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NEW TECHNIQUE

The role of comparative genomic hybridisation in prenatal diagnosis

Angela Thein[a,*], Adrian Charles[b], Teresa Davies[c], Ruth Newbury-Ecob[d], Peter Soothill[a]

The aims of this study were to assess the feasibility of using comparative genomic hybridisation instead of conventional cytogenetics in prenatal diagnosis and to determine the size of DNA loss that can be detected. Using comparative genomic hybridisation, six cases with standard aneuploidies were diagnosed correctly. This technique clearly identified a partial duplication of the long arm of chromosome 1 but was not capable of detecting the associated inversion. A small interstitial deletion on short arm of chromosome 10 also was detected precisely. Although the current comparative genomic hybridisation resolution is similar to the sensitivity of the highest resolution G banding, the latter is not a routine strategy in prenatal diagnosis. Comparative genomic hybridisation can allow full chromosome assessment equal to the highest resolution cytogenetic studies without the need for cell culture.

The reason why many antenatal and postnatal diagnostic cases with dysmorphology suspected of having a disorder of chromosomal aetiology have no detected abnormality by conventional cytogenetic techniques is likely to be because the techniques may not be sensitive enough to identify small aberrations such as micro-deletions. A range of molecular techniques has been developed to overcome this problem; some are used as an adjunct to conventional cytogenetic technique. Interphase fluorescent *in situ* hybridisation (FISH) using chromosome specific probes on uncultured amniocytes is rapid and accurate, and is used as a prenatal diagnosis for certain indications[1]. Indeed, a new five chromosome-probe cocktail is used to diagnose common numerical aneuploidies but these cannot detect structural rearrangements of any chromosome[2]. The use of chromosome painting to identify the origins of translocations and complex rearrangements is time consuming and requires several sequential hybridisations[3]. The use of locus-specific single sequence DNA probes to detect specific deletions requires prior knowledge of the region of interest. Availability of specific probes for some parts of the genome is limited. Spectral karyotyping is another FISH-based technique in which a mixture of 24 differentially labelled DNA sequences (originated from all 22 autosomes and both sex chromosomes) are hybridised to metaphases from a test sample. With the assistance of a fluorescent digital imaging system all the FISH signals are shown in different colours for each chromosome[4]. Spectral karyotyping is therefore useful in resolving complex translocations or when an abnormal chromosome is unidentifiable cytogeneticaly but the technique is expensive and very labour intensive.

A technique which might overcome these problems is comparative genomic hybridisation[5–8] in which an equal quantity of DNA from a test sample (labelled with green fluorochrome) and normal diploid reference (labelled with red fluorochrome) are hybridised onto a normal metaphase chromosome preparation.

The ratio of green and red hybridisation intensities at every point along the mid-line of the target chromosomes is calculated with the help of a digital image analysis system. Hence, deletions or duplications of part or all of a chromosome may be detected. Comparative genomic hybridisation requires only a DNA sample without the need for cell culture and so can be applied to clinical material such as frozen tissues, formalin fixed tissues and paraffin embedded archival materials. In contrast to FISH, comparative genomic hybridisation does not require DNA probes or any prior indication of the region of interest and offers a comprehensive approach to assess all unbalanced chromosomal gains and losses in a single experiment. We therefore undertook comparative genomic hybridisation in developmentally abnormal dysmorphic cases. This study received an ethical approval, and the scientist undertaking comparative genomic hybridisation knew none of these clinical details or cytogenetic results.

[a]*Fetal Medicine Research Unit, St. Michael's Hospital, University of Bristol, UK*
[b]*Paediatric Pathology, St. Michael's Hospital, University of Bristol, UK*
[c]*Regional Cytogenetic Department, Lewis Lab, Southmead Hospital, Bristol, UK*
[d]*Clinical Genetics Department, Department of Child Health, UBHT., UK*

* **Correspondence**: Dr A. Thein, Fetal Medicine Research Unit, University of Bristol, Southwell Street, St Michael's, Bristol BS2 8EG, UK.

