Comparative genomic hybridization analysis of fetal chromosomal aberrations

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Comparative genomic hybridization (CGH) can now be applied to detect the origin of extra or missing chromosomal material in cases with common unbalanced aberrations and in prenatal investigations. This method has been used in 13 cases of fetal samples for this study; 3 for amniocytes, 1 for cord blood and 9 for abortus tissues. These samples were previously subjected to GTG-banding. Our study showed aneuploidy in 9 cases, and partial monosomy, partial trisomy or marker chromosome in the remaining 5. The CGH disclosed further small genetic imbalances in 4 of all 13 cases: a prenatal sample showing del(20)(q13) by GTG confirmed a loss of the segment 20p13-pter by CGH; a marker chromosome manifested normal CGH profile; chromosome der(?)(?;15) found in an abortus sample by GTG turned out to be a loss of 15pter-q14 (partial monosomy) and a gain of 10pter-q22 (partial trisomy); the der(15) shown by GTG represented partial trisomy of 3q24-qter. These findings show that CGH is very useful and efficient for cytogenetic investigations of clinical cases.

Keywords: GTG-banding, comparative genomic hybridization, fetal samples, unbalanced aberrations

INTRODUCTION

The comparative genomic hybridization (CGH) method which has been developed recently allows the use of DNA extracted from fetal samples such as amniotic fluid cell, chorionic villi cell, and abortus, to find out the amplification and the deletion of DNA copies in the chromosomes (Kallioniemi *et al.*, 1992; Piper *et al.*, 1994). This method is a powerful new method for molecular cytogenetic tool in the field of clinical genetics. CGH experiment requires test DNA and reference DNA, labelled with fluorochrome as green and red respectively, equally mixed. Then, greater volume of unlabelled repetitive cot1 DNA is added to the mixture to block repetitive sequence region. Afterwards, the mixture is hybridized on a normal metaphase slide, making it possible for the analysis of the abnormal region by digital image analysis system which shows different fluorescent intensity for chromosomal abnormalities. After the CGH, metaphases are analyzed using a digital imaging microscope equipped with triple band pass splitter system that allows separate acqusition of images of blue, green, and red fluorescence. The blue fluorescence image shows the DAPI staining pattern used to identify each chromosome, and the green and red fluorescence images show the test and reference DNA hybridization patterns respectively. If the test DNA has amplification of long arm of chromosome 1, we would be able to see the intensity of green color in relation to the degree of the amplification (green/red ratio: >1.0). This method would make the analysis of genetic changes possible as well as chromosomal abnormalities in the single cell with minute DNA, in archival slides and in paraffin embedded tissues. In case of deletion of the reference DNA increased intensity of the red will be observed (green/red ratio: <1.0). This experimental procedure requires 36-48 h.

With the development of improved ultrasonography and triple marker test for the determination of fetal health, prenatal chromosome analysis is becoming important. So far, in order to analyse chromosomal abnormalities of fetus, the procedure required cell culture process in most cases. Generally, for prenatal diagnosis, 2-3 weeks are required for chromosome analysis of the fetus. In the case where chromosomal aneuploidy is suspected through the triple marker test and ultrasonography, although primed

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in situ labelling (PRINS) or fluorescence *in situ* hybridization (FISH) method is the fastest available, its limitation is that single experiment doesn't yield a confirmation of abnormalities in several genetic loci. However, because CGH can detect abnormalities of all chromosomes with a single experiment making it a more powerful tool for the chromosome analysis of a fetus. These CGH findings need to be confirmed by FISH with chromosome-specific probes to fetal metaphase spreads (Lichter *et al.*, 1988; Pinkel *et al.*, 1988). In addition, CGH can be applied to the abortus tissue, which has been autolysed or necrosed in the uterus, or for minute amount of fetal cells, both of which can not be cultured. In the case of first trimester abortion, chromosome analysis using abortus tissues in order to determine the cause of the abortion is necessary. This study aimed at future clinical application for the chromosome analysis of abortus tissues which has been autolysis and rendered unculturable, or for rapid prenatal chromosome analysis. We performed CGH for 13 cases of fetal cell, 9 for abortus tissues, 3 for amniotic fluid and 1 for cord blood.

In this study, we compared the chromosome analysis obtained by CGH with that of obtained by cell culture process.

