Less-Invasive Diagnostic Approaches for Infants with Suspected Differences of Sex Development: A Case Report of a 297-g Neonate with Ambiguous Genitalia

Takeshi Sato, Satsuki Nakano, Yosuke Ichihashi, Hisato Kobayashi, Mariko Hida, Tomohiro Ishii, Tomonobu Hasegawa

Department of Pediatrics, Keio University School of Medicine, Tokyo, Japan; The Center for Differences of Sex Development, Keio University Hospital, Tokyo, Japan

Established Facts
• Less-invasive diagnostic approaches for low-birthweight preterm neonates with suspected differences of sex development have not been established.

Novel Insights
• Endoscope-assisted inspection of the external genitalia and genetic testing using endotracheal aspirate collected during routine care are less-invasive diagnostic approaches in extremely low-birthweight preterm neonates with ambiguous genitalia.

Keywords
Ambiguous genitalia · Extremely low-birthweight infants · Less invasive · Sex assignment

Abstract
Less-invasive diagnostic approaches for low-birthweight preterm neonates with suspected differences of sex development have not been established. Herein, we describe our diagnostic approaches for a 297-g neonate with ambiguous genitalia. Using a fiberscope, the external genitalia were inspected in an incubator to minimize the risk of hypothermia and infection. Endotracheal aspirate, collected during routine care, was used for genetic testing to avoid anemia and vital signs fluctuations caused by peripheral blood sampling. Array comparative genomic hybridization indicated a 46,XY karyotype. No pathogenic variants of AR and SRD5A2 were found. Endocrinological data could not be evaluated owing to the absence of reference data. Identification and structural evaluation of the internal genitalia and gonads were difficult. On postnatal day 42, the parents assigned their baby’s sex as male. Our less-invasive diagnostic approaches of inspection and genetic testing are useful for management, including sex assignment in extremely low-birthweight preterm neonates with ambiguous genitalia.

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Correspondence to:
Tomonobu Hasegawa, thaseg@keio.jp
Introduction

Detailed inspection of the external genitalia and genetic testing are essential for sex assignment of neonates with ambiguous genitalia [1]. For small neonates, detailed inspection is associated with the risk of infection and hypothermia. Moreover, peripheral blood sampling for genetic testing can cause iatrogenic anemia and vital signs fluctuations. To date, less-invasive diagnostic approaches for extremely low-birthweight (ELBW) preterm neonates with ambiguous genitalia who are too small to survive without the aid of an incubator have not been established. Herein, we describe our less-invasive diagnostic approaches in a 297-g baby with ambiguous genitalia.

Case Presentation

The patient presented with fetal growth restriction at 21 weeks of gestation and was born by emergency cesarean section at 26 weeks of gestation due to fetal distress, with a birthweight of 297 g. Respiratory support was required at birth. On examination, the genitalia were ambiguous, and the sex assignment was postponed.

The baby’s external genitalia could not be observed in detail under direct visual inspection from outside the incubator, owing to the small size of the external genitalia. On postnatal day 3, the external genitalia were inspected using a 3.2-mm fiberscope (Ma-chida Endoscope Co. Ltd., Chiba, Japan) for 8 min, while the baby was still in an incubator (shown in Fig. 1a). Fiberscope images and videos revealed a small phallus, partial fusion and slight swelling of the labioscrotal folds, no laterality of the labioscrotal fold, and a urethral meatus in the perineum identified during voiding (shown in Fig. 1b–d). On postnatal day 10, DNA was extracted from endotracheal aspirate obtained during routine intratracheal suction (shown in Fig. 2). Polymerase chain reaction identified the presence of SRY. On postnatal day 17, array comparative genomic hybridization (aCGH) revealed no significant copy number variation of any chromosome, including X and Y, suggesting that the baby had a 46,XY karyotype. Additional Sanger sequencing revealed no pathogenic variants of AR and SRD5A2. Results of endocrinological examinations were inconclusive, owing to the absence of reference data and due to the effects of maternal betamethasone therapy and hydrocortisone administration for late-onset circulatory collapse. Repeated urine steroid profiling by gas chromatography-mass spectrometry implied that deficiencies of 21-hydroxylase, cytochrome P450 11β-hydroxylase, and 11β-hydroxylase, as well as congenital lipoid adrenal hyperplasia, were unlikely (shown in Table 1) [2]. The serum anti-Müllerian hormone level on postnatal day 6 was 92.6 ng/mL (reference for male infants born at term, 55.6 ± 21.3) [3]. Ultrasonography on postnatal day 30 revealed invisible uterus-like structures and in both labioscrotal folds, testes-like structures, 3–4 mm in diameter each. On postnatal day 36, chromosomal analysis of peripheral blood, obtained on postnatal day 26, confirmed the 46,XY karyotype. Our medical team for differences of sex development (DSD) discussed the clinical information and the results of the examinations, including images and videos of the external genitalia, with the following information shared with the parents on postnatal day 39: (i) small phallus and urethral meatus in the perineum, with testes-like structures in both labioscrotal folds with nonavailability of reference data for penile or clitoris size; (ii) ultrasound findings with insufficient information of the location and structure of the internal genitalia; (iii) endocrinological data with lack of reference norms by sex and gestational age; (iv) a 46,XY karyotype with the presence of the SRY gene; and (v) no pathogenic variants in the AR and SRD5A2 genes. On postnatal day 42, the parents assigned and registered their baby’s sex as male.

