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Array comparative genomic hybridization profiling of first-trimester spontaneous abortions that fail to grow *in vitro*

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Objectives Cytogenetic analysis of spontaneous abortion samples can be limited by culture failure. Failure to grow *in vitro* has traditionally been suspected to be due to *in vivo* death of tissue associated with spontaneous abortion (SAB) or simply technical factors of growth in culture.

Method We used array comparative genomic hybridization (array CGH) to investigate chromosomal imbalances in products of conception that failed to grow *in vitro*.

Results Our data on 26 cases of SABs that failed to grow in culture are compared and contrasted with published data on cytogenetic findings following *in vitro* culture. The results revealed abnormalities uncommonly seen by classic cytogenetic methods. These abnormalities include high rates of double aneuploidy and autosomal monosomy. The data taken together suggest that classic cytogenetics of spontaneous abortion may yield normal karyotypes or selected abnormal karyotypes that permit cell proliferation *in vitro* while Array CGH detects other abnormalities.

Conclusion Array CGH is becoming an important clinical assay for unbalanced chromosome abnormalities whether cells grow in culture or not and in cases of analysis on one or few cells. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS: first-trimester fetal loss; cell culture failure; array CGH; chromosome abnormalities; autosomal monosomy

INTRODUCTION

About 15% of clinically recognized pregnancies end up in spontaneous abortion (SAB) in the first trimester and the vast majority of those result from chromosome abnormalities (Warburton *et al*., 1979). Chromosomal abnormalities can result in much earlier failures of development including early arrest of cell division, lack of implantation, or early failure of an implanted embryo before clinically recognized pregnancy (Benkhalifa *et al*., 2003, 2004; Kahraman *et al*, 2004). Most of these earlier pregnancy failures escape patient and clinical detection. By contrast, patients and physicians are acutely aware of SABs in the first trimester or later developmental failure and developmental abnormalities (Gardner and Sutherland, 2003).

Most clinically recognizable SABs occur between 7 and 11 weeks of gestation and are related to: (1) *de novo* aneuploidy or polyploidy, (2) *de novo* unbalanced rearrangements, and (3) inherited unbalanced segregation outcomes (parent with balanced rearrangement). The latter accounts for a small number of cases but is highly suspected when there are multiple fetal losses in one family. Unbalanced chromosome constitution could affect placental development resulting in pregnancy failure (Qumsiyeh *et al*., 2000).

Understanding the mechanisms that cause an unbalanced chromosome constitution to lead to fetal loss is biologically interesting at many levels. Classic cytogenetic studies are offered in some institutions for SABs or intrauterine fetal demise and for parents who had a history of repeated SABs. These studies entail conventional tissue culturing and karyotyping and have limitations such as a high rate of culture failure and maternal cell contamination (Qumsiyeh, 1998; Lomax *et al*., 2000).

Some authors have utilized fluorescence *in situ* hybridization (FISH) and even classical comparative genomic hybridization CGH to screen for abnormalities in products of conception (Daniely *et al*., 1998; Bell *et al*., 2001; Fritz *et al*., 2001; Tabet *et al*., 2001). More recently, array CGH methods appear to be far better as they do not suffer from dependence on having metaphase preparations, nor do they require identification of chromosomes (counterstained with a DNA dyes in classic CGH), and have much higher sensitivity and specificity for subtle genomic changes (Solinas-Toldo *et al*., 1997;

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Pinkel *et al*., 1998; Schaeffer *et al*., 2004; Shaw-Smith *et al*., 2004).

Clinical applications of array CGH were made possible by developments in the human genome project and associated technologies. Such developments now allow for identification of sequences mapped to specific regions, arraying them on a slide, and using this array for comparing test and control DNA. This made high-resolution analysis feasible as a diagnostic tool for the comprehensive analysis of detailed chromosomal alterations of DNA copy numbers. In the case of SABs, this may also be extremely useful as the failure rate is high in trying to grow products of conception for classic cytogenetic analysis. Further, unlike classic CGH, array CGH is more amenable to automation and thus significant cost cutting. In the present study, we investigated the use of the array CGH technique to identify unbalanced chromosome abnormalities in 26 first-trimester SABs (before 11 weeks). These cases were chosen because they failed to grow in culture to yield routine G-band analysis.

