Am. J. Hum. Genet. 66:1516–1521, 2000

Comparative Genomic Hybridization in Combination with Flow Cytometry Improves Results of Cytogenetic Analysis of Spontaneous Abortions

Brenda Lomax,[1] Steven Tang,[1] Evica Separovic,[1] Don Phillips,[2] E. Hillard,[2] Tom Thomson,[2] and Dagmar K. Kalousek[1]

[1]Department of Pathology and Laboratory Medicine, University of British Columbia, and British Columbia Research Institute for Children's and Women's Health and [2]Department of Laboratory Medicine, Analytic Cytology Department, British Columbia Cancer Agency, Vancouver

More than 50% of spontaneous abortions (SAs) have abnormal chromosomes; the most common abnormalities are trisomy, sex chromosome monosomy, and polyploidy. Conventional cytogenetic analysis of SAs depends on tissue culturing and is associated with a significant tissue culture failure rate and contamination by maternally derived cells. Comparative genomic hybridization (CGH), in combination with flow cytometry (FCM), can detect numerical and unbalanced structural chromosomal abnormalities associated with SAs while avoiding the technical problems associated with tissue culture. Routine cytogenetic and CGH analysis was performed independently on tissue from 301 SAs. Samples shown to be chromosomally balanced by CGH were analyzed by FCM to determine ploidy. Of 253 samples successfully analyzed by both approaches, there was an absolute correlation of results in 235 (92.8%). Of the 18 cases with discrepancies between cytogenetic and CGH/FCM results, an explanation could be found in 17. Twelve samples produced a 46,XX karyotype by cytogenetics, whereas CGH/FCM demonstrated aneuploidy/polyploidy or a male genome, indicating maternal contamination of the tissue cultures. In two cases, where tetraploidy was demonstrated by cytogenetics and diploidy by FCM, tissue culture artifact is implied. In three cases, CGH demonstrated an aneuploidy, and cytogenetics demonstrated hypertriploidy. In one unexplainable case, aneuploidy demonstrated by CGH could not be detected by repeat CGH analysis, conventional cytogenetic, or FISH analysis. These results demonstrate that CGH supplemented with FCM can readily identify chromosomal abnormalities associated with SAs and, by avoiding maternal contamination and tissue culture artifacts, can do so with a lower failure rate and more accuracy than conventional cytogenetic analysis.

Introduction

More than 50% of all spontaneous abortions (SAs) have abnormal chromosomes, mainly numerical chromosomal defects such as trisomy of autosomes (29%), monosomy X (10%), polyploidy (10%), and mosaicism or structural abnormalities (2%) (Hassold 1986; Kalousek et al. 1993). Cytogenetic analysis of an aborted conceptus provides valuable information regarding the recurrence risk and possible therapies for couples experiencing recurrent SAs. Cytogenetic studies of SAs rely on obtaining viable tissue, establishing primary cultures, and harvesting metaphase chromosomes for analysis. The methodology of cell culture and analysis of chromosomes is well established and has been accepted as a clinical diagnostic technique for the past 4 decades (Rooney and Czepulkowski 1997). It is well recognized that this approach has limitations—specifically, the relatively high rates of tissue culture failure (10%–40%) and selective overgrowth of maternally derived cells such that the finding of a normal female karyotype may not be representative of the conceptus (Bell et al. 1999).

Comparative genomic hybridization (CGH) is a technique that offers a molecular approach to cytogenetic analysis and allows the entire genotype to be screened in a single hybridization (Kallioniemi et al. 1992). The CGH technique involves the simultaneous hybridization of genomic test and reference DNAs, each labeled with a different fluorochrome, to normal target metaphase chromosomes. By comparing the relative intensities of the two fluorochromes along the length of each target chromosome, one can detect variations in DNA copy number between the test and reference genomes. CGH can readily identify numerical and unbalanced structural chromosomal abnormalities. It has been successfully applied to the evaluation of postnatal cases in which traditional cytogenetic analysis yielded ambiguous results (Levy et al. 1998). To date, the application of CGH to the analysis of conceptuses has been limited (Bryndorf et al. 1995; Atkins et al. 1997; Daniely 1998),

Received January 25, 2000; accepted for publication February 21, 2000; electronically published March 30, 2000.