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Methods

DNA samples from six cases with standard aneuploidies were extracted from frozen fetal liver tissues obtained at the time of post-mortem examination after the termination of pregnancies because of cytogenetic abnormalities. Four cases with segmental aneuploidies of various sizes were used to assess the size of a deletion detectable by comparative genomic hybridisation. These are described in more detail in Table 1. Case 7 presented with hydrops in the second trimester of pregnancy and underwent an amniocentesis for karyotyping. The cytogenetic result showed that there was additional material on the long arm of chromosome, and the cytogenetic banding pattern suggested that it was a partial duplication with an inversion of the region 1q42-25. DNA was extracted for comparative genomic hybridisation from stored frozen cultured amniocytes. In Case 8, as a result of recurrent miscarriages, a paternal balanced translocation (46,XY, *t* (2:12)(q37;q24)) was detected, and a chorionic villous sampling was undertaken in the next pregnancy and DNA was extracted for comparative genomic hybridisation. In Case 9 an ultrasound examination in third trimester identified features suggestive of Beckwith-Wiederman syndrome, and the baby was born prematurely at 34 weeks because of polyhydramnios. Blood samples were taken from the parents and the baby at delivery and the paternal sample showed a balanced translocation (46XY, *t*(5:11)(p15;p15), and the baby had a paternally derived abnormal chromosome 5 resulting in partial trisomy of chromosome 11 in the region of Beckwith-Wioedemann gene. DNA was extracted from the neonate's blood for comparative genomic hybridisation. Case 10 was referred for genetic opinion because of mental retardation, short stature and dysmorphology.

Genomic DNA from test cases and control samples (peripheral blood from normal male or female volunteers) were obtained. Usually male reference DNA is used for any test cases, but in the first part of this study (Cases 1-6), female reference DNA was used for female trisomy 13, 18, 21 and monosomy X (Cases 1, 2, 4 & 6). Test and control DNA samples were incubated with DNase enzyme to obtain DNA fragments optimal for comparative genomic hybridisation hybridisation (about 600–1000bp). These were then labelled with fluorochrome, biotin-16-dUTP for test DNA and digoxigenin-11-dUTP for controls.

Equal amounts of differentially labelled test and control DNA were hybridised on to target metaphases for comparative genomic hybridisation with human Cot 1 DNA to suppress hybridisation in regions of repetitive DNA, as described by Kallioneimi *et al.*[5–7]. The biotin-labelled test DNA was detected with avidin fluorescein iso thiocyanate (Vector Laboratories, Burlingame, CA, USA), and the digoxigenin-labelled control DNA with a mouse anti-digoxigenin antibody, followed by rabbit anti-mouse and goat anti-rabbit antibody attached to (Sigma, Wimborne, UK).

The target metaphase spreads were made from male blood samples by phytohemagglutinin-stimulated and synchronised peripheral blood cultures. Target chromosomes were counterstained with diamidino phenylindole (DAPI) to generate a G band-like patterns by computer for karyptyping. Fluorescein iso thiocyanate and tetramethyl rhodamine iso thiocyanate filters (Chroma, Brattleboro, VT, USA) were used to visualise test and control DNA colours. Chromosomal aberrations involving the whole chromosomes were visualised directly under the fluorescence microscope (Leica Microsystems Imaging Solution Ltd, Cambridge, UK). However, a

Table 1. Please supply table caption.

Case	Cytogenetic results	Tissues from which DNA were extracted for CGH	CGH results
1.	47,XX, +13	Frozen fetal liver	Chromosome 13 profile was beyond the G:R ratio 1.25 showing chromosome 13 DNA sequences gain in test sample)
2.	47,XX, +18	Frozen fetal liver	Chromosome 18 profile was on the G:R ratio 1.25 showing chromosome 18 DNA sequences gain in test sample
3.	47,XY, +18	Frozen fetal liver	Same as above (not shown)
4.	47,XX, +21	Frozen fetal liver	Chromosome 21 profile was o the G:R ratio 1.25 showing chromosome 21 DNA sequences gain in test sample
5.	47,XY, +21	Frozen fetal liver	Same as above
6.	45,XO	Frozen fetal liver	Chromosome X profile was beyond the G:R ratio 0.75 showing Chromosome X DNA sequences loss in test case
7.	46,XY, Inv dup (1)(q42q25)	Amniocytes culture	The region chromosome 1q25-42 showed a DNA gain confirming the duplication
8.	46,XY,der (2) *t*(2;12)(2q37;12q24.3)	CVS culture	The distal end of chromosome 2 long arm showed a DNA loss and the distal end of chromosome 12 long arm showed a DNA gain
9.	46,XY,der (5) *t*(5;11)(p15;p15)	Neonatal blood culture	Distal end of the short arm of chromosome 5 showed a loss of DNA and the distal end of the short arm of chromosome 11 a gain
10.	46,XY, del (10)(p11.23p12.3)	Neonatal blood culture	The segment of chromosome 10 showed DNA loss confirming interstitial deletion of the short arm of chromosome 10.

smaller deletion may not be seen because of the limited ability of the human eye to detect subtle change in colour balance.

