

Twenty-four hour, Non-invasive, Neonatal Chromosome Analysis—Application in a Case of Mixed Gonadal Dysgenesis

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Abstract

Introduction: With the advent of interphase molecular fluorescent in-situ hybridisation (FISH), buccal mucosa can be used to provide an highly accurate assessment of those chromosomes most commonly causing abnormality in live-born children. **Clinical Picture:** A newborn child presented with ambiguous genitalia. The phallus-looking enlarged “clitoris” had a urethral opening at the ventral surface near the tip and the “labial” folds were completely fused. No definite gonads were palpable. Differential diagnostic possibilities included sex chromosome or a single gene abnormality such as congenital adrenal hyperplasia. Thus, one initial objective was to investigate the sex chromosomes. Buccal mucosa was used in conjunction with fluorescent molecular probes for the X and Y. This methodology enabled a firm diagnosis of a 45,X/46,XY mosaic to be made within 24 hours. Decisions could then be made concerning gender assignment. **Treatment and Outcome:** Intervention by means of reconstructive surgery of the external genitalia would be made available at a later date. **Conclusions:** The use of buccal mucosa is non-invasive, easy to obtain and, when combined with molecular techniques, is reliable and accurate. The clinical implication of this methodology is that it will be especially useful in gender assignment or when rapid decisions on live-saving surgery have to be made in cases of possible aneuploidy.

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Key words: Buccal mucosa, FISH, Gonadal ambiguity, Probe, Mosaic, Sex chromosomes

Introduction

Chromosome analysis in neonates requires venisection and this is sometimes both difficult and distressing. In addition, results can at best take 3 days but longer if the initial 48-h culturing period proves unsuccessful. When rapid results are needed, such as those when decisions concerning corrective surgery have to be made, these time periods can become unacceptable.

In the past, Barr body¹ and Y (quinacrine) fluorescence² have been used for foetal sexing using either amniotic fluid or chorionic villi cells. Buccal mucosa was the tissue used for analysis in neonates if the genitalia were ambiguous. Both these techniques were unreliable and were limited in that only either the X or the Y were quantified.

With the advent of interphase molecular fluorescent in-situ hybridisation (FISH),³ buccal mucosa can again be used. However, the analysis is now more extensive and highly accurate, and unlike the older methods, the results are rapid and can be available within 24 h. In the present

report, a newborn child presented with ambiguous genitalia. Genotyping was urgently needed both to distinguish between such differential diagnostic possibilities as a single gene or chromosomal defect and to relieve parental anxiety by giving them an informed gender assignment.

Case Report

A Chinese neonate with ambiguous genitalia was referred at birth. Parents were non-consanguineous. Two elder brothers were normal. The baby was born at 37 weeks of gestation by vaginal delivery with body weight, length and head circumference of 2210 g, 45 cm and 32 cm, respectively. The Apgar score was 9 at 1 min and 10 at 5 min. At 19 weeks of gestation, female external genitalia were noted by routine obstetric sonography. A repeat scan at 32 weeks showed clitoromegaly and a left cystic kidney. The femur length was also noted to be less than the mean, minus 2 standard deviations for gestation age. Physical examination at birth revealed a small-for-date term baby with left accessory ear tags and ambiguous external genitalia

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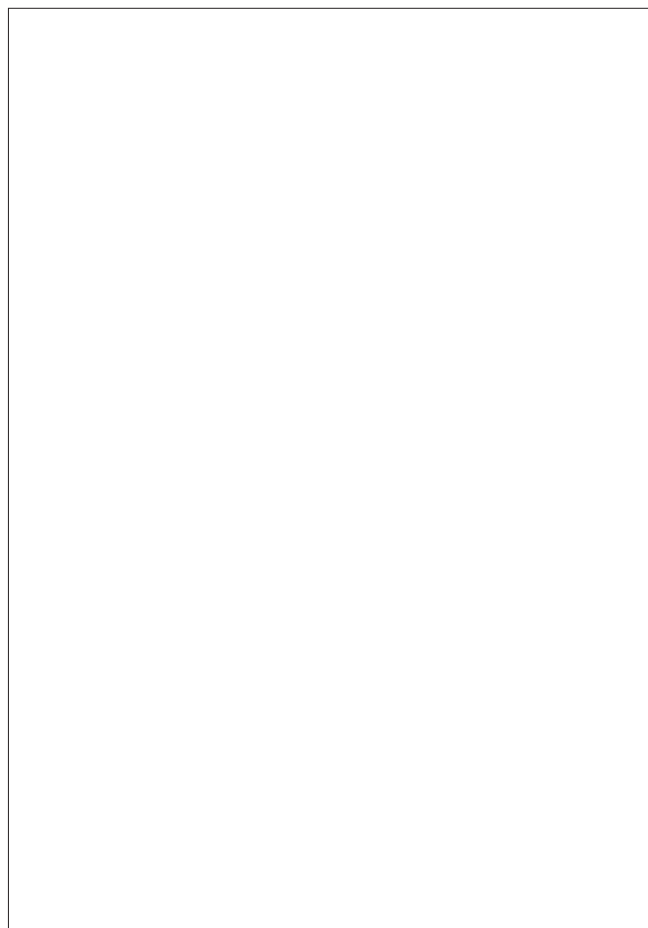


Fig. 1. External ambiguous genitalia demonstrating enlarged "clitoris" and fused labial folds.