The parents were informed from the beginning that the DSD team would support them throughout the clinical course. Psychia-trists evaluated parents’ psychological status. Once parents’ coping was ascertained, pediatric endocrinologists provided information regarding diagnostic approaches and management.

Table 1. Results of urine steroid profiling

| Urinary steroids | Local referencesa | Postnatal day |
|------------------|-------------------|---------------|
|                  |                   | 2b  | 7  | 14c | 21c | 27c | 34c | 41c |
| 3α,17α,20α-pregnenetriolone, mg/g creatinine | 0.000–0.003 | 0.002 | <0.002 | 0.003 | <0.003 | 0.008 | 0.005 | <0.002 |
| 5β-tetrahydro-11-deoxycortisol, mg/g creatinine | 0.029–0.326 | 0.032 | 0.214 | 0.959 | 0.521 | 1.328 | 1.788 | 1.069 |
| 16α-hydroxy-pregnenolone, mg/g creatinine | 0.591–77.943 | 0.236 | 9.276 | 12.515 | 66.051 | 25.924 | 68.689 | 22.158 |
| 3α,17α,20α-pregnanetriolone, mg/g creatinine | 0.000–0.102 | 0.131 | 0.453 | 0.936 | 0.251 | 0.908 | 1.286 | 0.281 |
| 16α-hydroxy-dehydroepiandrosterone, mg/g creatinine | 0.262–46.716 | 3.892 | 17.509 | 106.667 | 95.376 | 65.097 | 228.554 | 67.288 |
| Tetrahydroaldestosterone, mg/g creatinine | 0.029–1.077 | <0.014 | <0.017 | 0.118 | 0.045 | 0.229 | 1.552 | 0.779 |
| 5β-tetrahydrocortisone, mg/g creatinine | 1.180–6.411 | 0.194 | 1.853 | 15.114 | 37.591 | 42.831 | 25.499 | 24.753 |

aThese references are for term infants at postnatal day 4. b The mother underwent intramuscular injection of 12 mg of betamethasone, in two doses, at 91 h and 67 h before birth. c The neonate received hydrocortisone supplementation, intravenously or orally, from postnatal day 9, due to late-onset circulatory collapse.
Discussion

We described the usefulness of endoscope examination of the external genitalia and genetic testing using endotracheal aspirate as less-invasive diagnostic approaches for management, including sex assignment, in a 297-g baby. Our procedures have low-to-no risk for infection, anemia, and vital sign fluctuations.

Endoscope-assisted inspection of the external genitalia is superior to conventional inspection as it allows for

**Fig. 1.** Inspection of the external genitalia using a fiberscope. **a** Examination scene. A fiberscope is placed in front of the genitalia, along with a ruler for measurements (marks at every 1 cm and 1 mm are present). **b–d** Images and videos of patient’s external genitalia. **b, c** This picture shows a small phallus, partially fused labioscrotal folds, with slight swelling and no laterality. **c, d** Urethral meatus, identified during voiding, is located in the perineum.