Images from at least 10 metaphases were acquired by the fluorescence microscope attached to a high resolution "Sensys" cooled CCD camera (Photometrics, Tucson, AZ, USA). A combined image was obtained by computer to calculate the green to red ratio and displayed as Leica QCGH software profiles. A green to red ratio close to 1 denotes test and control DNA hybridisation in approximately equal proportions without neither gain nor loss. A green to red ratio ≥ 1.25 indicates a gain of test DNA when compared with the control sample. A green to red ratio ≤ 0.75 denotes a loss of DNA sequences in test sample (Figs. 2–5). Pericentromeric and heterochromatic regions cannot be evaluated by comparative genomic hybridisation since DNA sequences there are highly polymorphic in copy number between individuals[7].

Results

The six cases with standard aneuploidies were all correctly diagnosed by comparative genomic hybridisation (Cases 1-6). Fig. 1 shows the acquired images from Case 6 (monosomy X) as an example. The pink colour dots seen in Figs. 1a, b & c were centromeres of chromosomes 4, 8, 14, 19 & 22 shown by the alpha-satellite probes from these areas, and these pink dots were not due to incomplete suppression by Cot 1. Cocktails of selective centromeric alpha-satellite probes were added to the test and reference genomic DNA with Cot 1 DNA at the time of comparative genomic hybridisation hybridisation, and they were detected by special Cy 5 filter (the idea of adding Cy 5 labelled selective centromeric probes in comparative genomic hybridisation is to assist untrained beginners for easy karyotyping). Comparative genomic hybridisation profiles of case 6 are shown in Fig. 2. The green to red ratio profile of X chromosome fell