MATERIALS AND METHODS

We studied 26 samples of first-trimester SAB (gestational age between 9–11 weeks) by array CGH. Material was collected in RPMI 1640 medium and conserved at 4 °C until use. The analyzed specimens were duplicate pieces of tissue kept in storage from SAB that failed to divide in culture in routine cytogenetic studies. The tissue was subjected to classical methods of genomic DNA extraction using phenol/chloroform.

Our study followed the policies of the institutional review board of women's health, Infertility and Genetic Research Center, Ankara, Turkey. For sample assay ATL R&D, Reproductive Biology & Genetics Laboratory, Voisins Le Bretx, France; Consultorio Di Genetica&Genoma Laboratories, Rome, Italy; Cytogenetics Services, Department of Genetics, Yale University, USA; and siParadigm, Oradell, NJ, USA.

We used human genomic microarrays containing 2600 BAC/PAC clones (Human BAC Array-1MB system, Spectral Genomics Inc., Houston, TX). This array included subtelomeric regions as well as critical areas spaced roughly 1 Mb along each of the human autosomes as well as the X and Y chromosomes. The company can provide a list of BAC probes arrayed (http://www.spectralgenomics.com) and the details of each BAC/PAC clone can be found at http://www.ncbi.nlm.nih.gov/genome/cyto/hbrc.shtml.

For consistency and increasing sensitivity and specificity, the arrayed clones were printed in duplicate. The printed arrays were denatured and the repeated sequences of the human inserts of printed BACs were blocked by prehybridizing with Human Cot-1 DNA and the slide surface was blocked with shared salmon testes DNA in appropriate conditions.

Test and control (pooled normal male or female DNA from Promega, Madison, WI) DNA samples (2 μg each) were digested overnight with 80 units of EcoRI at 37 °C and then purified by Zymo Research's column (Orange, CA, USA). The test and reference DNAs were labelled with Cy3 and Cy5 using a random prime labelling kit (Invitrogen, Carlsbad, California) to obtain a labelled probe size averaging 100 to 500 bp in length.

For the hybridization solution, Cy5 labelled test DNA and Cy3 labelled reference DNA samples were mixed with 65 μg of Cot-1 DNA and 35 μg of Salmon sperm DNA. Then the mix was precipitated and washed with ethanol. The same experiment was repeated with Cy3 labelled test and Cy5 labelled reference test. This forward and reverse hybridization switching of dyes helps address issues related to dye specificity and strength. The pellets were dissolved in 10 μl of distilled water and mixed with 50 μl of hybridization solution (50% formamide, 10% dextran sulphate in 2 × SSC). The hybridization mix was denatured at 73° for 12 min and followed by 40 min at 37 °C for annealing.

The forward and reverse hybridization reactions were added on duplicate array slides and placed at 37 °C for 16 h. After hybridization, slides were washed at RT in 2 × SSC for 5 min and then for 20 min in 50% formamide/2 × SSC at 50 °C with shaking. The washing steps were repeated at 50 °C with shaking in 0.1% NP40/2 × SCC for 20 min and 0.2 × SCC for 20 min. Finally, the slides were rinsed briefly with distilled water at RT and centrifuged for 3 min at 500 rpm until dry.

Hybridized arrays were analyzed with a GenPix scanner (Axon Instruments, Union City, CA). Cy 3 and Cy5 images were scanned separately through two different channels. Two 16 bit TIFF images were created per array. Then the obtained data were analyzed by Spectralware software (Spectral Genomics, Houston, TX). The software recognizes the regions of fluorescent signal, determines signal intensity and compiles the data into a spreadsheet that links the fluorescent signal of every clone on the array to the clone name, its duplicate position on the array and its position in the genome. The software was also used to normalize the Cy5 : Cy3 intensity ratios for each slide and each data point. Normalization was such that the summed Cy5 signal equals the summed Cy3 signal. The normalized Cy3 : Cy5 intensity ratios were computed for each two slides and plotted together for each chromosome. The linear order of the clones is reconstituted in the ratio plots consistent with an ideogram, such that the p terminus is to the left and the q terminus is to the right of the plot.