(Fig. 1). The phallus-looking enlarged "clitoris" measured 1.5 cm with the urethral opening at the ventral surface near the tip. The "labial" folds with slight pigmentation and little rugosity were completely fused. No definite gonads were palpable in the "labial" folds, inguinal regions and the perineum. The anus was normal in the usual position. The kidneys were not palpable. Initial investigations included buccal smears using FISH analysis; karyotyping of peripheral lymphocytes; biochemical (serum urea, electrolytes and creatine) and endocrinologic (serum cortisol, 17 hydroxyprogesterone, alderone and dehydroepiandrosterone) screening for congenital adrenal hyperplasia (which subsequently proved normal). Echocardiography was performed to exclude associated congenital heart defect, although there was no abnormal cardiovascular sign. This revealed the presence of small fenestrated atrial septal defects with left to right shunt. The electrocardiogram was normal. The buccal smear results demonstrating 45,X/46,XY mosaicism were available within 24 h. The parents and family members were duly informed and the assignment of female gender was affirmed.

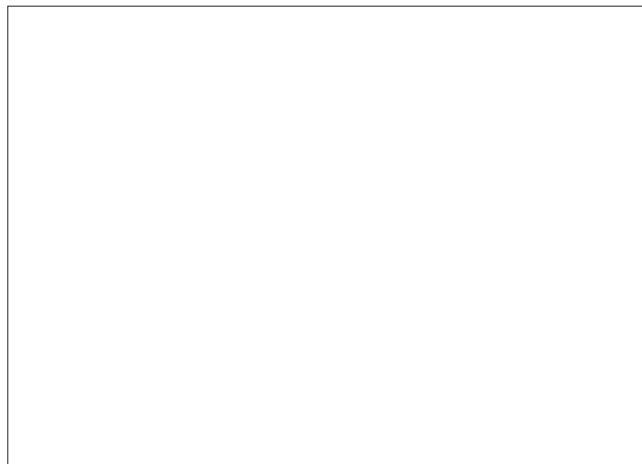


Fig. 2. FISH signals demonstrating two 18 signals in each nucleus; one nucleus also has both an X and a Y (signals green and red respectively), whereas the other has only a green signal representing monosomy X.

As the paradigm for assignment of gender in neonates with ambiguous genitalia is in transition,⁴ the decision was made after careful discussion of the future problems in reproductive and sexual function, sexual dimorphism of the brain and potential risk of malignancy of the dysgenic gonads. Formal karyotyping of the peripheral lymphocytes confirmed the buccal smear result. Renal sonography revealed the presence of a left multicystic kidney with absence of intrarenal blood flow by colour Doppler imaging, normal right kidney and normal adrenal glands. Pelvic sonography showed no well-formed uterus and absence of gonads in the inguinal regions, "labial" folds and perineum. She will be referred to a paediatric surgeon for laparoscopic removal of the gonads and reconstructive surgery of the external genitalia.

Materials and Methods

Buccal mucosa was obtained in the usual manner by means of a wooden spatula drawn against the inner cheek. The spatula end was then placed in Ham's F10 culture media (although normal saline would also be suitable) in a conical centrifuge tube. The top of the spatula was then broken off so that the tube could be sealed for transport to the laboratory. The tube was then shaken to dislodge the cells and the spatula removed. The tube was then centrifuged at 1300 rpm for 5 min and the supernatant discarded.

Once the buccal mucosa cells were recovered, they were then treated as standard for FISH analysis. The methodology has been previously described,⁵ but briefly: the slide preparation was as it would be for amniotic fluid cells and denaturation achieved by means of 70% formamide in 2x sodium chloride/sodium citrate solution (SSC) followed by immediate ethanol washes (70% to absolute). The probes used (Vysis) were for chromosomes 18, X and Y

since these were the chromosomes relevant, with the 18 acting as an internal control against false negativity. (In a separate experiment we were later also able to demonstrate the efficacy of additional probes for chromosomes 13 and 21, again using buccal mucosa). Both the 18, X and Y probes are alphoid sequences and are used with blocking DNA to suppress non-specific hybridisation. They are supplied in a single-stranded state and hybridisation takes place at 37°C for at least 6 h, but usually overnight. The probes are labelled with fluorochromes such that the X fluoresces green; the Y orange/red and the 18 aqua/blue. Diamidinophenylindole (DAPI) is used as a counter stain. We used a single excitation filter suitable for the maximum emission peak of each fluorochrome. These are housed in a rotating turret in an Olympus BX50 fluorescent microscope using UPLAPO x10 and x100 Universal objectives. The acquisition, processing and analysis software was the MacProbe (version 4.0) multi-colour imaging with a Power-Mac 8600 computer.