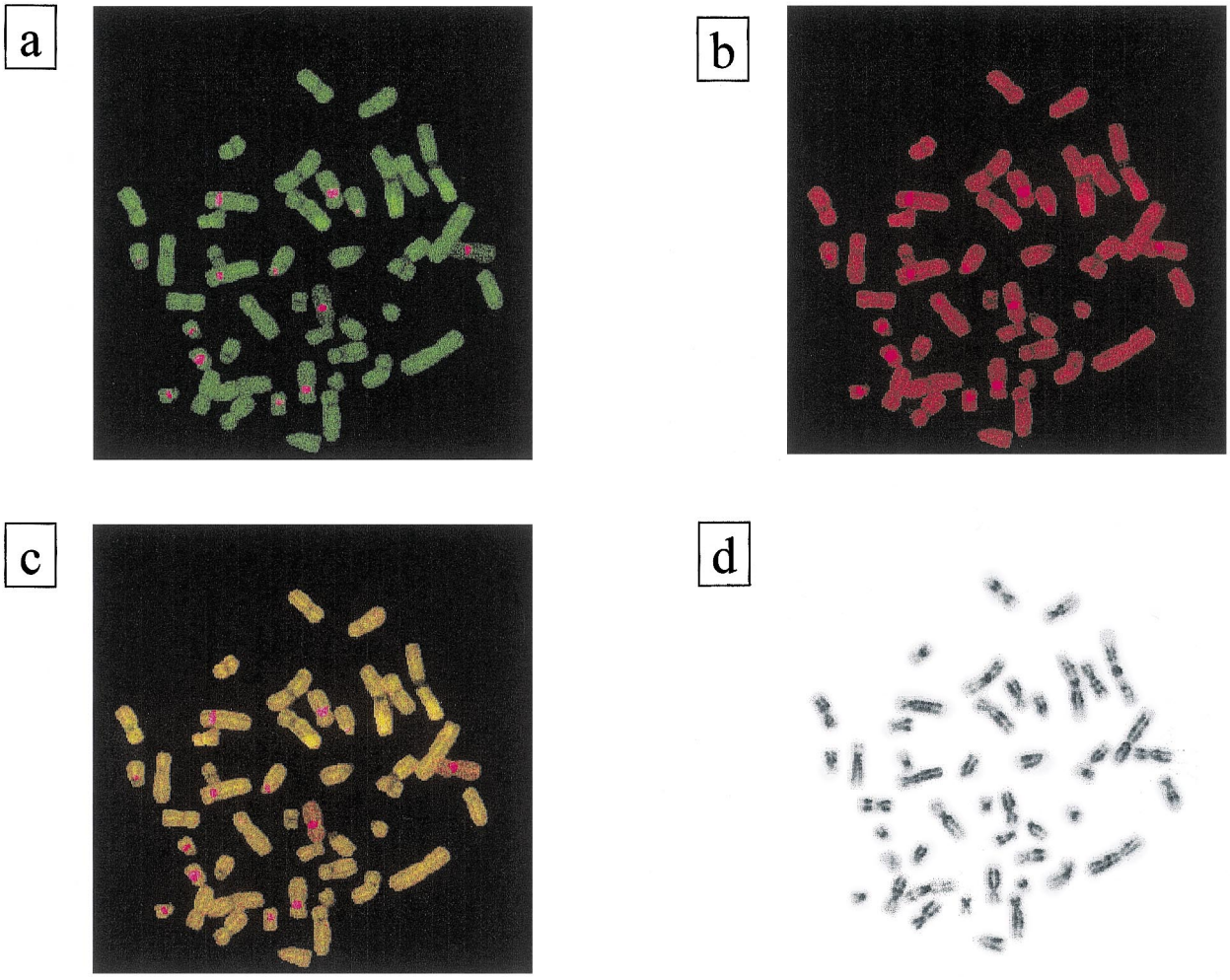

Fig. 1. Images acquired for CGH analysis: (**a**) is the test DNA hybridisation image acquired by FITC filter; (**b**) is the control DNA hybridisation image acquired by TRITC filter; (**c**) the computer merged image of test and control hybridisation; (**d**) a computer-converted image from DAPI counter stained image. Pink dots on images (**a**), **b** and **c** are alpha-satellite probes from chromosomes 4, 8, 14, 19 & 22 detected by Cy5 filter.