For data analysis, the average-Log-ratios of normalized data are plotted such that gains in DNA copy number at a particular locus are observed as the simultaneous deviation of the ratio plots from a modal value of 1.0, with the blue ratio plot showing a positive deviation (upward) while the red ratio plot shows a negative deviation at the same locus (downward). Small variations between individual clones are expected because of DNA content variation. Deviations of 1.2 are considered significant in array CGH studies. Chromosomal areas are interpreted as overrepresented when the ratio exceeds 1.2, which shows DNA copy number gains or 1.5, which shows DNA copy number amplifications and reversely for copy number losses or deletions. As CGH recognizes only proportional changes in copy number, the

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ratio profiles do not indicate the absolute copy number changes. A ratio of 1.5 indicates at least a 100% increase in the copy number of an entire chromosome arm or of a region of a chromosome the size of a chromosome band (e.g. chromosomal trisomy). When a DNA copy number increase is restricted to a small chromosome area representing, for example, amplification of a single gene, then the copy number increase has to be at least 1 Mb which is the resolution of the micro array used.

DNA copy number losses show the opposite pattern. In selected cases, FISH was used to confirm the array CGH findings in selected cases.

RESULTS

The data we obtained from A- CGH shows that 15 of 26 cases (57.7%) of POC samples that failed to grow in culture had abnormal profiles (Table 1). Three of the 15 cases had apparently structural abnormalities. More than chromosomal aneuploidies, two significantly amplified clones on chromosome Xp were observed on one case of monosomy 21. One case showed a gain of one clone in 1pter with normal genome profiling. A third case revealed a deleted clone on 22q13. Those observations were not confirmed because of the limitation or the quality of the material. Our further studies will be focused on performing a comparative study on a group of samples where the use of classical cytogenetics, molecular biology and array CGH techniques could be possible. An example profile for chromosomes X and 21 in a case with abnormalities on these two chromosomes is shown in Figure 1. Figure 2 shows a second case with double trisomy (for chromosomes 8 and 21).

DISCUSSION

While some of the noted abnormalities in this study are similar to those found in POCs that grow in culture (e.g. trisomy 13, monosomy X), other abnormalities were unexpected. Four of the 15 abnormal cases in our series had double aneuploidy (double trisomy or trisomy plus monosomy). Such findings are found in only 0.7% of routine cytogenetic studies of products of conceptions grown *in vitro* (Reddy, 1997). Another finding not normally noted in studies of growing cells from products of conception is autosomal monosomy. We observed autosomal monosomy in 5 of the 15 cases. The most common findings in cultured products of conception that divide to produce analyzable metaphases are trisomy 16, monosomy X, and triploidy. These three findings account for over 55% of all cytogenetically abnormal findings in routine studies by G-banding (Warburton *et al.*, 1979; Qumsiyeh, 1998). Further, we found structural abnormalities such as amplification of a small area of the X chromosome and deletion of autosomal material. It is not possible to determine if these would have allowed cells to grow in culture or would have been detected by routine cytogenetics if cells grew in culture. But in either case, they add to the significance of studying samples by array CGH. The variety of chromosomes involved in abnormalities that we detected and the patterns of these abnormalities are thus comparable by means of major cytogenetics findings, plus other abnormalities that are different than those noted in classic cytogenetic studies with <1 Mb changes in the genome.

Recently, a few studies used CGH (not array based) on cases already studied by classic cytogenetic methods (i.e. those that grew in culture). Bell *et al.* (2001) looked at nine such cases of SABs and reported that CGH failed to confirm three cases of abnormalities: trisomy 22, triploidy, and monosomy X. Fritz *et al.* (2001) reported on 57 cases successfully studied by CGH and routine cytogenetics: 72% showed abnormalities (mostly trisomies). Tabet *et al.* (2001) used CGH for 21 second-trimester cases already studied by classic cytogenetics. They showed concordance in all cases except in the detection of triploidy and the classic polymorphic pericentric inversion on chromosome 9. Lomax *et al.* (2000) used classic CGH to look at 300 cases studied by classic cytogenetic methods (i.e. those that grew in culture). Correlation was noted in 92.8% of the 253 cases that were analyzable by CGH. Two thirds of the discordant cases could be explained by maternal cell contamination leading to normal female findings by classic cytogenetics.

