments of Tehran University of Medical Sciences affiliated hospitals. Using a dissecting microscope, maternal decidua, mucus and blood clots were removed from all samples and then washed with PBS (Phosphate buffer solution). 100 mg of tissue sample was used for DNA extraction.

Metaphase spreads

Metaphase spreads were prepared from phytohaemagglutinin (PHA) stimulated, methotrexate synchronised peripheral blood lymphocytes from healthy males using standard procedures of hypotonic treatment and methanol/acetic acid fixation (3:1, v/v).

Genomic DNA probes and labeling procedures for CGH experiments

Test and control DNA was extracted by proteinase K and RNase digestions. Control genomic DNA was prepared from the blood of healthy males (46,XY)

or females (46,XX). Test (patient) human genomic DNA was directly labeled with FITC-12-dUTP (Roch), and control DNA was labeled with Texas Red-5-dUTP (Roch) by standard nick translation reaction. DNase I concentration was adjusted to result in an average fragment size of 500-1000bp.

Hybridization and post-hybridization washings

Metaphase chromosomes from normal males were denatured for two minutes at 70 ° Celsius in 70% formamide, 2 x SSC, pH 7.0 (2 x SSC: 3 mol/l NaCl, 30 mmol/l Na sub 3 -citrate); thereafter, slides were put through an ice cold ethanol series (70%, 90%, 100%) and air dried. Ten microlitres of hybridization solution contained 1 micro gram of labelled test DNA, 1 micro gram of labelled control DNA, and 50 micro gram of unlabelled human Cot 1 DNA (BRL Life Sciences) in 50% formamide, 1 x SSC, and 10% dextran sulphate. After denaturation at 74 ° Celsius for seven minutes, the hybridization mix was applied to the slide with the denatured metaphase chromosomes, covered by a 15 x 15 mm coverslip, and sealed with rubber cement. After 72 hours' hybridization at 37° Celsius, slides were washed 3 x five minutes with 50% formamide and five minutes with 2 x SSC at 42° Celsius and another five minutes with ST (4 x SSC, 0.05% Tween 20) at room temperature. Slides were then dehydrated in an ethanol series (70%, 90%, 100%) and air dried. Finally, they were counterstained with 4,6,diamino-2-phenylindole (DAPI, 0.1 pg/ml), resulting in coarse banding of the chromosomes, allowing individual chromosomes to be identified.

Digital image analysis

Images for CGH analysis and FISH were obtained using an epifluorescence microscope (Leica) equipped with a CCD camera (Leica DM6000 B) controlled by an image analysis system (Leica CW4000). For standard CGH analysis, green, red, and blue fluorescence images were captured from each high intensity, uniformly hybridised metaphase and were analysed as separate grey scale images. The image representing the blue DAPI counterstain was inverted and used for chromosome identification based on its coarse banding pattern. The mean of the individual ratio profiles of at least 10 and generally 20 metaphase spreads was calculated. The green and red fluorescence intensities were calculated and the green to red ratio profiles along the chromosome axis were displayed. For normalization of the ratio profiles, the model value of the green to red ratio for the entire metaphase was set to 1.0. Finally, the individual ratio profiles were combined to yield the average ratio profiles, which were displayed next to the chromosome diagrams with significance intervals of 0.8 and 1.2.

Interpretation of CGH results and quality control

Chromosomal regions with a green to red ratio above 1.2 were considered to be over-represented (gained), whereas regions with a ratio below 0.8 were considered to be under-represented (lost). These limit values were slightly different in each experiment depending on the thresholds deduced from the analysis of negative control experiments where two sets of differently labeled normal DNA were hybridized against one another. Reliability of the results was controlled in different ways. Negative and positive controls were included in each CGH experiment. Hybridizations of directly FITC labeled normal female DNA and Texas-Red labeled normal male DNA were used as negative controls and DNA samples with known gains and losses as positive controls.

Results

Forty samples were included in this study. The samples had mainly been selected among those which the villi were too old and did not have enough viable cells for the tissue to grow. Summary of results are shown in Tables I and II. Overall, CGH and standard cytogenetics revealed an abnormality in 45% (18/40) of the samples. Of the 40 samples evaluated, 8 were assessed both by conventional cytogenetic analysis and CGH. Results of the conventional cytogenetics and CGH analysis performed in 8 samples are summarized in Table I. In all 8 cases, cytogenetic analysis and CGH analysis were in agreement and consistent. Five out of 8 samples analysed both by CGH and standard cytogenetics were found to be chromosomally balanced.

