

The Importance of High Resolution Chromosome Analysis in the Diagnosis of Birth Defects: Case Reports of Holoprosencephaly and Cystic Hygroma

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Abstract

Introduction: The goal of cytogenetics is the detection of chromosomal abnormalities, achieved by the analysis of adequate numbers of metaphases at the appropriate bands per haploid set (BPHS). **Clinical Picture:** Two cases presented here include a foetal blood sample (FBS) of a 33-week-old referred with holoprosencephaly by ultrasonography, and an amniotic fluid (AF) specimen of a 14-week-old foetus with cystic hygroma, cardiac and renal defects. **Outcome:** The FBS had a deletion at 18p11.31. Another laboratory had earlier given a normal cytogenetic result on its AF sample. In the second case, an unbalanced 46,XY,der(5)ins(5;3) (q33.1;q26.2q27)mat karyotype was obtained with the AF sample. In both cases, the abnormalities were more obvious when band levels were ≥ 450 BPHS. **Conclusion:** This report underscores the importance of obtaining longer chromosome preparations above the current recommended 400 BPHS for prenatal specimens. This is particularly important in cases with abnormal ultrasound findings suggestive of an underlying chromosomal pathology.

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Introduction

Optimal chromosome preparation is a function of many factors. These include cell density culture initiation, optimal time for harvest, concentration and exposure duration to a mitotic arrestant, appropriate hypotonic treatment and adequate fixation with modified Carnoy's fixative.^{1,2} The latter 2 factors are essential to chromosome spreading, which is in turn essential to good metaphase preparation. In a hypotonic solution, the chromosomes move toward the periphery of the cell, become stretched and spread out during mitotic swelling. As the fixative evaporates from the slide, the mitotic cells swell and cause the chromosomes to spread out.³ Long chromosome preparations are obtained through synchronisation of the cell cycle and/or the use of various chromosome anti-contraction reagents.² Other equally important factors are relative humidity, air-flow, and ambient temperature during the slide making process.⁴ After harvesting and slide making, the slides are usually baked in an oven to speed up the drying process of the preparations before performing banding and staining techniques. Deficiencies in any of the above steps may lead to preparations with less than acceptable quality which may lead to a misdiagnosis.

The present study highlights the importance of achieving longer chromosome preparations with optimal banding qualities, particularly when the ultrasound findings are suggestive of a chromosomal defect. Two case studies detected prenatally by ultrasound are discussed. In Case 1, ultrasound scan of the foetus showed holoprosencephaly and clawed hands, while in Case 2, the foetus had cystic hygroma, heart defects, and renal hydronephrosis.

Case Reports

Case 1

A foetal blood sample (FBS) was obtained from a 34-year-old Chinese gravida 3 para 1, who was referred at 33 weeks' gestation. The ultrasound findings showed holoprosencephaly and clawed hands. A genetic amniocentesis performed at 16 weeks' gestation by another laboratory showed a normal karyotype.

Case 2

An amniotic fluid sample was obtained from a 28-year-old Chinese gravida 1 para 0 at 14 weeks' gestation. Ultrasound findings included cystic hygroma and multiple other anomalies including heart and renal defects.

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Culture Set-up

The heparinised foetal blood specimen in sodium heparin was established in 3x T-25 flasks: a 48-hour culture in Chang Medium® MF (Irvine Scientific, Santa Ana, CA, USA) supplemented with PHA (GIBCOBRL, Grand Island, NY, USA), and 2 synchronised 72-hour cultures in complete RPMI 1640 (GIBCOBRL) and Chang Medium® MF, respectively. ½ mL of whole blood was used to inoculate each 10 mL flask of medium.

Because only 8 mL of amniotic fluid sample were available, only 2 in situ coverslip cultures were established. Both cultures were established in a medium comprised of a 40:60 mixture of α MEM:Amniomax-II™ (GIBCOBRL). The cultures were flooded with 1.5 mL of medium the following day. A complete medium change was performed 3 days later.