Schaeffer *et al.* (2004) was the first article to use array CGH to look at SABs (albeit not at cases that failed to grow and yield routine cytogenetic findings). The array methodology was concordant with classic cytogenetic findings and additionally revealed other abnormalities missed by classic cytogenetics. The four abnormalities detected by array CGH but not classic cytogenetics included: additional trisomy 20 (on a background of trisomy 21), deletion 9 (on a background of trisomy 13), dup(15q) (on a background of trisomy 16), and duplication 10q. The fact that in three of these four cases the missed abnormality occurred on a background of other chromosome abnormalities noted by classic cytogenetics may be related to the laboratories focusing on the obvious finding that explains the SAB (e.g. a

Table 1—Summary results of Array CGH for the 26 spontaneous abortion samples studied

Finding	Number of cases
Normal genome profile	11
Abnormal genome profile	15
Trisomy 13	2
Trisomy 21	2
Trisomy 13, 14, Monosomy 1	1
Trisomy 8, Trisomy 21	1
Trisomy 16, Trisomy 22	1
Trisomy 18, Monosomy 21	1
XXY	1
Monosomy X	1
Monosomy X, Monosomy 16	1
Monosomy 21	1
Monosomy 21, amplification on X	1
Loss on 22q13	1
Gain on 1p Terminal	1

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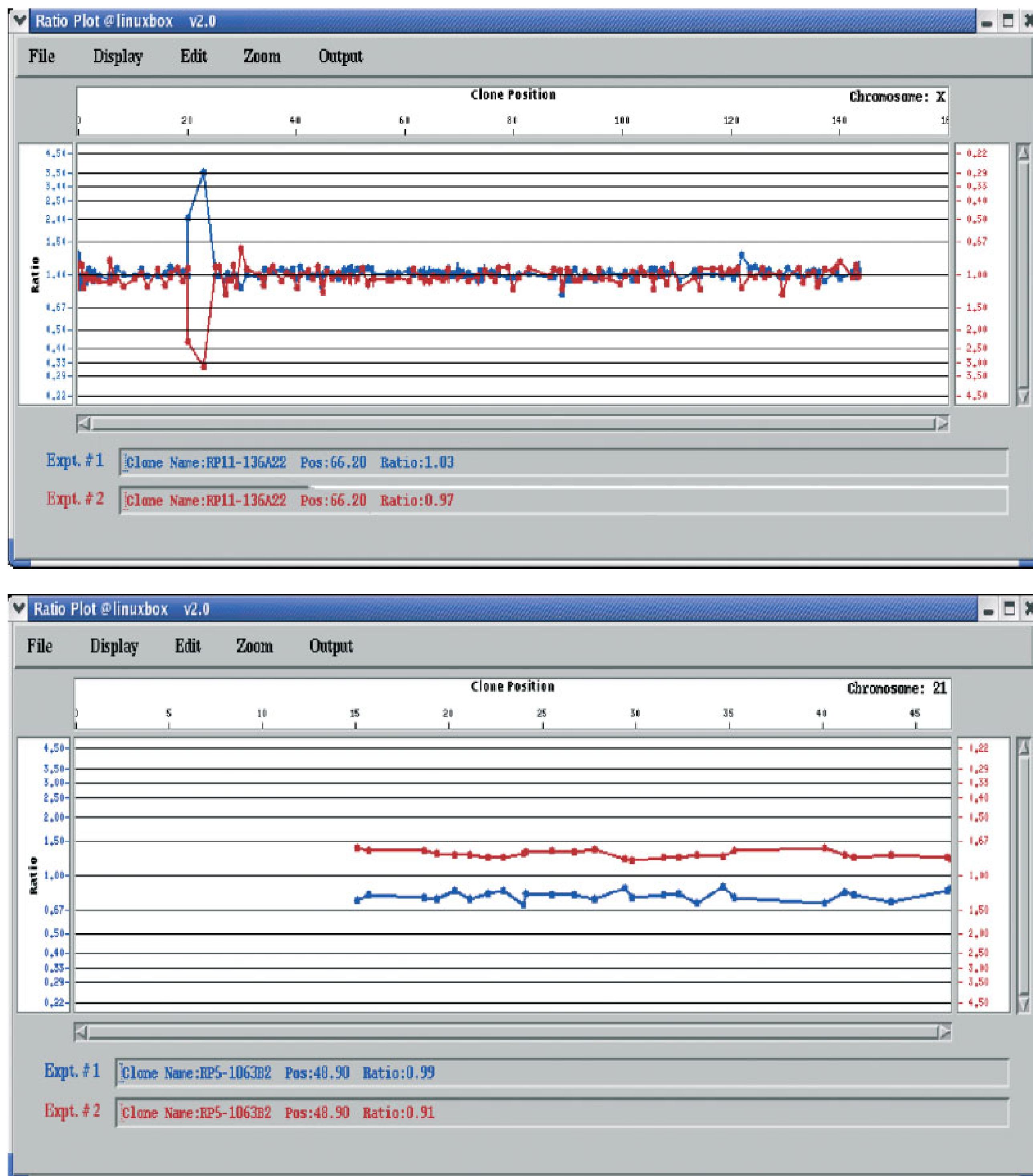