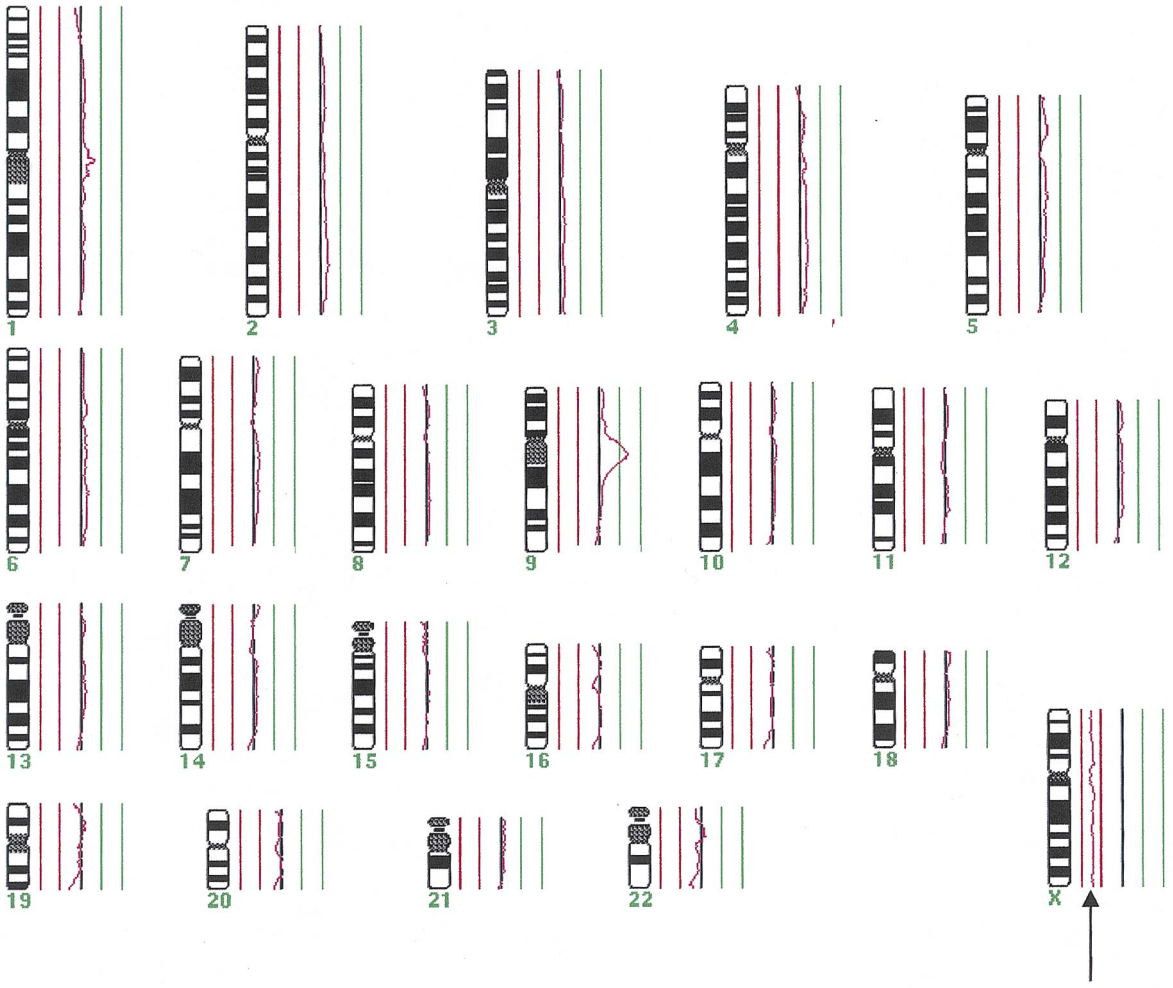

Fig. 2. Shows the green to red ratio profile of X chromosome below 0.75 cutoff line due to DNA loss in monosomy X case compare to diploid reference DNA. The remaining profiles of the rest of the chromosomes appear normal, close to ratio profile 1.

below the 0.75 cutoff line due to DNA loss in the monosomy X case compare to the diploid reference DNA. However, the remaining profiles of the rest of the chromosomes (apart from the heterochromatic area of 9[7]) were normal showing ratios close to 1. Fig. 3 shows the comparative genomic hybridisation profiles shift beyond the green to red ratio 1.25 line, representing DNA gain in whole length of chromosomes 13, 18 and 21 in standard trisomies.

Also the partial chromosomal abnormalities were detected while being blinded to the cytogenetic result. DNA gain due to a large partial duplication on chromosome 1 (Case 7) was indicated by the profile shift towards the green to red ratio 1.25 line on the distal part of chromosome 1 long arm (Fig. 4: image on the left side). However, comparative genomic hybridisation is not capable of detecting the inversion of the duplicated segment, 1q42-q25, proximal to the telomere. DNA loss due to a small interstitial deletion on short arm of chromosome 10 (Case 10), was also shown by the profile shift towards the green to red ratio 0.75 line at 10p11.2-12.3 (Fig. 4-image on the right). A paternally-derived unbalanced translocation between chromosome 2 and 12, which was cytogeneticaly estimated to be about 1/2 a large G band (at a 450 band resolution Fig. 5a)[9] (Case 8) gave a profile of chromosome 2 with a green to red ratio of 0.75, but only in the telomeric region of its long arm. Similarly a profile of chromosome 12 has a green to red ratio of 1.25 in its long arm.

Therefore the fetus inherited an abnormal chromosome 2 with a small translocation from chromosome 12 (der (2) *t* (2:12)) from its father, with a normal copy of chromosome 2 from its mother. This resulted in an unbalanced translocation of chromosomes 2 and 12, with a partial trisomy of chromosome 12 and partial monosomy of the long arm of chromosome 2. In case 9 there was again a paternally-derived unbalanced translocation between chromosomes 5 and 11 of about 1/2 a large G band in size (at a 450 band resolution, Fig. 5b)[9]. The comparative genomic hybridisation profile showed a DNA loss on the short arm telomeric region of chromosome 5 and DNA gain in the telomeric area of the short