Address for correspondence and reprints: Dr. Dagmar K. Kalousek, British Columbia Children's and Women's Hospital, 4480 Oak Street, Vancouver, B.C., Canada V6H 3V4. E-mail: dkalousek@cw.bc.ca

0002-9297/2000/6605-0006$02.00

because of the fact that, although CGH can readily detect chromosomal imbalances, it is unable to differentiate between diploid, triploid, and tetraploid states.

Flow cytometry (FCM) can be used for the quantitative determination of cellular DNA content. FCM has been used extensively for ploidy analysis of molar pregnancies and has gained widespread popularity because it is easy to perform, rapid, accurate, and inexpensive (Berezowsky et al. 1995). By combining CGH analysis with FCM, it is feasible to screen a test DNA for chromosomal imbalances with CGH and, when no imbalance is detected, to determine the ploidy status with FCM. With this approach, it is possible to detect virtually all numerical and unbalanced structural abnormalities that are associated with SAs. In the present report, we describe the first controlled study of SAs, comparing conventional cytogenetic analysis to a novel approach that uses CGH analysis supplemented with FCM.

Material and Methods

Study Population

The study population includes products of 301 first- and second-trimester SAs which were examined at the embryofetopathology laboratory of the British Columbia (B.C.) Children's and Women's Hospital. The majority of samples were obtained from abortions in which there was a maternal history of advanced maternal age or repeated pregnancy loss or from those with phenotypic abnormalities of the embryo or fetus. The samples were taken from amniotic membrane and/or chorionic sac and varied in size from 10–100 mg. All samples were submitted for routine cytogenetic analysis to the clinical cytogenetics laboratory of the B.C. Children's and Women's Hospital, with duplicate tissue samples being submitted for CGH/FCM analysis.

Tissue Preparation and DNA Isolation

All tissues were grossly examined, freed of any maternal decidua, and washed clean of blood. High–molecular-weight DNA was isolated from the tissue by the high salt extraction method described by Miller et al. (1988). After extraction, DNA was precipitated in two volumes of cold ethanol and dissolved in 10 mM Tris, 0.2 mM EDTA, pH 8.

DNA Labeling and CGH Technique

Labeling and hybridization protocols were performed as described by Lestou et al. (1999). Test DNA was labeled with FITC-12-dUTP and the 46,XX reference DNA with TRITC-6-dUTP.

Preparation of Metaphase Chromosomes

Diploid metaphase spreads were obtained from peripheral blood lymphocyte cultures of normal male donors by use of standard protocols. Male target metaphases were used to provide copy number information about all chromosomes, including Y. Slide preparation was optimized to minimize residual cytoplasm and to generate well-spread metaphases of 350–400 band-length resolution.

Digital Image Analysis

Image capture and analysis were performed by means of a Zeiss Axioplan epifluorescence microscope with selective filters, a COHU charge coupled device camera, and Perceptive Scientific Instruments POWERGENE software. The criteria used for assessing the quality of CGH preparations are described by Kallioniemi et al. (1994). A minimum of four high-quality metaphase spreads were analyzed from each hybridization, and average red-to-green fluorescence intensity ratio profiles were generated for each chromosome.

Interpretation of CGH Ratio Profiles

A shift in the average red-to-green ratio value (plus 2 SD) above 1.1 was considered an indication of increased copy number (i.e., trisomy), and any shifts below 0.9 were considered an indication of reduced copy number (i.e., monosomy). Heterochromatic regions near the centromeres of chromosomes 1, 9, and 16, the q arm of the Y chromosome, and the satellite regions of the acrocentric chromosomes were not considered in our interpretation of the CGH profile analysis, because these regions are polymorphic and are largely suppressed by the Cot-1 DNA. CGH ratio profiles for chromosomes 1pter, 16p, 19, and 22 were interpreted with caution, because these regions are subject to labeling artifacts that can result in variations in the ratio profiles (Kallioniemi et al. 1994; Lestou et al. 1999). Although it may be theoretically possible to differentiate between a 46,XY genotype and a 69,XXY genotype by the degree of shift in X-chromosome profile (0.5 vs. 0.66, respectively), in light of the inherent variability of CGH profiles and the possibility of maternal contamination of the test DNA, for the purposes of this study we have not used CGH profiles to determine ploidy.