Figure 1—Example of output ratio plots from chromosomes X and 21 for a case showing monosomy 21 and amplification of two BACS on the X chromosome. This shows normalized data in which test sample was labelled with Cy3 and is shown here in blue while the control sample was labelled with Cy5 and shown in red. The left most clone is most distal on the p arm and the right most clone is most distal on the q arm. Data was confirmed using the reverse labelling (test sample in Cy5 and control in Cy3) and the monosomy 21 was confirmed by FISH (data not shown). A loss, amplification, or duplication of a particular clone is manifested as simultaneous deviation of the ratio plots from a modal value of 1.0 (of both test and control values) that exceeds 1.2. Chromosomal areas are interpreted as overrepresented when the ratio exceeds 1.2 which shows DNA copy number gains or 1.5 which shows DNA copy number amplifications and reversely for copy number losses or deletions

trisomy) and missing other abnormalities. In all these studies, the authors limited their analysis to cases already studied by classic cytogenetic methods.

To our knowledge, there has been only one study using classic CGH dealing with SABs that fail to grow in culture (Daniely *et al*., 1998) and another study using

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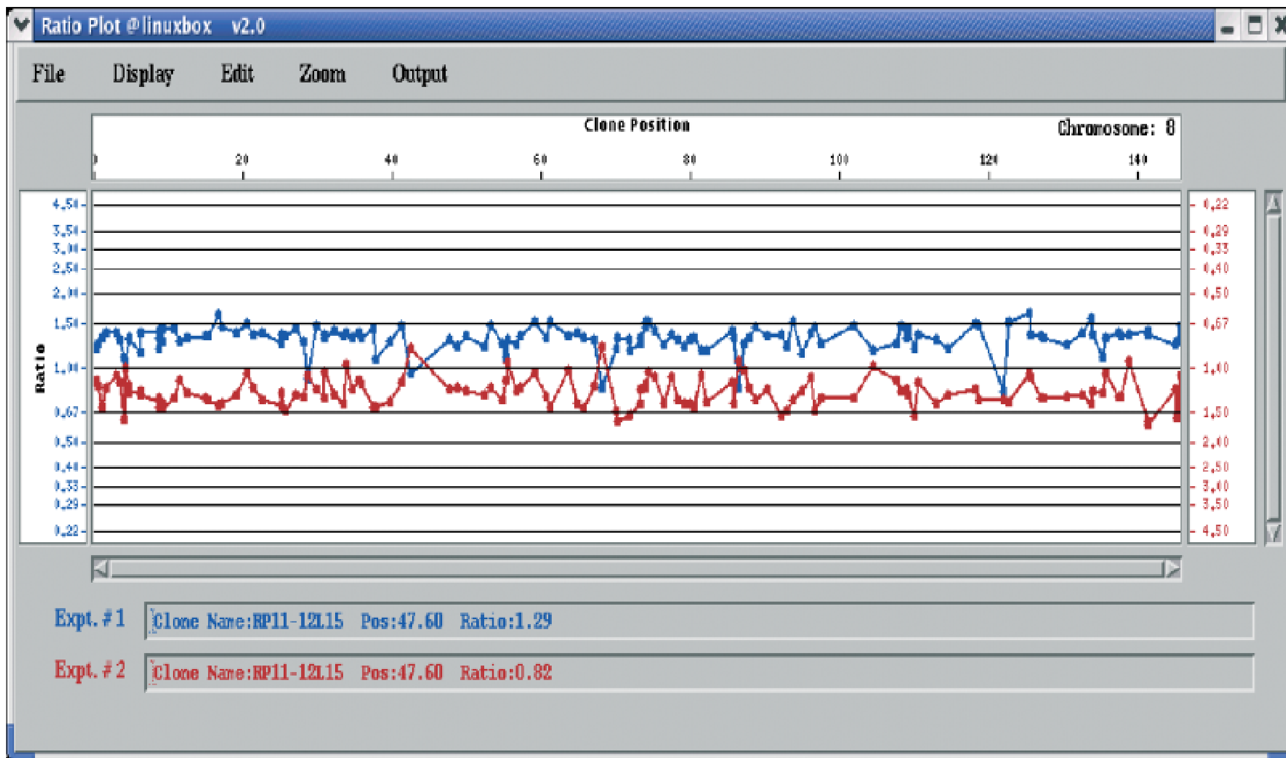

