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Research Article

Array-CGH testing in spontaneous abortions with normal karyotypes

Cleide L. Borovik[1], Ana Beatriz A. Perez[2], Luciana R.J. da Silva[1,2], Ana Cristina V. Krepischi-Santos[3], Silvia S. Costa[3] and Carla Rosenberg[3]

[1]*Setor de Genética Laboratorial, Serviço de Técnicas Especiais, Departamento de Patologia Clínica, Hospital Israelita Albert Einstein, São Paulo, SP, Brazil.*
[2]*Departamento de Morfologia e Pediatria, Centro de Medicina Genética, São Paulo, SP, Brazil.*
[3]*Departamento de Genética e Biologia Evolucionária, Instituto de Biociências, Universidade de São Paulo, São Paulo, SP, Brazil.*

Abstract

In about 50% of first trimester spontaneous abortion the cause remains undetermined after standard cytogenetic investigation. We evaluated the usefulness of array-CGH in diagnosing chromosome abnormalities in products of conception from first trimester spontaneous abortions. Cell culture was carried out in short- and long-term cultures of 54 specimens and cytogenetic analysis was successful in 49 of them. Cytogenetic abnormalities (numerical and structural) were detected in 22 (44.89%) specimens. Subsequent, array-CGH based on large insert clones spaced at ~1 Mb intervals over the whole genome was used in 17 cases with normal G-banding karyotype. This revealed chromosome aneuplodies in three additional cases, giving a final total of 51% cases in which an abnormal karyotype was detected. In keeping with other recently published works, this study shows that array-CGH detects abnormalities in a further ~10% of spontaneous abortion specimens considered to be normal using standard cytogenetic methods. As such, array-CGH technique may present a suitable complementary test to cytogenetic analysis in cases with a normal karyotype.

Key words: spontaneous abortion, chromosomal aberrations, array-CGH.

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Introduction

Numerical chromosome anomalies are the major cause of recognized early gestational loss, with autosomal trisomies being the most frequent and representing about 50% of all abnormalities, followed by triploidies and monosomy X (Nagaishi *et al.*, 2004; Ljunger *et al.*, 2005). Countless efforts have been made to discover the causes of such cell division errors, particularly in couples presenting recurrence, and several hypotheses to explain them have been proposed (Turleau & Vekemans, 2005).

The main purpose of performing routine anatomic-pathological examinations in all cases of early abortion is to identify hydatidiform moles, although, from the histological point of view, it is very difficult to distinguish a complete from a partial mole (Lai *et al.*, 2004). Identification of possible fetal malformations responsible for the abortion is generally not possible, because in most cases the material available is maternal decidua and fetal fragments (Fulcheri *et al.*, 2006).

Send correspondence to Cleide Largman Borovik. Laboratório de Genética, Departamento de Patologia Clínica, Hospital Israelita Albert Einstein, R. Ernesto Nazareth 782, 05462-001 São Paulo, SP, Brazil. E-mail: cleide.borovik@gmail.com.

In clinical laboratories routine, cytogenetic analysis is carried out by two methods: short-term culture of chorionic villi and long-term culture. The short-term culture only yields metaphase cells if the sample contains viable cytotrophoblasts, with spontaneous division, usually presenting a lower success rate (Eiben *et al.*, 1990). Long-term cultures generally contain cells both of the fetus and the maternal decidua, and frequently the latter presents preferential growth. Thus, in addition to being a time-consuming test, karyotyping does not always lead to a result; furthermore, in about 4.4% to 29% of cases the result does not correspond to the actual fetal karyotype, mainly due to maternal cell contamination as shown by other techniques such as fluorescence in situ hybridization (FISH) and others (Bell *et al.*, 1999; Lomax *et al.*, 2000; Diego-Alvarez *et al.*, 2005; Karaoguz *et al.*, 2005; Nikitina *et al.*, 2005)