FCM Analysis

All samples shown by CGH analysis to be balanced were subsequently sent for FCM analysis to the analytic cytology laboratory at the British Columbia Cancer Agency. Nuclear suspensions were prepared from formalin-fixed, paraffin-embedded chorionic villous tissue,

as described by Hedley et al. (1983). FCM analysis was performed on a Beckman/Coulter Epic Elite Flow cytometer. Data were analyzed with Beckman/Coulter ELITE software and MULTICYCLE software (Phoenix Flow Systems).

FISH Analysis

FISH was performed on trophoblast suspension by means of unique sequence and α-satellite DNA probes (Oncor), as described by Henderson et al. (1996). At least 100 interphase nuclei were analyzed per sample.

Sex Determination

Sex determination by the use of primers that flank a region of the Y chromosome–specific SRY gene (BioCan Scientific/GENOSYS) was performed on four samples for which maternal contamination of the tissue cultures was suspected and for which CGH analysis indicated a male genome. PCR amplification was done under standard conditions, and the resulting male-specific 239-bp band was visualized on a 1% agarose gel.

Results

A total of 301 samples were submitted for both conventional cytogenetic and CGH/FCM analysis. Thirty-five samples failed to produce metaphase chromosomes of sufficient quality for cytogenetic analysis. Extraction of DNA from seven samples did not yield adequate DNA for CGH analysis. In three samples, both tissue culture and DNA extraction failed. Thirteen samples that demonstrated a balanced CGH profile could not be subsequently analyzed by FCM, because no formalin-fixed, paraffin-embedded chorionic villous tissue was available, and four of the same samples also failed tissue culture. Therefore, 48 cases could not be analyzed by one or more method(s).

We successfully analyzed 253 samples by both conventional cytogenetic and CGH/FCM analyses (table 1). Identical findings were obtained by the two approaches for 235 cases, a 92.8% correlation rate. Discrepancies between the cytogenetic and CGH/FCM results occurred in 18 samples (table 2). Twelve samples (4.7%) with a 46,XX karyotype by cytogenetic analysis demonstrated aneuploidy/polyploidy or a diploid male genome by CGH/FCM, thereby suggesting maternal contamination of the tissue cultures. In addition, sex determination of cases 3, 4, 7, and 10 (table 2) demonstrated a PCR product for the SRY marker, which indicates the presence of a Y chromosome. FISH analysis, by means of a chromosome 22–specific DNA probe of trophoblast from case 6, confirmed the trisomy 22 detected by CGH analysis. In two cases (13 and 14) in which tetraploidy was demonstrated by cytogenetics and diploidy by FCM, tissue culture artifact is implied. Additional FISH analysis of case 14, by means of α-satellite DNA probes for chromosomes 7 and 2, demonstrated only two copies of each chromosome pair. In three cases, a chromosomal imbalance was demonstrated by CGH, and, therefore, the samples were not initially analyzed by FCM. Cytogenetic analysis of these three samples demonstrated triploidy in combination with the same chromosomal imbalances that were detected by CGH. Subsequent FCM analysis confirmed polyploidy in all three samples. In one final case, CGH analysis demonstrated aneuploidy that was not detected on repeat testing or by conventional cytogenetic analysis and subsequent FISH analysis.

Table 1

Summary of 253 Cases Analyzed by both Conventional Cytogenetic and CGH/FCM Analyses

Finding	Cytogenetic Analysis	CGH/FCM Analysis
Diploidy	98 (38.7)	92 (36.4)
Polyploidy	25 (9.9)	21 (8.3)
Aneuploidy	111 (43.9)	121 (47.8)
Monosomy X	12 (4.7)	12 (4.7)
Structural imbalance	7 (2.8)	7 (2.8)

NOTE.—Numbers in parentheses are percentages.