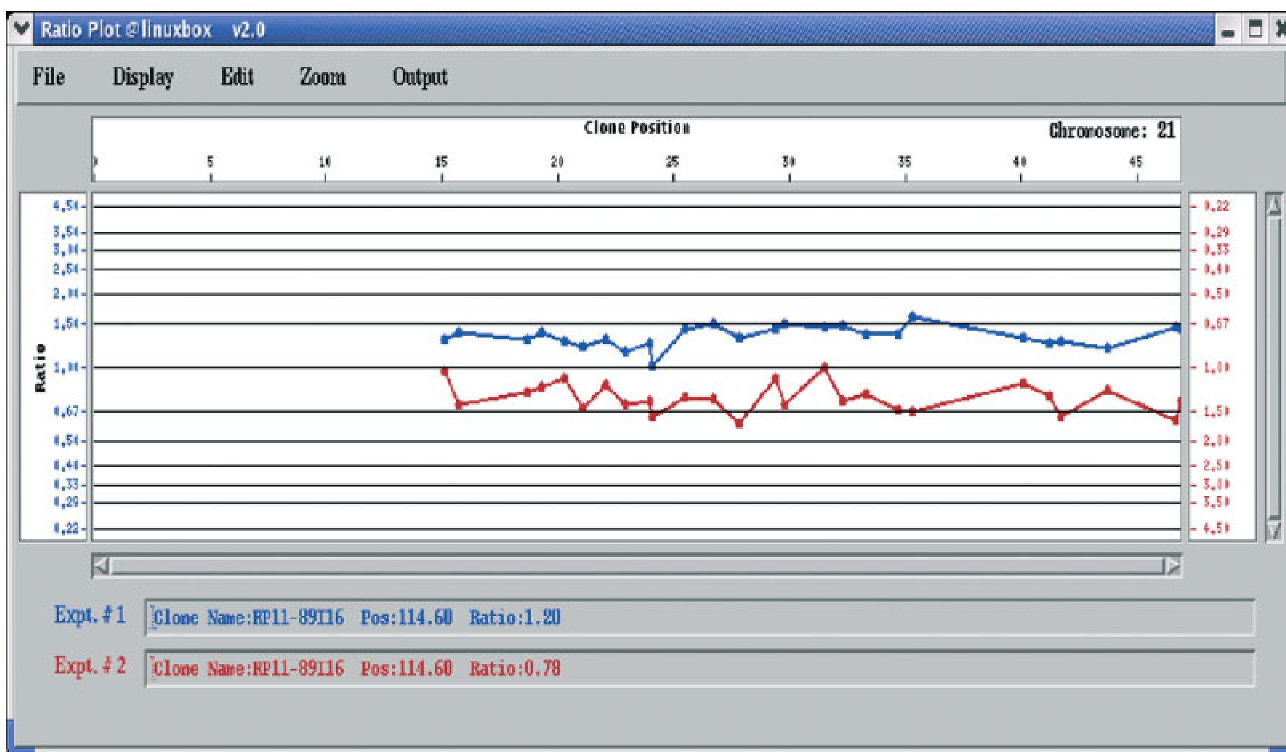

Figure 2—Double trisomy (8 and 21) of 9 weeks spontaneous abortions case. The small variation between specific loci is normal and relates to DNA content and technical variations