MATERIALS AND METHODS

Preparation of fetal samples and DNA extractions

We have taken 13 cases of numerical and structural chromosomal aberrations from the samples referred to our laboratory for prenatal diagnosis or for detecting the causes of unwanted termination between the years 1996 and 1998. Of a total of 13 cases, 9 were abortus tissues, 3 were amniotic fluid cell, and the remaining 1 was the cord blood. For the purpose of CGH experiments, for the 3 amniotic fluid cell samples 1 was from 1 ml of amniotic fluid kept at -70˚C for further study, and the remaining 2 cases of amniotic fluid cells came from culture samples. 50 to 100 mg of the abortus tissues and the lysis pellet of 300 µl cord blood kept at -70˚C were used. DNA was extracted from two amniotic fluid cell cultured samples, eight abortus tissue samples, and normal male and female lymphocyte samples followed by Sambrook (1989). The case of cord blood was used by the gentle kit (Takara shuzo Co.). While 2 cases of amniotic fluid cell cultured samples and 8 cases of abortus tissue samples yielded sufficient DNA. When the yield of DNA of cord blood and amniocyte was insufficient, DOP-PCR was performed and the products were precipitated in 95% ethanol and centrifugated at 14,000 *g* (Telenius *et al.*, 1992). After the spin, the precipitated DNA was washed with 75% alcohol and dried. 100-200 µl of distilled water was put to the dried DNA. Then, we applied the CGH shaking the tube for solving.

Target metaphase slides

Spreads of the metaphase on the slide were prepared according to standard protocols from phytohemagglutinin-stimulated peripheral blood in MEM medium (Gibco Co.), supplemented with 10% fetal calf serum (Hyclone Co.) from a karyotypically normal male and female. About 20-30 slides were prepared at a time. Slides with high index of metaphases were used for the CGH. For the target slides, before hybridization, the slides were denatured at 70˚C in 70% formamide, 2X SSC solution for 3-4 min.

CGH and digital imaging

All samples of both test and reference DNAs were indirectly labeled by nick-translation kit (Vysis Co.) with digoxigenin-11-dUTP and biotin-16-dUTP respectively (Boehringer Mannhein Co.) as described by Kallioniemi *et al.* (1992). After nick translation, we were able to obtain the DNA fragments of approximately 500-1500 base pair (Kallioniemi *et al.*, 1994) as measured on the agarose gels. Chromosomal DNA on the target slide was denatured prior to hybridization by incubated metaphase slides in deionized 70% formamide, 2X SSC (1X SSC: 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7.0) at 72˚C for 3 min and dehydrated in 100% ethanol. For the hybridization, 500 ng of labeled test DNA, 500 ng reference DNA and 10 µg of unlabeled Cot1 DNA (Boehringer Mannhein Co.) were mixed and precipitated in 100% ethanol, dried and dissolved in the hybridization buffer (50% formamide/ 2X SSC/ 10% dextran sulphate).

The probe mixture was denatured at 70˚C for 5 min and dropped on the denatured target metaphase slide. A cover slip was overlaid and sealed with rubber cement. Slide was hybridized at 37˚C in a moist chamber for 36-48 h. After taking the slides out of the wet chamber and carefully removing the coverslips, slides were washed in 4X SSC/ 0.1% Tween, three times for 5-10 min at 42˚C in a couplin jar placed in a shaking water bath. To avoid hybridization of repeated sequences, slides were in blocking solution (3% bovine serum albumin(BSA), 4X SSC, 0.1% Tween) at 37˚C for 30 min. Hybridized test and reference DNA with chromosome was stained with 200 µl of detection solution containing the 5 µg/ml of anti-digoxigenin-fluorescein Fab fragment (green) and 6 µg/ml of streptoavidine-

sulforhodamin 101 (red) prior to counterstaining with 4',6-diamidino-2-phenylindole (DAPI, 40 ng/ml) in antifade agent (Vectashield, Vector Laboratories, Inc., Burlingamem CA) (Florijn *et al.*, 1995).

The acquisition of images is carried out through the entire slide to find the best metaphase cells. Generally, images of 13-15 metaphases of the good quality are collected from each slide. Images were captured with uncooled charge-coupled device (CCD) camera controlled by a Cytovision image analysis system (Applied Imaging Co.). Applied Image system is based on a Zeiss Axioscope fluorescence microscope equipped with triple band pass beam splitter and emission filters by computer-controlled filter-wheel.