Discussion

Our aim was to determine whether the CGH technique is as effective as traditional cytogenetics for analysis of SAs. As anticipated, the rate of failure for CGH (2%) was lower than that for cytogenetic analysis (12%). We found that only 7 of 301 samples were too small for extraction of adequate DNA for CGH analysis, as compared with 35 of 301 samples that failed to grow or produce sufficient metaphase chromosomes for conventional analysis. This finding indicates that the collection of samples for CGH analysis is easier than for conventional methods, because the main limiting factor is the size of the sample. We found that with experience, when 20 mg of the tissue was provided, CGH results could be guaranteed. The fact that, among 35 tissue culture failures, CGH analysis provided results in 32 cases represents a significant improvement from the reproductive counseling perspective. Among these 32 cases, the diagnosis of aneuploidy was made in 20 and balanced karyotype in 12.

Maternal contamination represented the main explainable discrepancy between conventional cytogenetic analysis and CGH analysis. In 12 women diagnosed as

Table 2

Summary of 18 Cases with Discrepancy between Results Obtained by Conventional Cytogenetic and CGH/FCM Analyses

Case	CGH Result	FCM	Cytogenetic Karyotype	Additional Analysis	Interpretation
1	XX, gain for 19	...	46,XX	NA	Maternal contamination
2	XX, gain for 3 and 10	...	46,XX	NA	Maternal contamination
3	XY, gain for 21	...	46,XX	SRY+ve	Maternal contamination
4	XY, gain for 4q	...	46,XX	SRY+ve	Maternal contamination
5	XX, gain for 15	...	46,XX	NA	Maternal contamination
6	XX, gain for 22	...	46,XX	FISH: trisomy (D22S75)	Maternal contamination
7	XY, balanced	Diploid	46,XX	SRY+ve	Maternal contamination
8	XX, gain for 22	...	46,XX	NA	Maternal contamination
9	XX, gain for 22	...	46,XX	NA	Maternal contamination
10	XY, gain for 20	...	46,XX	SRY+ve	Maternal contamination
11	XX, balanced	Tetraploid	46,XX	NA	Maternal contamination
12	XX, balanced	Tetraploid	46,XX	NA	Maternal contamination
13	XY, balanced	Diploid	92,XXYY	NA	Tissue culture artifact
14	XY, balanced	Diploid	92,XXYY	FISH: disomy D7Z1/2 and D2Z1	Tissue culture artifact
15	XY, gain for 8, loss for 18	Triploid	69,XXY,+8,−18	NA	Hypertriploidy
16	XX, gain for 14	Triploid	70,XXX,+14	NA	Hypertriploidy
17	XY, gain for 4	Triploid	70,XXY,+4	NA	Hypertriploidy
18	XY, gain for 18	...	92,XXYY,−1,+idic(1)(p31)	FISH: tetraploidy D18Z1 and D8Z1	Sample mislabeling

NOTE.—Ellipses points (...) indicate that FCM was not performed because CGH analysis indicated an unbalanced karyotype. SRY+ve indicates that microsatellite analysis demonstrated presence of Y chromosome–specific marker. NA indicates that insufficient tissue was available for additional analysis.

having a normal female karyotype by traditional cytogenetics, a male karyotype with aneuploidy was detected three times, a normal male karyotype once, a female karyotype with aneuploidy six times, and tetraploidy twice. The detection of aneuploidy is especially important for reproductive counseling, because the determination of a normal karyotype is usually interpreted as a poor prognostic sign and leads to more-intensive investigation of the cause of pregnancy loss. In an effort to confirm our interpretation of maternal contamination, additional analyses were performed when possible. Sex determination by the use of SRY-specific sequences was performed on four cases that demonstrated a male karyotype by CGH analysis. All four cases showed the presence of the Y chromosome–specific marker. FISH analysis by means of chromosome 22–specific DNA probes confirmed the trisomy 22 diagnosed by CGH. Together, these additional analyses support our interpretation of maternal contamination.