Harvest

Forty-eight hours after culture initiation, the 72-hour blood cultures were synchronised with 100 μ L excess thymidine and returned to the incubator for another 24 hours. Harvesting the 48-hour culture was initiated by first adding 100 μ L of ethidium bromide (Sigma Chemical Co., St Louis, MO, USA) and incubating for 1 hour. Following this, 100 μ L of Colcemid® (stock concentration 10 μ g/mL, GIBCOBRL) was added to the culture and incubated for a further 30 minutes. The culture was treated with Ohnuki's hypotonic for 15 minutes at room temperature and pre-fixed with cold 3:1 methanol: acetic acid before centrifugation. A total of 3 fixative washes were carried out before the supernatant became clear. To improve chromosome morphology, the cell suspension was placed overnight in the freezer at $-20 \pm 5^\circ\text{C}$. Slides were dropped the following day and oven baked at 90°C for 1 to 2 hours before the banding procedure. The harvest protocol used for the 72-hour cultures was identical to that used on the 48-hour culture. There was no release period proceeding synchronisation block. Parental peripheral bloods were set up as 72-hour synchronised cultures only.

The in situ amniotic fluid cultures were dispersed with 1X trypsin-EDTA (GIBCOBRL) and sub-cultured on 4 new coverslips. When the cultures were judged to be ready for harvest, 25 μ L 5-bromo-2'-deoxyuridine (BrdU) (stock concentration 2.5 mg/mL, Sigma) and 25 μ L Colcemid® (1 in 12 dilutions) were added to each dish, and incubated overnight. The following morning, the medium was aspirated and gently replaced with 2 mL 0.8% sodium citrate hypotonic for 30 minutes. This was followed by a pre-fix step with the addition of 1 mL of cold 3:1 methanol:acetic acid fixative. The hypotonic-fixative mixture was aspirated and replaced with fresh cold fixative. After another 2 changes of fixatives, the fixative was aspirated completely from the dishes. The coverslips were

then blown dry with a gentle air current. Optimal cell spreading was ascertained with an inverted microscope. The ambient temperature was kept between 20°C and 23°C and the humidity was maintained between 50% and 60% with a dehumidifier. The coverslips were then removed, mounted onto microscope slides, and baked at 90°C for 1 hour before the banding procedure.

Banding

The banding procedure followed the standard GTG protocol with slight modifications.⁵

Analysis

The criteria for which cytogenetic analyses were carried out were in accordance to or exceeded the guidelines set up for genetic laboratories by the American College of Medical Genetics Laboratory.⁶ The method of band level estimation adopted was based on the Vancouver General Hospital's Banding Resolution System.⁷

With the 48-hour blood culture, a total of 5 metaphases were counted of which 2 were analysed and karyotyped to obtain a preliminary result. The purpose of the rapid 48-hour preliminary result was primarily to detect or exclude aneuploidies. A further 15 metaphases from the 72-hour cultures were counted the following day, including 5 metaphases that were fully analysed and karyotyped. A total of 20 metaphases were thus screened and 7 metaphases analysed and karyotyped to obtain the final result.

As the in situ amniotic fluid cultures were dispersed to obtain an adequate number of metaphases for a full cytogenetic analysis, the colony method of assessment could not be employed. Instead, 20 metaphases were counted from the two cultures, of which 5 were fully analysed and karyotyped.

Discussion

Chromosomes become progressively shortened as the cell progresses from interphase to metaphase. This behaviour allows the chromatin to be neatly packaged for segregation into 2 daughter cells at the end of telophase. As cell culture techniques evolve, cytogenetic analysis has gradually moved from being performed on mid-metaphase chromosomes to longer early metaphase or even late prophase chromosomes. This can be accomplished by synchronising the cell cycle with a block at the S-phase and subsequent release with a releasing agent. Several block and release reagents are available, such as a methotrexate block with a thymidine release, or an excess thymidine block with 2-deoxycytidine as a releasing agent. With the release, the cells then progress at the same stage of the cell cycle and many cells can be harvested at prometaphase and early metaphase after a brief exposure to Colcemid® at low concentrations. This mitotic arrestant acts by preventing polymerisation of the tubulin