Fluorescent *in situ* hybridization (FISH)

Fluorescence in situ hybridization with whole chromosome probes specific for chromosome 3 and 10 (Cytocell, Applied Image Co.) were used for the confirmation of cases the translocated chromosome. The slides were washed in 2X SSC for 2 minutes at room temperature, then dehydrated by soaking in 70%, 85% and 100% ethanol for 2 min each sequentially. Each probe was denatured on a hotplate at 75˚C for 5 min and covered on the target metaphase slide. The slides with covered probes were transferred to humid box and hybridized for overnight at 37˚C. After hybridization, coverslides with probe were removed and the slides were washed in 50% formamide/ 1X SSC for 5 min at 45˚C and ST buffer (4X SSC, 0.05% Tween) for 5 min at room temperature. The slides were counterstained with DAPI (40 ng/ml) in 10 µl of antifade solution under the coverslips and viewed by Zeiss Axioscope fluorescent microscope.

RESULTS

Of the total 13 fetal samples, CGH results for 8 abortus cases and 1 prenatal case, all having numerical aberrations, were in concordance with the results of cytogenetic analysis previously applied (Table 1, Fig. 1). However 4 cases with structural abnormalities showed greater accuracy with CGH when compared to the results of chromosome analysis. In the case of C1, which was thought to be the del(20)(q13) through chromosome analysis, was found to be del(20)(p13) by the CGH method (Fig. 2A). To determine the cause of the deletion, we performed the karyotyping of the parents

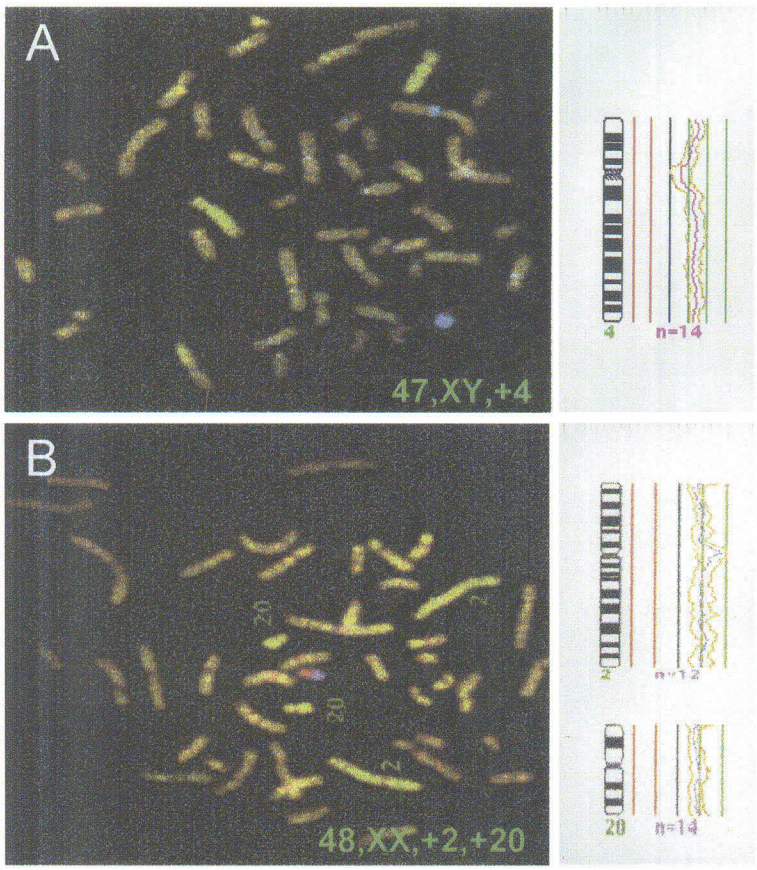


Fig 1. Metaphase Images and DNA profiles, after CGH. A; The image shows strong intensity of fluorescent for chromosome 4, a trisomic chromosome 4 (left). CGH profile reveals cut-off value of greater than 1.25. The result of profile confirms the image which shows trisomic chromosome 4 (right). B; The image shows strong intensity of fluorescent for chromosome 2 and 20 (left). CGH profiles reveal cult-off value of 1.25 for both. The result of profile confirm the image shows double trisomic 2 and 20 (right).