DNA-based technologies do not require dividing cells thus, overcoming one of the main limitations associated with conventional cytogenetic analysis of spontaneous abortion material. Several of these methods have been used as diagnostic tools, including fluorescent polymerase chain reaction (Diego-Alvarez *et al.*, 2005), interphase-FISH (Horiuchi *et al.*, 1997; Lebedev *et al.*, 2004, Vorsanova *et al.*, 2005),

chromosome-CGH (Daniely *et al.*, 1998, Fritz *et al.*, 2001), and multiplex ligation probe amplification (MLPA) (Diego-Alvarez *et al.*, 2006). More recently, array-CGH has been considered as a particularly useful alternative to conventional karyotyping in the field of diagnosis (Schaeffer *et al.*, 2004; Benkhalifa *et al.*, 2005; Shimokawa *et al.*, 2006), as it allows screening gains and losses in thousands of targets simultaneously. Major advantages of the array-based cytogenetic technologies are the high resolution and high throughput (Salman *et al.*, 2004). It also offers new possibilities for genetic pre-implantation analysis, opening the route toward aneuploidy screening and detection of unbalanced translocations in embryos (Le Caignec *et al.*, 2006).

To evaluate the efficiency of alternative methods for identification of chromosome anomalies in abortion material, we present the results of a study on 49 cases referred for cytogenetic analysis. In 17 of these cases in which the karyotype was normal by conventional analysis we also performed analyzes by a-CGH.

Material and Methods

Material

From September, 2005, to May, 2006, 80 products of conceptions (POCs) of first-trimester abortions were referred for cytogenetic analysis on medical request to the Genetics section of the Special Techniques Service of the Clinical Pathology Department of the Hospital Israelita Albert Einstein in São Paulo, Brazil (HIAE).

Initially, informed consent according to the Research Ethics Committees of the HIAE was obtained for 54 cases and a clinical chart was filled out for further research. The cases in which a normal karyotype was found and for which there was still material available were referred for investigation by array-CGH.

The mean maternal age of the entire patient group was 33.52 ± 4.50 years (range 23-43). Twenty-nine mothers were primigravidae, and in 14 cases there was at least one previous abortion. Assisted reproduction was reported in only one case.

Cytogenetic analysis

Whenever the material contained chorionic villi, both short (according to the technique described by Simoni *et al.*, 1983) and long-term culture were set up. If the short-term culture was successful the karyotype result was based on this analysis. If the short-term culture failed or yielded insufficient material for analysis, the long-term cultures were used. If the material did not contain chorionic villi only long-term cultures were used for analysis. Chromosome analyses were performed after G-banding using Wright stain. A minimum of five, but preferentially 20 cells, were analyzed per sample.

Comparative Genomic Hybridization (array-CGH)

DNA was extracted from uncultured abortion samples using the QIAmp DNA Mini Kit (Qiagen). Array -CGH was performed as previously described (Rosenberg *et al.*, 2006), using slides containing triplicates of 3,500 BAC/PAC DNA targets spaced at ~1 Mb intervals, produced at Leiden University Medical Center. As a reference, female DNA was used. Imbalances of the targets were determined based on log 2 intensity ratios of the test/reference sample. A sequence was considered as amplified or deleted when the average log 2 intensity ratio of the replicates was outside the ± 0.4 range.

Results

Of the 54 cases referred initially, five (9.26%) neither yielded metaphases for cytogenetic analysis nor DNA sufficient for array-CGH testing. In 26 cases only short-term cultures were analyzed, in seven cases only long-term cultures, and in 16 cases both types of cultures. Among the latter ones, two showed a discrepancy in the sex complement (in the short-term cultures: 46,XY and in the long-term cultures 46,XX). Of the 49 cases in which a cytogenetic result was obtained, 26 (53.06%) had a male chromosome complement and 23 (46.93%) had a female chromosome complement. Twenty-two (44.89%) of these 49 cases had an abnormal karyotype: 12 cases showed autosomal trisomy, two cases double autosomal aneuplody, four had triplody, one tetraploidy, two had structural alterations, and one case was 45,X. In one of the cases with a structural alteration, the chromosome imbalance was found to be inherited from a balanced alteration in the father (Table 1).