**Fig. 2.** Endotracheal aspirate collected during routine care. Endotracheal aspirate is suspended in normal saline, and pellets are seen in several tubes.
sharing of recorded videos and pictures among different physicians for consultation. Moreover, recorded video during voiding permits identification of the location of the urethral meatus, which is sometimes missed during conventional examinations due to the limited examination time and small external genitalia. We must recognize however that endoscope-assisted inspection is not a complete substitute for conventional inspection. Endoscope-assisted inspection provides only a rough estimation of phallus size and width and the degree of labial fusion. Thus, while conventional inspection remains important, endoscope-assisted inspection is useful to inspect the external genitalia in ELBW preterm neonates.

Genetic testing using endotracheal aspirate provides practical advantages. Foremost, the use of endotracheal aspirate avoids iatrogenic anemia and vital signs fluctuations associated with blood sampling. Endotracheal aspirate is easily collected during routine intratracheal suction and provides sufficient genomic DNA, even in very small neonates. The DNA in endotracheal aspirate allowed us to perform diagnostic genetic testing, namely, Sanger sequencing and aCGH, and obtained the results in a week. These two methods have indispensable advantages, as follows. Sanger sequencing is inexpensive and rapid to perform, compared to next-generation sequencing. aCGH, and results are often modified by maternal betamethasone therapy and hydrocortisone administration for late-onset circulatory collapse. Therefore, to interpret results of the urine steroid profile in premature infants, clinicians must closely cooperate with specialists to analyze the urine steroid profiles of DSDs.

Even in very-premature infants, the urine steroid profile is a potential diagnostic tool, allowing us to identify which step in steroid metabolism is impaired without relying on references as ratios and concentrations of metabolites are directly associated with metabolic defect. However, it is important to note that interpretation of results of the urine steroid profile requires specialized knowledge, especially for premature infants where results are often modified by maternal betamethasone therapy and hydrocortisone administration for late-onset circulatory collapse.

In conclusion, for ELBW preterm neonates with ambiguous genitalia, endoscope-assisted inspection is useful to inspect the external genitalia and genetic testing using endotracheal aspirate is beneficial for ELBW preterm neonates with ambiguous genitalia. In the process of identifying the underlying diseases or clarifying the pathogenesis in ELBW preterm neonates with ambiguous genitalia, there are three potential hurdles that our procedures cannot overcome. First, gonadal and adrenal function could not be accurately evaluated due to the absence of reference data by sex and gestational age and the effect of exogenous steroid therapy. Second, the uterus and gonads may sometimes be difficult to identify by imaging studies due to the small organ size or physiologically undescended testes. For the diagnosis for 21-hydroxylase deficiency, XX testicular DSD, mixed gonadal dysgenesis, or ovotesticular DSD, functional assessment of gonadal or androgen hormone and structural evaluation of the internal genitalia are mandatory [5–7]. Third, genetic diagnosis may be confirmed in only a low proportion of small-for-gestational-age infants with ambiguous genitalia [8, 9]. Therefore, we must investigate each patient individually from various angles and decide on the management strategy carefully, including steroid therapy and sex assignment. In particular, sex assignment may be delayed for neonates suspected of having above-mentioned disorders until functional assessment or structural evaluation is available.
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Statement of Ethics

This study has been complied with all the relevant national regulations, institutional policies, and in accordance the tenets of the Helsinki Declaration and has been approved by the Institutional Review Board at the Keio University School of Medicine (Institutional Review Board number 20170130). We obtained written informed consent from the patient’s parent for publication of this report, including photos.

Conflict of Interest Statement

Tomonobu Hasegawa has the following financial relationships to disclose: research funding from AMED (22ek0109464h0003), Novo Nordisk Pharma Ltd., and JCR Pharmaceuticals Co., Ltd. The other authors declare no conflict of interest.

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Author Contributions

Dr. Takeshi Sato, Dr. Tomohiro Ishii, and Dr. Tomonobu Hasegawa conceptualized and designed the study, collected data, carried out the analyses, drafted the initial manuscript, and reviewed and revised the manuscript. Dr. Satoki Nakano, Dr. Yosuke Ichishashi, Dr. Hisato Kobayashi, and Dr. Mariko Hida conceptualized and designed the study, collected data, carried out the analyses, and critically reviewed the manuscript. All the authors approved the final manuscript for submission and agree to be accountable for all aspects of the study.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.