It was interesting to note that maternal contamination is not the only tissue culture artifact observed in cytogenetic analysis of SAs. In two separate cases, we had traditional cytogenetic diagnosis of tetraploidy, whereas CGH/FCM demonstrated diploidy. High levels of tetraploidy or even complete tetraploidy have elsewhere been described in cultured amniotic fluid in pregnancies with a normal diploid fetus (Kohn and Robinson 1970). Additional tissue was available for FISH analysis of one of our cases, and the results showed only two copies of each chromosome pair tested.

Hypertriploidy detected by traditional cytogenetic analysis was interpreted from CGH results as aneuploidy. When the study was finalized and the results of traditional analysis and CGH compared, it was obvious that three cases with hypertriploidy were interpreted on CGH as aneuploidy and were not submitted for FCM analysis. Retrospective FCM analysis has confirmed the presence of triploidy. From a reproductive-counseling point of view, the knowledge of existing aneuploidy and the lack of knowledge about the triploidy does not change the management of the future pregnancy.

Only human error (i.e., sample mislabeling) can explain one discrepancy, in which the original CGH analysis showed unquestionable trisomy 18 in a male karyotype. Traditional cytogenetic analysis showed tetraploidy, which was confirmed by FISH analysis (table 2). After having repeated the CGH analysis several times, we could demonstrate only a balanced male karyotype.

The advantages of CGH analysis of SAs are broader than diagnostic accuracy. Technically, CGH analysis allows collected tissues to be stored in the freezer and batching of DNA extraction, labeling, and hybridization, thereby increasing technical efficiency. Because one

technologist can process twice as many samples per unit of time, the entire procedure is more cost-effective than traditional cytogenetic analysis combined with tissue culture. An additional FCM analysis is required for all diploid samples, however, because the distinction between polyploidy and diploidy is an important one for reproductive counseling. In the future, we plan to perform FCM on a portion of frozen tissue sample set aside prior to DNA extraction, rather than on formaldehyde-fixed and paraffin-embedded placental tissue.

Of critical importance to the accuracy of the analysis is the correct identification of the aborted tissues, such that maternal tissues are not used for analysis. Although a DNA-based approach avoids the problems associated with tissue culturing, the integrity of the analysis depends on extracting DNA from an appropriate tissue. Although CGH has been shown to be capable of detecting placental mosaicism as low as 32% (Lomax et al. 1998), because of the inherent variability of the technique (Lestou et al. 1999), the presence of high levels (60%–70%) of maternal cells in a tissue sample may render CGH analysis ineffective in the determination of fetal aneuploidy or a male fetus. Proper identification and separation of maternal and fetal/placental tissues is essential if this approach is to be effective.

It would be of interest to repeat cytogenetic studies of SAs with elimination of culture failure, culture artifact, and maternal contamination. Some classic articles in this field do not mention the rate of culture failure (Bouié et al. 1976), whereas others describe failure rates of 47% (Creasy et al. 1976) and slightly less than one-third (Kajii et al. 1980). If our CGH results from cases with tissue culture failure represent a general trend, then about two-thirds of the failed specimens would have shown chromosomal aneuploidy. Further, by eliminating maternal contamination, the rate of chromosomal aneuploidy (trisomy) would be, in general, higher than is currently accepted. The repetition of earlier studies would allow even greater accuracy in karyotype-phenotype correlation, because of the availability of ultrasound morphology of missed abortions.

In summary, the CGH/FCM analysis provided accurate information on the chromosomal complement of SAs in 99.7% of analyzable cases. In only one case (case 18), the result obtained by CGH could not be confirmed in repeated CGH experiments or explained by cytogenetic or FCM findings. It was finally attributed to human error. We do, however, believe that the advantages of CGH overcome the difficulties we experienced in explaining this one case. CGH/FCM analysis not only identified cases with maternal contamination and tissue culture artifacts that provided misleading clinical information but also revealed, for the first time, the chromosomal composition of tissues from SAs that failed to grow in culture. Future extensive CGH analysis of tissue-culture failures will provide additional insight and may even change our knowledge on the frequencies of chromosomal abnormalities in SAs.