Of the 27 cases with a normal karyotype, DNA was extracted from 17 for analysis by the array-CGH technique;

Table 1 - Chromosomal abnormalities detected among 49 first-trimester spontaneous abortions.

Case	Cytogenetic analysis karyotype and number of cells		Array-CGH	Maternal age (years)	GPA
	Short-term culture	Long-term culture			
1	69,XXY[15]	NA	NA	30	G1P0A1
2	46,XY[10]	NA	XY+7	30	G1P0A1
3	47,XY, +15[4]/46,XY[8]	NA	NA	28	G1P0A1
4	69,XXX[5]	69,XXX[5]/46,XX[10]	NA	37	G2P1A1
5	NA	46,XX, [20]	XX	34	G2P1A1
6	NA	46,XX, [20]	XX	43	G1P0A1

Table 1 (cont.)

Case	Cytogenetic analysis karyotype and number of cells		Array-CGH	Maternal age (years)	GPA
	Short-term culture	Long-term culture			
7	46,XX[5]	NA	XX	32	G1P0A1
8	46,XY[3]/45,X[3]	47,XY, +?[2]/46,XY[7]	XY+13	43	G2P0A2
9	46, XX[10]	46, XX[10]	XX	32	G1P0A1
10	46, XX[20]	NA	XX	32	G2P1A1
11	46, XX[5]	46, XX[15]	XX	38	G2P1A1
12	NA	47,XY, +22[20]	NA	37	G1P0A1
13	47,XY, +22[20]	NA	NA	32	G1P0A1
14	47,XY+14[2]/48,XY, +14,+18[17]	NA	NA	40	G1P0A1
15	48,XY, +8,+21[18]	NA	NA	42	G4P2A2
16	46, XX[4]	46, XX[15]	NA	41	G3P2A2
17	46,XY[3]/45,XY, -3[2]/45,XY, -15[2]	NA	XY	29	G4P0A4
18	46, XX[17]	NA	NA	33	G3P2A1
19	46, XY[20]	NA	NA	31	G1P0A1
20	NA	46,XX[20]	NA	34	G1P0A1
21*	46,XY[8]/45,XY, -?[2]	46,XX[9]	XX	31	G1P0A1
22	46, XX[15]	NA	NA	33	G3P1A2
23	46, XX[5]	46, XX[15]	NA	41	G3P2A2
24	NA	46,XX[5]/92,XXXX[2]	XY+18	31	G1P0A1
25	69,XXY[7]/46,XY[2]	NA	NA	40	G2P0A2
26	F	F	NA	34	G1P0A1
27	F	F	NA	29	G1P0A1
28	47,XY, +16[13]	NA	NA	28	G1P0A1
29	47,XY, +16[5]	47,XY, +16[15]	NA	30	G1P0A1
30	46,XY[3]	46,XY[17]	NA	29	G1P0A1
31	F	F	NA	29	G1P0A1
32	47,XX, +10[17]	NA	NA	32	G2P1A1
33	46,XX[18]	NA	NA	38	G2P0A2
34	46,XY, DER(7), t(7;13)(p13;q14[10]pat	NA	NA	28	G2P1A1
35	46,XX[16]	NA	NA	23	G1P0A1
36	46,XY[9]	46,XY[2]	XY	36	G1P0A1
37	F	F	NA	35	G2P1A1
38	F	F	NA	26	G1P0A1
39	46,XY[13]	NA	XY	36	G3P1A2
40*	46, XY[5]	46,XX [5]	XY	33	G3P1A2
41	47,XY, +16[15]	NA	NA	28	G1P0A1
42	47,XY, +16[20]	NA	NA	33	G1P0A1
43	46,XX[5]	46,XX[15]	NA	35	G2P1A1
44	47,XY, +22[7]	47,XY, +22[13]	NA	36	G3P1A2
45	47,XX, +16[4]/46,XX[2]	NA	NA	34	G1P0A1
46	46,XY, idic(8)(q10q10)[3]/46,XY, del(8)(q10)[6]/46,XY[11]	NA	NA	31	G1P0A1
47	47,XX, +16[15]	NA	NA	34	G2P0A2
48	47,XX, +16[5]	47,XX, +16[15]	NA	31	G2P1A1
49	69 XXY[10]/46,XX[4]	NA	NA	28	G1P0A1
50	46,XY[20]	NA	NA	38	G1P0A1
51	90,XXXX, -? [2]	46,XX[10]/92,XXXX[4]	XX	39	G2P0A2
52	NA	46,XX[20]	XX	35	G3P1A2
53	45,X[6]	45,X[6]	NA	32	G1P0A1
54	46,XY[5]	NA	XY	35	G2P1A1