Table 1. Cytogenetics and CGH comparison of 13 fetal samples and parental karyotypes

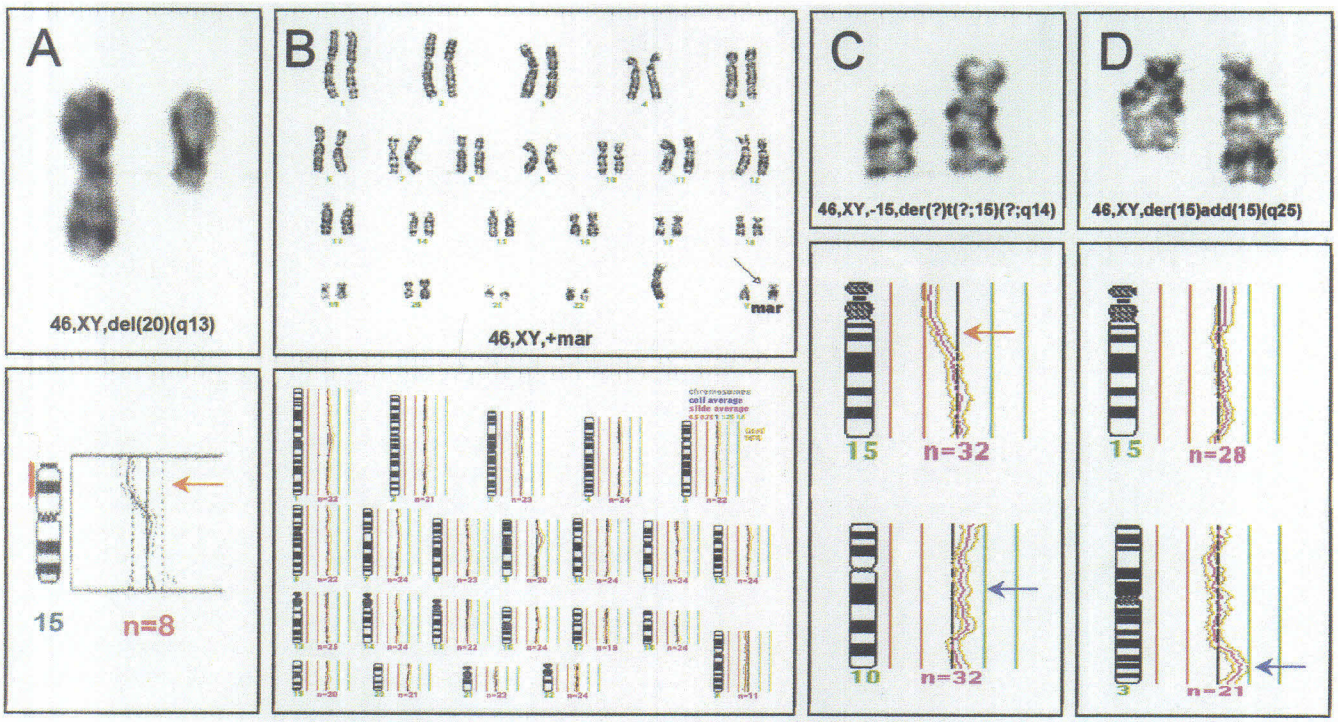
No.	Samples	Reason	Cytogenetics	CGH	Parental karyotypes[a]	Remarks
C1	Amniotic Fluid	prenatal diagnosis	46,XY,del(20)(q13)	loss of 2p13-ter	normal	descrepancy
C2	"	"	47,XY,+mar	normal	father : 47,XY,+mar	descrepancy
C3	"	"	46,XY/47,XY,+22	gain of 22		
C4	Trophoblast Cell	abortion	47,XY,+11	gain of 11		
C5	"	"	48,XY,+8,+18	gain of 8 &18		
C6	"	"	48,XX,+2,+20	gain of 2 & 20		
C7	"	"	46,XX,+8,-21	gain of 8, loss of 21		
C8	"	"	47,XY,+6	gain of 6		
C9	"	"	47,XX,+4	gain of 4		
C10	"	"	47,XX,+7	gain of 7		
C11	"	"	47,XX,+21	gain of 21		
C12	"	"	46,XY,der(?)t(?;15)	gain of 10pter-q22 loss of 15pter-q14	mother: 46,XX,t(10;15)(q22;q14)	descrepancy
C13[b]	Cord Blood[c]	prenatal diagnosis	46,XY,der(15)add(15)(q25)	gain of 3(q24-qter)	father: 46,XY,t(3;15)(q24;q25)	descrepancy

[a] parental karyotyping was done after CGH when necessary

[b] used DOP-PCR to obtain sufficient whole genome

[c] cordocentesis was done at 22 weeks without previous amniocentesis and chorionic villi sampling