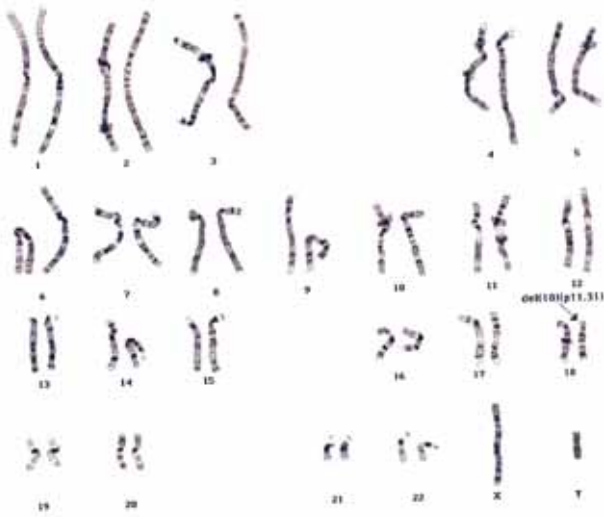


Fig. 1. GTG banded karyotype from the fetal blood specimen showing the terminal deletion of one chromosome 18p.

molecules into a microtubule that makes up the spindle fibres. This prevents the chromatids from aligning on the metaphase plate to be pulled apart during anaphase. The stoichiometric relationship between Colcemid® and tubulin needs to be borne in mind when deciding the amount of the mitotic arrestant to be added. This is because the concentration of Colcemid® has been shown to be critical as it affects the mitotic index, chromosome spreading, and chromosome length.^{8,9}

Another method of obtaining long chromosomes is to add chemical reagents that prevent chromosome condensation. These additives intercalate into the DNA so that the chromosomes do not shorten during their progress from interphase to metaphase, even in the presence of Colcemid®. Several chromosome anti-contraction agents are available and include ethidium bromide, the thymidine analogue BrdU, 5-azacytidine, actinomycin D, echinomycin, and others.^{2,10} Recently, 9-aminoacridine was used to increase chromosome spreads to 850 BPHS resolution, compared to 600-700 BPHS with ethidium bromide.¹¹

In our laboratory, long chromosomes with band resolution of at least 550 BPHS are routinely achieved with blood specimens. This is achieved with a combination of cell synchronisation and the addition of chromosome anti-contraction additives.¹² The protocol we employ comprises a 24-hour block with excess thymidine but without an accompanying release period. This strategy does not block DNA synthesis completely but instead prolongs the S-phase so that chromosome condensation is reduced as the cells proceed toward metaphase.¹³ The addition of ethidium bromide before Colcemid® exposure ensures even longer chromosomes are acquired. We prefer ethidium bromide to other anti-contraction agents because it intercalates between the base pairs without base-pair preferences. The result is

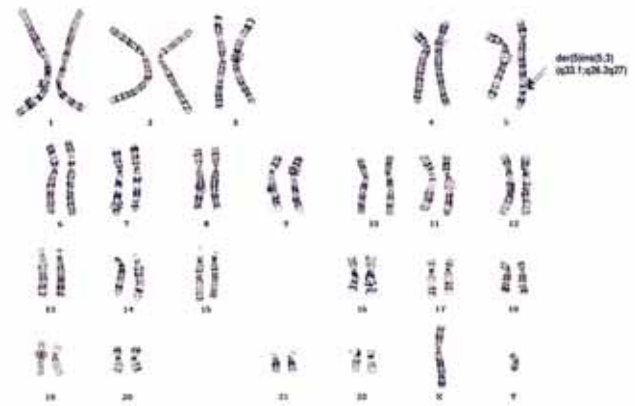


Fig. 2. GTG banded karyotype from the amniotic fluid specimen showing the insertion in the long arm of one chromosome 5.

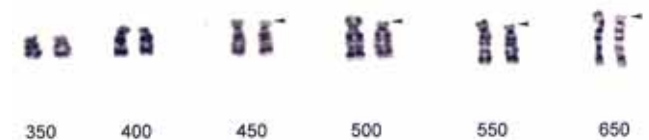


Fig. 3. Homologous chromosome 18 pairs obtained from 6 fetal cord blood metaphases of varying band levels 350 BPHS, 400 BPHS, 450 BPHS, 500 BPHS, 550 BPHS, and 650 BPHS. The deleted chromosome 18p is one on the right-hand-side (arrowhead). Note that the deletion becomes more obvious at ≥ 450 BPHS.