NA = not analyzed; F = culture failure; G = Gravity; P = Parity; A = Abortion.
*Discrepancies of results obtained by short and long-term cultures.

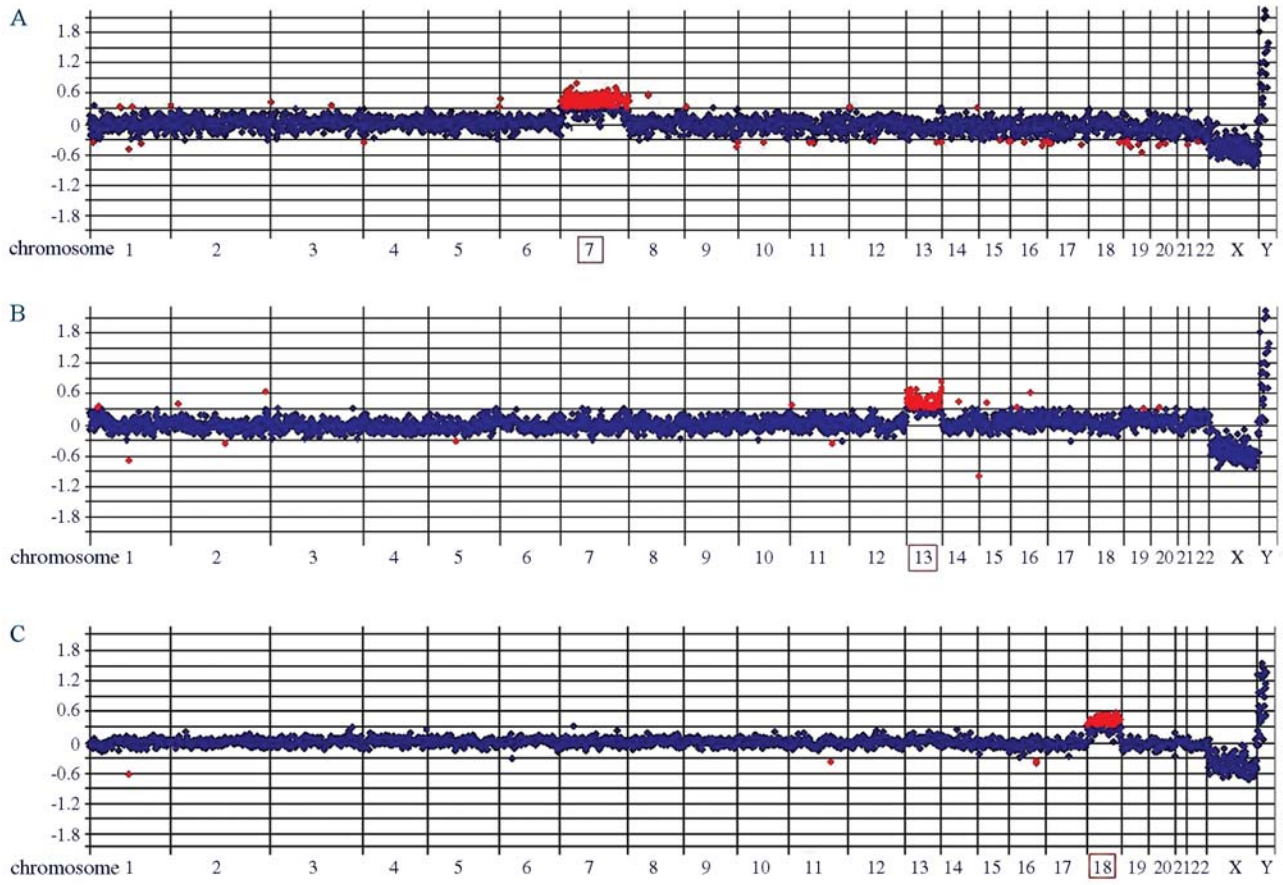

Figure 1 - Whole-genome array-CGH profiles of abortion samples which revealed whole chromosome copy-number changes. The Y axis is log ratio of the test/ reference DNAs intensities, and the X axis displays the whole-genome profile for ordered by chromosome from 1 to 22, X and Y, and from p to q arm. Genomic sequences with significantly increased or decreased copy number are represented by red dots, illustrating a gain of chromosome 7 (A), chromosome 13 (B) and chromosome 18 (C). The three cases are XY and were hybridized against female DNA, resulting the observed imbalances for the sex chromosomes.

from the other 10 cases with a normal karyotype no suitable material was available. In three of the 17 cases analyzed by array-CGH, chromosome alterations that had gone undetected were found: one trisomy 7, one trisomy 13 and one trisomy 18 (Figure 1). Discrepancy in the sex complement was detected in two cases: in the one with trisomy 18 and in one with a normal karyotype (Table 1).

Thus, 51% of the cases in which a result was obtained showed chromosomal alterations.

The results of the anatomic-pathological examination showed hydropic degeneration of the chorionic villi to a greater or lesser extent in 13 cases: one with a 46,XX karyotype, five 46,XY, five with trisomy of chromosome 16, and two with triploidy.

Discussion

Chromosome abnormalities detected by conventional karyotyping have been described in abortion material for a long time now. However, this material is often unsuitable for analysis with reliable results, either due to its poor quality or the small number of cells obtained in culture (Danielly *et al.*, 1998; Lomax *et al.*, 2000; Greenwold and Jauniaux, 2002).