Fig 2. Karyotypes and CGH profiles of 4 cases with chromosomal structural abnormalities. A; Partial karyotype shows fetus to be a del(20)(q13) by GTG -banding (upper), CGH shows a loss of 20p13-pter indicated by an arrow (lower). B; Idiogram of fetus shows an extra marker chromosome (upper), CGH profile shows normal (lower). C; Partial karyotype shows abortus to be a der(?)t(?;15) by GTG-banding (upper), CGH profiles show a loss of 15pter-q14 (red arrow) and a gain of 10pter-q22 (blue arrow). D; Partial karyotypes show a fetus to be a der(15) add(15)(q25) (upper), CGH profiles show a normal chromosome 15 and a gain of 3q24-qter (blue arrow).



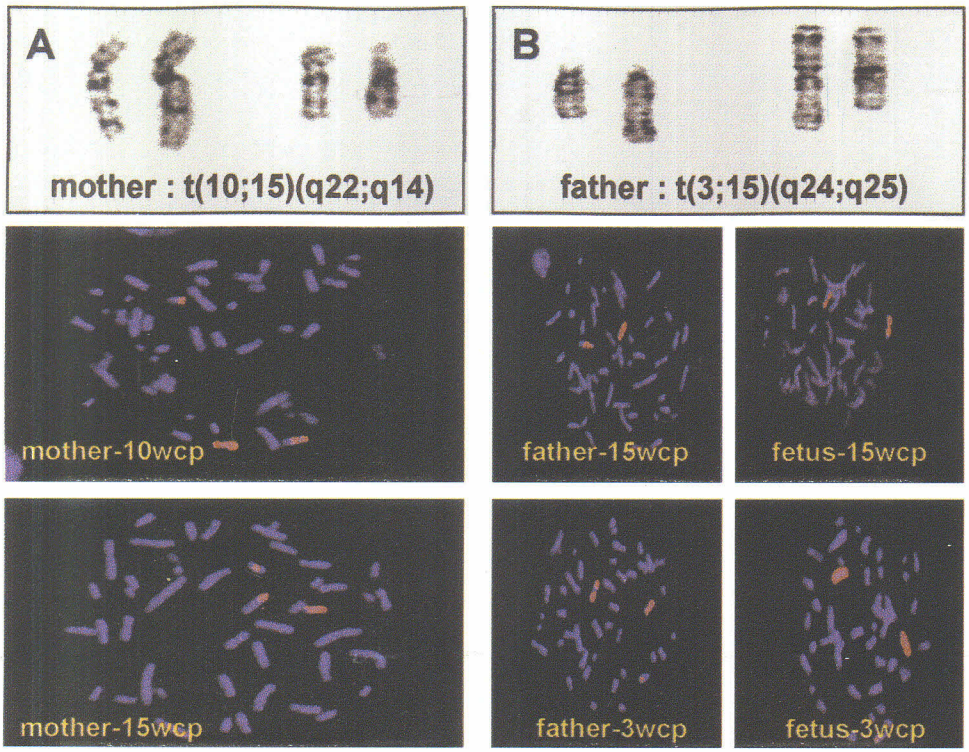


Fig 3. Partial karyotypes of one of the parents with reciprocal translocation and whole chromosome FISH paintings of mother-case 12 (A), and father and fetus-case 13 (B). A; Partial karyotype shows translocation of chromosome 10 and chromosome 15 (Top). Upper FISH painting shows one whole chromosome 10 and two the other reciprocal translocation of the other chromosome 10 with chromosome 15. Lower FISH painting Shows one whole chromosome 15 and confirms the reciprocal translocation of chromosome 10 and chromosome 15. B; Partial karyotype shows translocation of chromosome 3 and chromosome 15 (Top). Upper left FISH painting shows one whole chromosome 15 and the other reciprocal translocation of the other chromosome 15 with chromosome 3. Lower left FISH shows one whole chromosome 3 and two reciprocal translocation of the other chromosome 3 with chromosome 15. Upper right FISH painting of fetus shows one whole chromosome 15 and partial deletion of the other chromosome 15. Lower right FISH painting of fetus shows one whole chromosome 3 and partial gain of the other chromosome 3.