a pool of patients who elected to have their studies by classic CGH instead of cytogenetics (Tan *et al*., 2004) and no such studies using array CGH (on cases that did not grow *in vitro*). Daniely *et al*. (1998) showed correspondence in 8 of 12 cases examined by both classic cytogenetics and CGH (75%). The discordance was in failing to detect one case of mosaicism and three cases of structural abnormalities by classic cytogenetic findings. More interesting, for the cases without available classic cytogenetic findings, Daniely *et al*. (1998) found

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approximately half to have copy number variation by CGH. These included the following abnormalities: loss of Y, loss of 19, trisomy for 8, 15, 16, and 22, and six cases of structural abnormalities. These patterns of abnormalities are already different than noted in cases that grew in culture. Tan *et al.* (2004) similarly showed autosomal monosomies and double abnormalities in a series of SABs from IVF patients but his series was done by classic CGH on cases where apparently no classic cytogenetics was attempted.

Because of culture failure, and insufficient material from patients, it has not been possible to confirm all of the findings presented in the present manuscript by independent methods.

Our study used array CGH on cases that failed to grow in culture. Six of the 15 abnormal cases detected by array CGH contained two chromosome abnormalities each (double aneuploidy or one aneuploidy and a structural abnormality). Five cases had autosomal monosomy (Table 1) which is rarely reported from classic cytogenetic studies of POCs. Lebedev *et al.* (2003) using FISH on cases that failed to grow in culture also reported autosomal monosomies in POCs. The most likely explanation for these unusual abnormalities is that specimens containing these chromosomal abnormalities do not do well when cultured and thus fail to produce analyzable metaphases for classic cytogenetic studies. Indeed, it is common to find cell lines with single trisomies or triploidy or monosomy X but it is very rare to find cell lines with autosomal monosomy or double aneuploidy (e.g. cell lines available through the American Type Culture Collection). This is significant since our findings now suggest that part of the failure to get routine cytogenetic studies on some products of conception are perhaps due to having the kinds of abnormalities detectable here by array CGH. These findings complement studies done by classic cytogenetics and will likely lead to revising our estimates of incidence and distribution of chromosome abnormalities in first-trimester fetal loss.

Array CGH use in a clinical cytogenetics is not without limitations. These include the inability to detect polyploidy or balanced chromosome abnormalities. Polyploidy can be easily detected by FISH, microsatellite analysis, or flow cytometry. Balanced translocations are rarely associated with an abnormal phenotype but will still be examined using classic cytogenetics in parents of children with unbalanced translocations or cases of recurrent pregnancy loss or by FISH in known recurrent cancer translocations. For most other clinical cytogenetic studies, array CGH is likely to become the method of choice because of the ability to apply it to nondividing cells or even very few cells. Successful procedures were developed recently for assaying single cells by array CGH for preimplantation genetic diagnosis; for example, see (Handyside *et al.*, 2004; Hellani *et al.*, 2004).

There are other advantages for array CGH. One advantage of array CGH is the increase in resolution that can be achieved compared to chromosome-based CGH. For BAC arrays, the limit of resolution is on the order of 100 to 200 kb with full genome coverage using a minimal tiling path of overlapping clones. Constitutional deletions as small as 40 kb have been detected using an array encompassing a 7 Mb interval of chromosome 22 with 90% coverage (Bruder *et al.*, 2001). Array CGH can also provide a technically less demanding and more sensitive assay than classic CGH or even routine cytogenetics. This is because it is more amenable to automation and provides finer resolution and better quality controls. Thus, it appears likely that in the next few years, array based CGH will become routinely used in clinical cytogenetics in areas ranging from preimplantation diagnosis to fetal losses to cancer cytogenetics and beyond. This study showed that chromosomal analysis on POC samples can be detected without cell culture now by a high-resolution technique like array CGH for molecular karyotyping with a 1 Mb resolution level.

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