In an attempt to overcome these problems other techniques such as fluorescent polymerase chain reaction (Diego-Alvarez *et al.*, 2005), interphase-FISH (Horiuchi *et al.*, 1997; Lebedev *et al.*, 2004, Vorsanova *et al.*, 2005), chromosome-CGH (Daniely *et al.*, 1998; Fritz *et al.*, 2001), MLPA (Diego-Alvarez *et al.*, 2006) and array-CGH have been introduced in the study of POCs.

To our best knowledge, there are so far three published studies using the array-CGH technique in abortion material. Schaeffer *et al.* (2004) examined 41 POC samples by a 1 Mb resolution array-CGH, and compared the results with those obtained by G-band analysis. In this study, 37/41 POC cases were in exact concordance with the karyotype results. Moreover, the array identified additional anomalies that had not been detected by routine chromosome analysis, in the four remaining cases: a clone with trisomy 20, besides non-mosaic trisomy 21; an interstitial microdeletion

of chromosome 9p in addition to trisomy 13; an interstial microduplication of chromosome 15 in addition to trisomy 16; and a submicroscopic duplication of chromosome 10 long arm. Benkhalifa *et al.* (2005) studied 26 samples of abortion material with no growth. They found 12 cases with numerical anomalies and two with possible submicroscopic anomalies. Shimokawa *et al.* (2006) analyzed 20 POC samples with normal karyotype and identified two cases with microdeletions. These studies show that POC material can be analyzed by array-CGH, and that it can detect additional alterations when compared to classical cytogenetics.