which were found to be normal. The C2 which had marker chromosome cytogenetically was found to be normal by CGH. Parental karyotyping of both parents were done and the father was found to have the same marker chromosome cytogenetically. As such, the origin for marker chromosome could not be confirmed (Fig. 2B). C12, which showed a der(?)t(?;15) cytogenetically, was shown to be a deletion from the long arm(q14) to terminal region of the short arm on the chromosome 15 (Fig. 2C). Furthermore, the parents were karyotyped and the mother turned out to be a carrier with 46,XX,t(10;15)(q22;q14); The origin of derivative chromosome was confirmed by parental karyotype and FISH (Fig. 3A). Chromosome analysis showed C13 has an addition of long arm of the chromosome 15. Our CGH analysis showed C13 to be partial trisomy (q24-qter) of the chromosome 3 (Fig. 2D). This led us to karyotyping the parents and the father was found to be a carrier with 46,XY,t(3;15)(q24;q25). These results were confirmed by FISH (Fig. 3B).

DISCUSSION

CGH has been applied in the field of cancer cytogenetics extensively, but there has been a lack of enthusiastic application of CGH in chromosomal analysis of fetuses or abortuses. With the future goal of clinical application, the purpose of this study was to make possible the rapid prenatal chromosomal analysis of the fetuses or the abortuses which had been autolysed, necrosed or contaminated rendering the tissues unculturable. The CGH analysis of fetuses or abortuses can be much more difficult than analysis of cancer cells due to the fact that gain or loss of

genomic DNA sequence copies for cancer cells are much greater than that of abortuses or fetuses. CGH, having the advan-tage of providing complete chromosome analysis with single hybridization, we expect its application for clinical cytogenetic analysis of fetal cell to be highly useful. When abnormality are found by triple test, prenatal diagnosis is necessary. In such cases, general chromosome analysis requires cell culturization for 3 weeks to process. However CGH process requires about 60-72 h after sampling of those suspected of chromosome abnormalities, information obtained by the process could be applied effectively in clinical prenatal diagnosis.

Currently, FISH is used for rapid detection of chromosome aneuploidy, however in cases which require simultaneous analysis of several chromosomes, it has some limitation. As such CGH can take the place of FISH for the prenatal diagnosis, and also can provide genetic information in culture failure cases of abortus tissues. In CGH experiment, slide preparation was an important factor since we were able to obtain good results by using our home-made slides. Also, for the labelling method, we have chosen indirect method instead of direct method which fades more quickly, thus providing more effective analysis of numerous metaphases.

For cases 3 and 13, where gDNA were unobtainable, we put a minute quantity of fetal cells of about 5-10 cells through DOP-PCR. The amplicon of DOP-PCR was used for CGH analysis. Small numbers of the cells would be necessary because degenerated-oligonucleotide PCR (DOP-PCR) could be used to amplify gDNA for use in CGH (Speicher *et al.*, 1993). C1, suspected of chromosome abnormality after ultrasonography, showed hydronephrosis, hydroureter, micrognathia, cleft palate, undescended testes and inguinal hernia etc., was sent to us with request for chromosome analysis. Through general GTG banding, we found a deletion of long arm on the chromosome 20. However, the application of CGH showed the deletion of short arm on the chromosome 20. To confirm the deletion, whether long arm or short arm, we analyzed the chromosome of the parents but found them to be normal, confirming that case 1 was sporadic occurrence. And, unfortunately, due to lack of sample, FISH couldn't be applied. The prenatal case of C2 with a marker chromosome was found to have normal by CGH and father's karyotype also had the same marker chromosome. We assume that marker chromosome might be a non-functional gene derived from heterochromatic region. In abortus case C12, chromosome analysis result showed der(?)t(?;15) but the CGH result showed a partial monosomy loss of the segment from the terminal region of the short arm(pter) to the long arm (q15) on the chromosome 15 and a partial trisomy a gain of the segment from the terminal region of the short arm to the long arm (q22) of the chromosome 10. We were able to confirm the origin by parental karyotypes and FISH. We, therefore, conclude that one of the parents is a reciprocal translocation carrier leading to the partial trisomy or monosomy of fetus during meiosis. Generally, since the chromosome analysis of abortus tissues is more difficult, we often face difficulties in obtaining high quality metaphase slides. And also due to limited samples obtained during curretage, we often face impossibility of further experiments for greater accuracy.