Once the slides are prepared, the initial analysis assesses the signal intensity and shape. Each should be bright, distinct and oval in shape. More than 90% of cells should demonstrate suitable signals with little or no background fluorescence. Below 10% aneuploidy is disregarded.

Results

A total of 100 interphase cells were examined. Of these, 57% were found to have a single X signal and none for the Y chromosome. In each of these cells, the signal representing the 18 chromosome was present, suggesting that hybridisation of each probe was being achieved. In the remaining 43% of cells, a Y signal was present as was that for the X and again the 18 (Fig. 2).

A subsequent full karyotype analysis using peripheral blood confirmed this preliminary result and the percentage of each cell type was 52% monosomy X and 48% 46,XY (a total of 105 cells were analysed allowing 5% mosaicism or greater to be revealed within 99% confidence limits). The final karyotype was thus: 45,X/46,XY.

Discussion

The use of buccal mucosa allows a non-invasive, rapid method by which chromosome abnormalities causing defects in live-born children can be detected. Probe combinations currently used in prenatal diagnosis (13 and 21, as well as those used here) would allow detection of 67% of chromosome abnormality causing birth defects. In those situations where a major aneuploidy is suspected (or as in this case of ambiguous genitalia), it may be the initial method of choice in the future. Thus for example, where urgent corrective surgery is appropriate in cases of multifactorial cleft lip/palate and heart defect, it may not be

so when the cause is Patau's syndrome.

In addition, parents of newborn babies with ambiguous genitalia urgently need to have an accurate genetic sex, not only to relieve their anxiety but also to be able to give informed answers to relatives and others. In the present case, a rapid (24 h) analysis allowed a firm diagnosis to be made. Not only was the methodology accurate but it later allowed the examination of a tissue from a different embryological origin. Since buccal mucosa is ectodermal, whilst peripheral blood is mesodermal, both between and within tissue mosaicism can be detected. This may allow a more definitive prognosis with a particular phenotype, or where a negative result seems at odds with the diagnosis. Without a direct skin culture, this would not otherwise be possible. Furthermore, mosaicism can now be directly detected within gonadal tissue. Buccal mucosa has also been used for microdeletion syndromes and a variety of polymerase chain reaction-based single gene detection methods using extracted DNA.⁶

It has been shown that a wide spectrum of phenotypes are possible with a 45,X/46,XY karyotype. Thus, Turner syndrome, mixed gonadal dysgenesis, male pseudohermaphroditism and apparently normal male have been reported. The correlation between genotype and phenotype was not apparent in one cohort.⁷ Nonetheless, in that report, 3 males apparently normal at birth later developed dysgenic testes and so such correlations may not be valid until at least puberty. With the availability of the extensive accurate analysis used here, following phenotype development may provide a worthwhile prognostic aid for the future. In addition, observed mosaicism could be correlated with the situation at the single gene level.⁸ Thus, a patient with a Turner phenotype but karyotypically 45,X/46,X,idic(Y)(p11.2) was negative for the sex determining genes (known as SRY and ZFY), whereas a mixed gonadal dysgenesis patient with the same karyotype was SRY and ZFY positive.

Perhaps the major source of influence derived from mosaicism is that within the gonads. Paraffin embedded sections have been analysed in gonads of patients with 45,X/46,XY karyotype presenting with either gonadal dysgenesis, Turner's, ambiguous genitalia, mixed gonadal dysgenesis and atrophic testis.⁹ Using FISH dual colour X and Y probes, it was demonstrated that in mixed gonadal dysgenesis the testis had a higher percentage (x2) of XY cells compared to the ovary but that the highest percentage of XY cells was in the atrophic cells of the infertile male. The Turner females with dysgenic gonads had predominately 46,XY cells in the coelomic epithelium whereas the Leydig and hilius had a lower percentage and least in the ovarian stroma.

Additional prognostic information can be obtained using FISH on buccal mucosa. It is known that patients with

Turner's syndrome phenotype who also possess a Y chromosome, are at risk from gonadoblastoma estimated to be at 15% to 25% of cases.¹⁰ When this is suspected (or even as a precautionary measure in all Turner's), it may be appropriate to perform a secondary analysis using FISH, thereby allowing any such cryptic mosaicism to be detected.

We have shown a favourable comparison in accuracy between the two methods using different tissues. There was only a 5% difference and this is acceptable for levels of above 10% mosaicism. However, it has been shown that in breast-fed infants, maternal cells can contaminate the buccal mucosa especially if sampling is within 5 min of feeding.⁶ Precautions such as cleaning the buccal mucosa and sampling an hour after feeding reduces this problem to almost zero. In addition, in infants with mouth infections such as oral thrush, sampling difficulties may be apparent.

Finally, although FISH is a powerful analytical method, it is nonetheless limited. It cannot detect rearrangements nor aneuploidy for which it was not designed. Therefore, it is best used in context and not in isolation; otherwise the ambiguity that it can help to eliminate may be retained or even exacerbated.

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