In the present study we employed an array with 3,500 BAC/PAC DNA targets spaced at ~1 Mb intervals and identified aneuploidies in three cases in which conventional cytogenetic analysis had shown a normal karyotype. In two cases in which a normal male karyotype (46,XY) had been found, array-CGH revealed the presence of an additional chromosome 7 in one of them and of an additional chromosome 13 in the other. Although the presence of an undetected mosaicism cannot be ruled out, considering that the presence of mosaicism is a rather frequent finding in abortion material (Lebedev *et al.* 2004; Schaeffer *et al.*, 2004; Vorsanova *et al.* 2005), it is also possible that it went undetected by the conventional cytogenetic analysis because of the previously mentioned difficulties usually presented by abortion material. In the third case in which conventional analysis of cells from a long term culture revealed a 46,XX karyotype and the array-CGH analysis showed a male complement with an additional chromosome 18, very likely the analyzed cells were of maternal origin.

In one case in our study the cytogenetic analysis based on a short-term culture was 46,XY but the array revealed a female DNA. It is possible that the extracted DNA was mainly of maternal origin, once the karyotype from the long-term culture was 46,XX.

After applying the array-CGH technique, the overall rate of detected chromosome abnormalities was 51%. This figure is similar to the frequency of chromosome aberrations usually reported in the literature. Complementarily, if only array-CGH had been performed, probably all alterations except the ploidy changes would have been detected. Although the array-CGH technique is not a suitable tool for detecting ploidy changes, Ballif *et al.* (2006) described the use of a control DNA of a Klinefelter individual as an approach to identify possible ploidy alterations. All papers in the literature on studies using complementary techniques to cytogenetic analysis showed that the contribution of chromosome anomalies to first-trimester fetal losses is much greater than found by conventional cytogenetic analysis, being estimated in up to 70% (Fritz *et al.*, 2001). For instance, when Daniely *et al.* (1998) used chromosome-CGH in cases in which the karyotype found by cytogenetics was normal or could not be obtained due to culture failure, they detected additional numerical and structural anomalies in 8% of the POC from couples with recurrent abortions.

In the present study, trisomy of chromosome 16 was predominant, followed by triploidy and trisomy 22. Trisomy 16 is the most frequently encountered trisomy in early abortion specimens, presenting an incidence of ~1.5% of all clinically recognized gestations. Most of these embryos undergo spontaneous abortion or a developmental arrest between the 8th and 15th weeks (Yong *et al.*, 2003).

Double trisomy was detected in two cases of our sample (4.08%). In both of them, maternal age was high (40 and 42 years), supporting the implication of a maternal age-related mechanism, as previously reported (Reddy, 1997; Diego-Alvarez *et al.*, 2006).

In two of our cases, structural anomalies were detected. In one of them, the father carried the balanced form of a t(7;13) translocation. The gestational history revealed a previous abortion and a normal child. However, there was a history of multiple fetal losses in the paternal grandmother and great-grandmother, suggesting that the translocation may be segregating in the family for some generations. In the other case it was not possible to obtain the parental karyotypes, but the kind of alteration found, ie, a mosaicism involving an idic(8), a del(8) and a normal karyotype suggests that the events were not inherited.

Triploidy occurs in approximately 2% of conceptuses. Its occurrence is not associated with an increase in maternal age, but whenever the additional chromosome complement is of paternal origin, the placenta exhibits histological characteristics of a partial hydatidiform mole (Brancati *et al.*, 2003). In fact, in one of the cases with triploid karyotype, in which the anatomic-pathological study showed hydropic degeneration of the villi, we were able to demonstrate the paternal origin of the additional chromosome complement (data not shown).

The anatomic-pathological findings show that hydropic degeneration of villi is not a feature exclusive of hydatidiform moles (complete or partial). Although half of the cases in which it was observed presented a chromosome alteration, inferences regarding the karyotype based on pathological examination should be avoided (Lescoat *et al.*, 2005).

The data of abortion investigations reported so far show an encouraging picture as to the applicability of array-CGH, at least as a complementary method to the traditional cytogenetic techniques. The results of the present study, confirm that the use of array-CGH to complement classical cytogenetics in the chromosome analyses of abortion material is highly recommended.

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