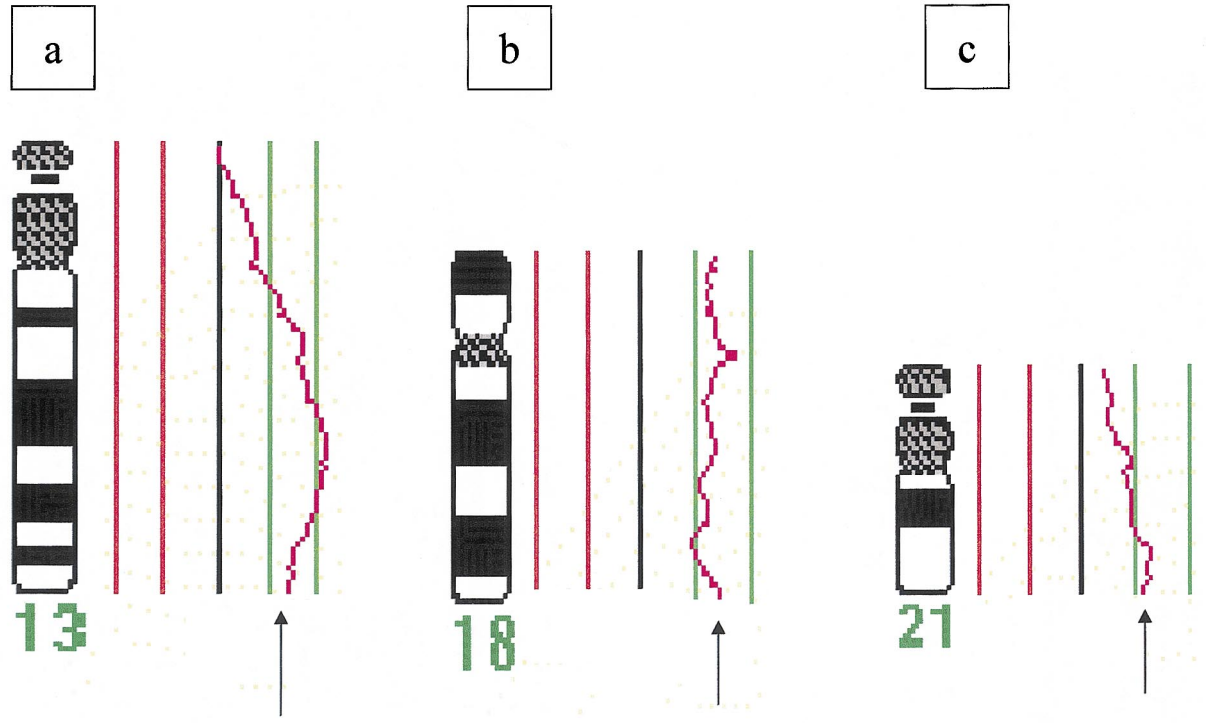

Fig. 3. Shows CGH profiles of chromosome 13, 18 and 21 representing DNA gains in the whole length of chromosomes in standard trisomies.

arm of chromosome 11. The Beckwith-Wioedemann gene is located in 11p15.5 so the fetal karyotype contains three copies of telomeres of short arms of chromosome 11, which resulted in a partial trisomy in the region of Beckwith-Wioedemann gene and partial monosomy of the telomeric end of the short arm of chromosome 5.