Acknowledgments

This study was supported by the Vancouver Foundation grant BCM98-0004 and the March of Dimes grant FY97-0275. Sex determination was performed in Dr. Wendy Robinson's laboratory. We are very grateful to Dr. Deborah McFadden, Dr. Tapio Pantzar, Tamiko Suzuki, Sui-Imm Tay, and Kent Neuert, from the Ermbryofetopathology Laboratory of the B.C. Children's and Women's Hospital, for their assistance and cooperation with this project.

References

Atkins D, Storkel S, Brinkschmidt C, Simon R, Yelbuz M (1997) Comparative genomic hybridization (CGH) in fetal pathology (abstract). Pediatr Pathol Lab Med 17:707

Bell KA, Van Deerlin PG, Haddad BR, Feinberg RF (1999) Cytogenetic diagnosis of "normal 46,XX" karyotypes in spontaneous abortions frequently may be misleading. Fertil Steril 71:334–341

Berezowsky J, Zbieranowski I, Demers J, Murray D (1995) DNA ploidy of hydatiform moles and nonmolar conceptuses: a study using flow and tissue section image cytometry. Mod Pathol 8:775–781

Bouié J, Phillippe E, Giroud A, Boué A (1976) Phenotypic expression of lethal chromosomal anomalies in human abortuses. Teratology 14:3–20

Bryndorf T, Kirchhoff M, Rose H, Maahr J, Gerdes T, Karhu R, Kallioniemi A, et al (1995) Comparative genomic hybridization in clinical cytogenetics. Am J Hum Genet 57: 1211–1220

Creasy MR, Crolla JA, Alberman ED (1976) A cytogenetic study of human spontaneous abortions, using banding techniques. Hum Genet 31:177–196

Daniely M, Aviram-Golding A, Barkai G, Goldman B (1998) Detection of chromosomal aberration in fetuses arising from recurrent spontaneous abortion by comparative genomic hybridization. Hum Reprod 13:805–809

Hassold T (1986) Chromosome abnormalities in human reproductive wastage. Trends Genet 2:105–110

Hedley DW, Friedlander ML, Taylor IW, Rugg CA, Musgrave EA (1983) Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. J Histochem Cytochem 31:1333–1335

Henderson KG, Shaw TE, Barrett IJ, Telenius AHP, Wilson RD, Kalousek DK (1996) Distribution of mosaicism in human placentas. Hum Genet 97:650–654

Kajii T, Ferrier A, Niikawa N, Takahar H, Ohama K, Avirachan S (1980) Anatomic and chromosomal anomalies in 639 spontaneous abortuses. Hum Genet 55:87–98

Kallioniemi A, Kallioniemi OP, Piper J, Isola J, Waldman F, Gray JW, Pinkel D (1994) Optimizing comparative genomic hybridization for analysis of DNA sequence copy number

changes in solid tumors. Genes Chromosomes Cancer 10: 231–243

Kallioniemi A, Kallioniemi OP, Sudar D, Rudovitz D, Gray JW, Waldman F, Pinkel D (1992) Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258:818–821

Kalousek DK, Pantzar T, Tsai M, Paradice B (1993) Early spontaneous abortion: morphologic and karyotypic findings in 3,912 cases. Birth Defects 29:53–61

Kohn G, Robinson A (1970) Tetraploidy in cells cultured from amniotic fluid. Lancet 2:778–779

Lestou VS, Lomax BL, Barrett IJ, Kalousek DK (1999) Screening of human placentas for chromosomal mosaicism using comparative genomic hybridization. Teratology 59:325–330

Levy BL, Dunn TM, Kaffe S, Kardon N, Hirschhorn K (1998) Clinical applications of comparative genomic hybridization. Genet Med 1:4–12

Lomax BL, Lestou VS, Barrett IJ, Kalousek DK (1998) Confined placental mosaicism for chromosome 7 detected by comparative genomic hybridization. Prenat Diagn 18:752–754

Miller SA, Dukes D, Polesky HF (1988) A simple salt procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215

Rooney DE, Czepulkowski BH (1997) Essential techniques series: human chromosome preparation. Wiley, Chichester, West Sussex, UK, pp 1–2