Table 1: The results of 8 spontaneously aborted samples by standard cytogenetic analysis and CGH technique.

Sample No	CGH result	Standard cytogenetics	Comment
1	Balanced	46,XX	Normal
10	Balanced	46XY	Normal
14	Balanced	46,XY	Normal
18	Gain of ch. 22	47,XY,+22	Trisomy 22
19	Balanced	46,XY	Normal
24	Balanced	46,XX	Normal
30	Gain of ch. 21	47,XY,+21	Trisomy 21
37	Loss of ch X	45,X	Chromosome X monosomy

Table 2: Results of CGH analysis in 32 samples, for which no conventional cytogenetic evaluation was possible.

Sample No	CGH result	Comment
2	Balanced	Normal
3	Balanced	Normal
4	Balanced	Normal
5	Gain of ch. 21	Trisomy 21
6	Balanced	Normal
7	Balanced	Normal
8	Gain of ch. 13	Trisomy 13
9	Balanced	Normal
11	Loss of ch X	Chromosome X monosomy
12	Balanced	Normal
13	Gain of ch. 16	Trisomy 16
15	Gain of ch. 13	Trisomy 13
16	Gain of ch. 22	Trisomy 22
17	Balanced	Normal
20	Loss of ch X	Chromosome X monosomy
21	Gain of ch. 22	Trisomy 22

Sample No	CGH result	Comment
22	Balanced	Normal
23	Gain of ch. 21	Trisomy 21
25	Balanced	Normal
26	Loss of ch X	Chromosome X monosomy
27	Balanced	Normal
28	Balanced	Normal
29	Gain of ch. 21	Trisomy 21
31	Balanced	Normal
32	Balanced	Normal
33	Gain of ch. 16	Trisomy 16
34	Balanced	Normal
35	Gain of ch. 13	Trisomy 13
36	Balanced	Normal
38	Balanced	Normal
39	Loss of ch X	Chromosome X monosomy
40	Gain of ch. 18	Trisomy 18

The other three were diagnosed to be aneuploidies including trisomies 21, 22 and monosomy X. No discrepancy was found between the two approaches.

In 32 additional samples, chromosomal constitution was determined solely by CGH because the tissue culture was not possible due to the sample quality.

Table II presents the results of CGH analysis in the remaining 32 samples, for which no cytogenetic evaluation was possible. In 17 out of 32 samples (53%), chromosomal constitution was balanced, and in 15 samples (46%), gains and losses of various chromosomes were detected (Table I)

Discussion

In the present study, CGH technique was successfully used for detecting chromosomal aberrations in aborted fetal material from 40 cases of recurrent spontaneous abortions. Results in all 8 cases which were analyzed using both CGH and conventional cytogenetics were in agreement and consistent. Similar studies have also shown similar results. Our study was however, mainly focused on samples which conventional cytogenetic studies were not possible due to insufficient viable cell numbers for culture. The application of CGH which is a DNA-based method can overcome the limitations associated with conventional cytogenetic analysis. There are also several promising possibilities including the increase of the sensitivity of the technique up to a single cell resolution. Despite these promising results, on the other hand, there are limitations of widespread use of the CGH which must be emphasized. Using CGH for detection of abnormalities in telomeric and pericentromeric regions needs special technical considerations. Moreover, CGH cannot detect balanced chromosomal rearrangements. However, since these kinds of abnormalities are not a known cause of recurrent abortions this limitation is not considered as a pitfall in this area of clinical diagnosis. While, contamination of normal cells in theory may interfere with reliable detection of chromosomal aberrations, this may not be the case if reliable separation of fetal and maternal cells are performed. Furthermore, unlike PCR-based techniques, low percentage contamination will not prevent reliable analysis.

In summary, these results of this study show that collecting samples for CGH analysis is much more feasible than that of the conventional cytogenetic approach using cultured fetal cells. Moreover, in cases which viable cells are not available CGH analysis provides help to achieve a chromosomal diagnosis paving the way for a comprehensive genetic counseling to help the couple for their future reproductive options.

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