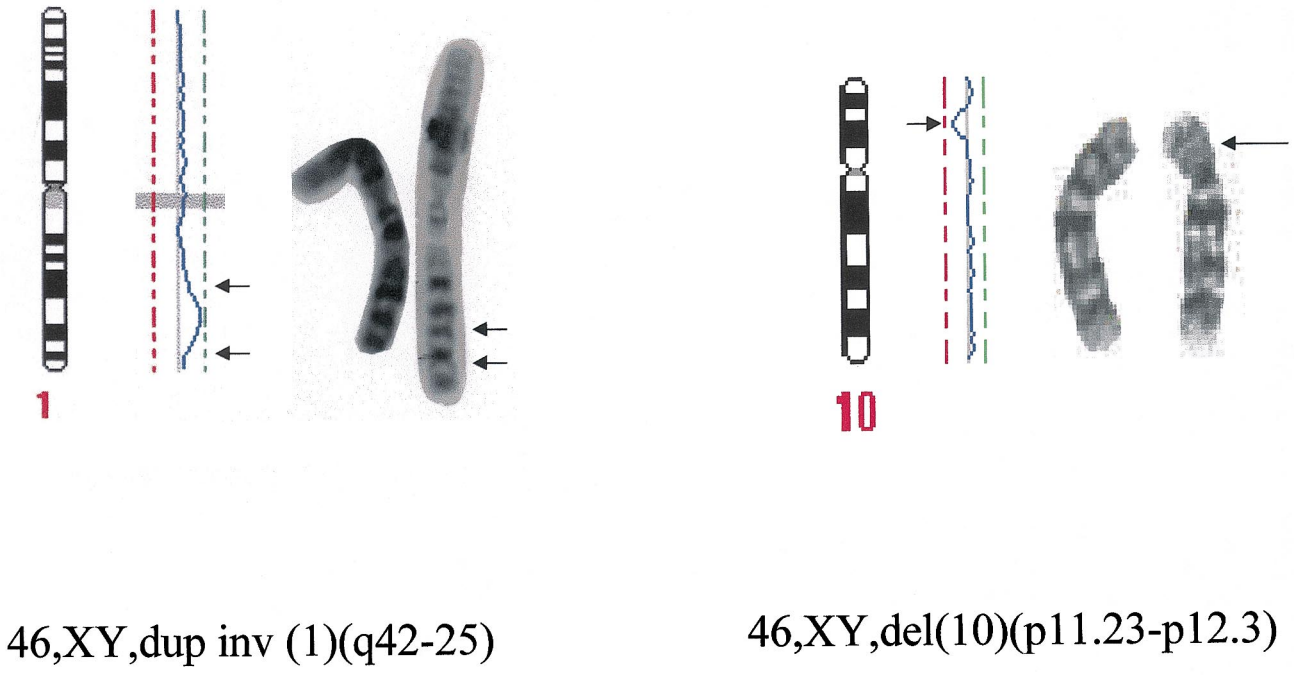

Fig. 4. (**a**) CGH profile shift towards the green to red ratio 1.25 line indicates DNA gain on the duplicated part of chromosome 1 long arm. Both the normal copy of chromosome 1 and the copy with duplication represent the 450-band resolution G banding karyotype; (**b**) CGH profile shift towards the green to red ratio 0.75 line indicates DNA loss of an interstitial part of chromosome 10 short arm. Both normal copy of chromosome 10 and a copy with deletion represent the 450-band resolution G banding karyotype.