So far, we could confirm whether one of the parents was a reciprocal translocation carrier or not. When analysing the final DNA profile from the CGH, there are certain precautions that must be taken. In case of insufficient blocking during hybridization, there is a variability of centromere region. In addition, even during normal hybridization, we often observe variability in both of the telomeric regions. Without much experience and without sufficient control study, above two obstacles can hinder proper determination of gain and loss. In these experiments, we have applied the CGH method retrospectively for the fetal cells and compared them with the conventional chromosome analysis which we had already obtained.

The purpose of such comparative analysis was to find out how effective the CGH could be applied in the field of clinical cytogenetics. We have found the CGH is an effective method for genetic analysis, providing accurate chromosome analysis, and it can be done quickly. It will provide rapid and accurate results for fetal cell analysis. Furthermore, we believe it will also be effective in the area of abortus and/or single fetal cell analysis.

REFERENCES

Bryndorf, T., Kirchhoff, M., Rose, H., Maahr, J., Karhu, R., Kallioniemi, A., Christensen, B., Lundsteen, C. and Philip, J. (1995) Comparative genomic hybridization in clinical cytogenetics. *Am J Hum Genet* 57: 1211

du Manoir, S., Spreicher, M. R., Joos, S., Schrock, E., Popp, S., Dohner, H., Kovacs, G., Robert-Nicoud, M., Lichter, P. and Cremer, T. (1993) Detection of complete and partial chromosome gains and losses by comparative genomic in situ hybridization. *Hum Genet* 90: 590-610

Florijn, R. J., Slats, J., Tanke, H. J. and Raap, A. K. (1995) Analysis of antifading reagents for fluorescence microscopy. *Cytometry* 19: 177-82

Lichter, P., Cremer, T., Borden, J., Manuelidis, L. and Ward, D. C. (1988) Delineation of individual human chromosomes in metaphase and interphase cells by in situ suppression hybridi-

zation using recombinant DNA libraries. *Hum Genet* 80: 224-234

Kallioniemi, A., Kallioniemi, O. P., Sudar, D., Rutovitz, D., Gray, J. W., Waldman, F. and Pinkel, D. (1992a) Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. *Science* 258: 818-821

Kallioniemi, A., Kallioniemi, O-P., Waldman, F. M., Chen, L. C., Yu, L. C., Fung, Y. K., Smith, H. S., Pinkel, D. and Gray, J. W. (1992b) Detection of retinoblastoma gene copy number in metaphase chromosomes and interphase nuclei by fluorescence in sity hybridization. *Cytogenet Cell Genet* 60: 190-193

Kallioniemi, A., Kallioniemi, O-P., Piper, J., Tanner, M., Stokke, T., Chen, L., Smith, H. S., Pinkel, D., Gray, J. W. and Waldman, F. M. (1994) Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *Proc Natl Acad Sci USA* 91: 2156

Kallioniemi, O.-P., Kallioniemi, A., Kurisu, W., Thor, A., Chen, L. C., Smith, H. S., Waldman, F. M., Pinkel, D. and Gray, J. W. (1992) ERBB-2 amplification in breast cancer analyzed by fluorescence in situ hybridization. *Proc Natl Acad Sci USA* 89: 5321-5325

Pinkel, D., Landegent, J., Collins, C., Fuscoe, J., Segraves, R., Lucas, J. and Gray, J. (1988) Fluorescence *in situ* hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. *Proc Natl Acad Sci USA* 85: 9138-9142

Piper, J., Rutovittz, D., Sudar, D., Kallioniemi, A., Kallioniemi, O.-P., Waldman, F. M., Gray, J. W. and Pinkel, D. (1994) Computer image analysis of comparative genomic hybridization. *Cytometry* 19: 10-26

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Chorionic villus culture for prenatal diagnosis of chrmosome defects: reduction of the long term cultivation time. *Prenat Dian* 9: 309-319

Spreicher, M. R., du Manoir, S., Schrock, E., Holtgreve-Grez, H., Schoell, B., Lengauer, C., Cremer, T. and Ried, T. (1993) Molecular cytogenetic analysis of formalin-fixed, paraffin-embedded solid tumors by comparative genomic hybridization after universal DNA-amplification. *Hum Mol Genet* 2: 1901-1914

Telenius, H., Pelmear, A. H., Tunnacliffe. A., Carter, N. P., Behmel, A., Ferguson-Smith, M. A., Nordenskjd, M., Pfragner, R. and Ponder, B. A. (1992) Cytogenetic analysis by chromosome painting using DOP-PCR amplified flow-sorted chromosomes. *Genes Chrom Cancer* 4: 257- 63