Fig. 4. Homologous chromosome 5 pairs obtained from 5 amniotic fluid metaphases of varying band levels 350 BPHS, 400 BPHS, 450 BPHS, 500 BPHS, and 550 BPHS. The derivative chromosome 5 is the one on the right-hand-side (arrowheads). Note that the insertion becomes more obvious at ≥ 450 BPHS.

a homogeneous inhibition of contraction along the whole length of the chromosome.¹⁴ A further strategy to obtain high-resolution chromosomes is to use Ohnuki's hypotonic instead of the standard 0.075M KCl hypotonic solution.¹⁵

With the in situ coverslip technique, our cultures are usually harvested after about a week of growth. The use of BrdU at the harvest step serves the dual function of synchronising the cell cycle and acting as a chromosome anti-contraction agent. Metaphase spreads derived from amniotic fluids and chorionic villi routinely achieve a banding level of between 450 and 550 BPHS with this method. Chromosomes with wider widths are obtained when 0.8% sodium citrate hypotonic is used instead of 75mM KCl.

In Case 1, the patient had a genetic amniocentesis performed at 16 weeks' gestation and the specimen was sent to another laboratory for analysis. The result was

reported as normal. No details concerning the banding level achieved or a copy of the karyotype was available. Nevertheless, the requesting clinicians remained suspicious because of the abnormal ultrasound findings and subsequently performed a cordocentesis. Preliminary results obtained from analyses of the 48-hour blood culture initially also suggested an apparently normal karyotype. However, longer chromosomes obtained from the 72-hour cultures showed a subtle terminal deletion in the short arm of one chromosome 18 and the karyotype was re-designated as 46,XY,del(18)(p11.31) (Fig. 1). The referring clinicians were alerted immediately about the abnormality. This deletion was obvious in every metaphase spread with a banding resolution of ≥ 450 BPHS but not with those below this level. The highest band level achieved with the 72-hour cultures was 650 BPHS. As the karyotypes of the parents were normal, the deletion in the foetus was assigned as *de novo*.

All 20 cells scanned from the amniotic fluid in situ cultures with a banding resolution of 450 BPHS or greater showed additional chromosomal material in the long arm of one chromosome 5 at band q33.1. Fluorescence in situ hybridisation (FISH) assays revealed that the additional material was of chromosome 3q origin and included the BCL6 gene locus at band 3q27. Analysis of the parental bloods showed that the mother was a balanced insertional carrier with a 46,XX,ins(5;3)(q33.1;q26.2q27) karyotype. Accordingly, the unbalanced fetal karyotype was designated as 46,XY,der(5)ins(5;3)(q33.1;q26.2q27)mat (Fig. 2).

Detection of subtle chromosomal rearrangements are possible only if the banding resolution is high enough to permit their visualisation. Figure 3 shows the morphology of the homologous chromosome 18 pairs of Case 1 at different band levels. Note that the deletion is apparent only at 450 BPHS and higher. The deletion is consistent with the recent mapping of the holoprosencephaly minimal critical region of chromosome 18 (HPE4) containing the TG-interacting factor (TGIF) gene to 18p11.3.^{16,17} Figure 4 shows the morphology of the homologous chromosome 5 pairs of Case 2 at the different band levels. Again, the insertion is apparent only at ≥ 450 BPHS. In both cases, labour was induced and the products delivered. However, the products of conception were not available for further testing and confirmation.

Conclusion

Cytogenetics laboratories have a responsibility to attain and maintain a high standard of competency. This includes instituting methodologies and technical protocols that will consistently yield long chromosome preparations so that subtle rearrangements can be picked up. The del(18p) and the der(5)ins(5q;3q) abnormalities may be missed with chromosome preparations that have a banding resolution

of ≤ 400 BPHS, or with those preparations that have a poor morphology and sub-optimal banding. Although the 48-hour blood culture had a banding resolution of 450 BPHS, the abnormality was not detected at first because of the somewhat fuzzy chromosome morphology. Essentially, 48-hour culture preliminary results are reported to simply exclude numerical abnormalities and not subtle structural rearrangements. Although a banding level of 400 BPHS is deemed adequate for most prenatal specimens, this report underscores the need for laboratories to implement procedures that will achieve even longer chromosomes for analyses for cases where ultrasound findings are suggestive of an underlying chromosomal pathology.

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