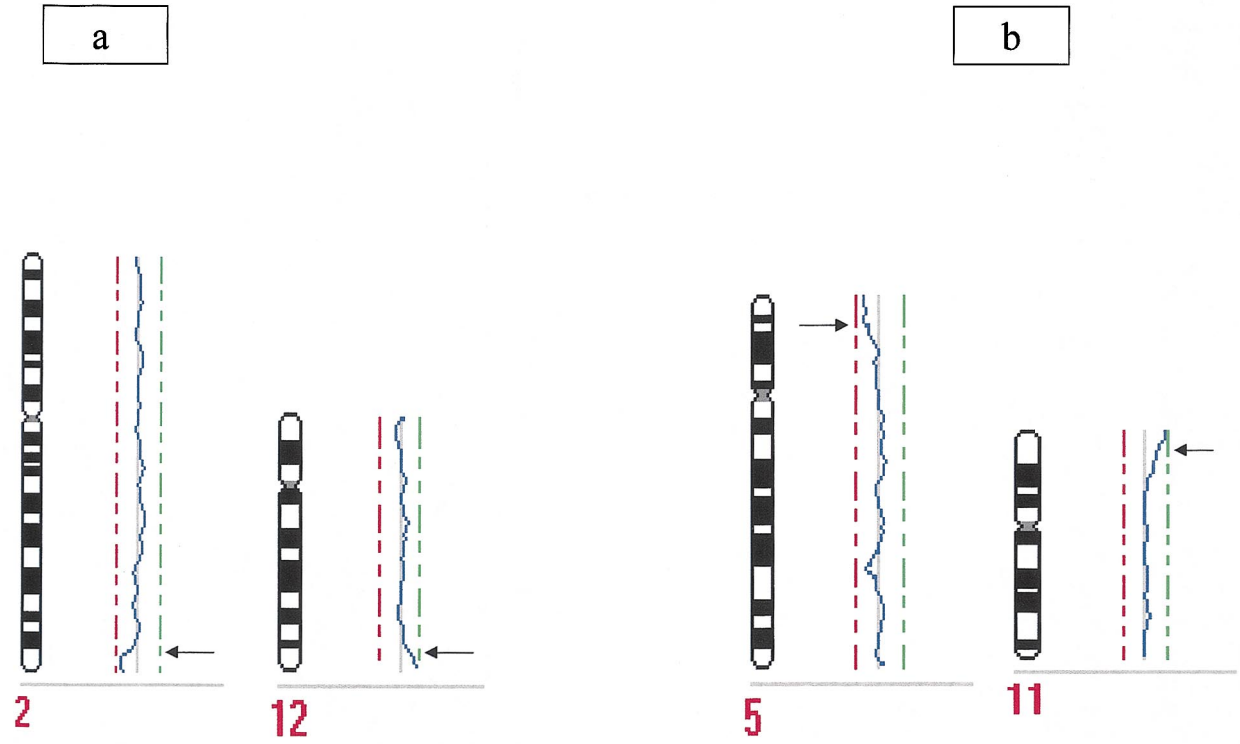

Fig. 5. (**a**) CGH profiles of chromosome 2 and 12 from Case 8; (**b**) CGH profiles of chromosome 5 and 11 from Case 9. Ideograms represent the 450-band resolution G banding.

These results indicate that we can detect loss and gain within the genome without prior knowledge of the areas affected in Cases 8 and 9 and suggests a sensitivity of comparative genomic hybridisation to detect deletion of about 5 Mb.

Discussion

Until now the comparative genomic hybridisation technique has been applied mainly in cancer genetics, especially in a variety of solid tumours and haematological malignancies. Comparative genomic hybridisation has several advantages in prenatal diagnosis. It requires only DNA, which can be obtained from clinical samples and can avoid cell culture and can prevent problems associated with culture failure. It has tremendous potential in prenatal diagnosis because comparative genomic hybridisation can be applied to DNA from clinical material, which cannot be cultured, such as frozen tissues. Archival clinical materials, which have been formalin-fixed and paraffin-embedded could be analysed retrospectively by comparative genomic hybridisation using DNA from such cases. Comparative genomic hybridisation can be applied on DNA extracted from products of conception either after spontaneous miscarriage or following surgical evacuation, which are not normally grow well in culture[16] and usually macerated; this could help detecting a genetic aetiology in recurrent miscarriages. In contrast to conventional FISH investigation, comparative genomic hybridisation does not require DNA probes or any prior indication of the chromosome or region of interest. With genomic polymerase chain reaction amplification, can generate sufficient DNA for comparative genomic hybridisation from a minute DNA sample, such as a single cell[10], its use in pre-implantation diagnosis may be possible. Comparative genomic hybridisation has the advantage of a comprehensive approach to the genome by assessing all unbalanced aberrations in one experiment. It therefore has the potential to be used as a genome scanning technique in prenatal diagnosis.

Before this study it was already known that comparative genomic hybridisation is an excellent technique for determining the origin of extra chromosomal material when its origin was not certain by a cytogenetic G banding technique[9]. It certainly can be used as a complementary test to standard conventional karyotyping in revealing the nature of unbalanced chromosomal aberration causing partial trisomy or monosomies of critical regions for dysmorphology. However, there are limitations to this technique. Comparative genomic hybridisation is not able to detect balanced chromosomal aberrations including inversions. It would therefore be inappropriate for the investigation of couples with recurrent miscarriage. Also comparative genomic hybridisation will not detect low-level mosaicism if the abnormal cells compose less than 50% of the total. Finally, comparative genomic hybridisation is not reli-

able in the interpretation of the centromeric, heterochromatic, acrocentric and telomeric regions and especially the distal end of short arm chromosome 1, the short arms of chromosome 16, 19 and 22[7].

Previously, others[11–14] have used comparative genomic hybridisation in prenatal samples and have diagnosed whole chromosome aneuploidies (standard trisomies and monosomy). However, although its use in detecting partial aneuploidies has been reported, the smallest deletion size detected was estimated to be between 10-20 Mb [7,12,14,15]. In our study the smallest size of chromosomal deletion detected by comparative genomic hybridisation was the size of half a large G band (a 450 band resolution[9]).

Conclusion

It is very important to note that although the current comparative genomic hybridisation resolution is similar to the sensitivity of the highest resolution G banding, this banding quality is not the routine strategy in prenatal diagnosis because the standard preparations may not allow this to be achieved. Comparative genomic hybridisation appears to be the molecular technique allowing full chromosome assessment equal to the highest resolution cytogenetic studies without the need for cell culture. In addition, comparative genomic hybridisation does appear to have the quality which would allow it to be applied usefully in certain selected circumstances, such as macerated clinical samples, frozen samples and formalin fixed paraffin embedded archival samples which are not appropriate and suitable for tissue culture.

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