Original Research

Fetal Sex Chromosome Testing by Maternal Plasma DNA Sequencing

Clinical Laboratory Experience and Biology

Diana W. Bianchi, MD, Saba Parsa, MS, Sucheta Bhatt, MD, Meredith Halks-Miller, MD, Kathryn Kurtzman, MD, Amy J. Sehnert, MD, and Amy Swanson, MS

OBJECTIVE: To describe the clinical experience with noninvasive prenatal testing for fetal sex chromosomes using sequencing of maternal plasma cell-free DNA in a commercial laboratory.

METHODS: A noninvasive prenatal testing laboratory data set was examined for samples in which fetal sex chromosomes were reported. Available clinical outcomes were reviewed.

RESULTS: Of 18,161 samples with sex chromosome results, no sex chromosome aneuploidy was detected in 98.9% and the fetal sex was reported as XY (9,236) or XX (8,721). In 4 of 32 cases in which the fetal sex was reportedly discordant between noninvasive prenatal testing and karyotype or ultrasonogram, a potential biological reason for the discordance exists, including two cases of documented co-twin demise, one case of a maternal kidney transplant from a male donor, and one case of fetal ambiguous genitalia. In the remaining 204 samples (1.1%), one of four sex chromosome aneuploidies (monosomy X, XXX, XXY, or XYY) was detected. The frequency of false positive results for sex chromosome aneuploidies is a minimum of 0.26% and a maximum of 1.05%. All but one of the discordant sex chromosome aneuploidy results involved the X chromosome. In two putative false-positive XXX cases, maternal XXX was confirmed by karyotype. For the false-positive cases, mean maternal age was significantly higher in monosomy X ($P<.001$) and lower in XXX ($P=.008$).

CONCLUSION: Noninvasive prenatal testing results for sex chromosome aneuploidy can be confounded by maternal or fetal biological phenomena. When a discordant noninvasive prenatal testing result is encountered, resolution requires additional maternal history, detailed fetal ultrasonography, and determination of fetal and possibly maternal karyotypes.

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LEVEL OF EVIDENCE: II

Noninvasive prenatal testing using massively parallel sequencing of circulating cell-free DNA in maternal plasma for detection of fetal aneuploidy has evolved rapidly since its introduction into clinical practice in 2011.[1] Before massively parallel sequencing, detection of Y chromosome sequences in maternal blood using quantitative polymerase chain reaction was offered by some laboratories to determine fetal sex in families at risk for sex-linked disorders.[2–4] Subsequent studies demonstrated the ability to analyze sex chromosome sequences using massively parallel sequencing to classify XY, XX, and monosomy X with a high degree of sensitivity and specificity[5,6] and to detect other sex chromosome aneuploidies, including XXX, XXY, and XYY.[7–15]

Sex chromosome aneuploidies are common. Monosomy X is present in 1–1.5% of recognizable gestations.[16] The prevalence of sex chromosome aneuploidies is on the order of 1.88 per 10,000 births.[17] With the exception of monosomy X, most affected individuals do not present with anomalies or symptoms in the neonatal period. They may,

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Corresponding author: Amy Swanson, MS, 800 Saginaw Drive, Redwood City, CA 94063; e-mail: aswanson@illumina.com.

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however, develop abnormalities in growth, sexual maturation, and learning later in childhood as well as infertility in adulthood. In 2012, noninvasive prenatal testing for monosomy X was first offered to women who had ultrasonographic detection of fetal cystic hygroma.[6] This was followed by clinical noninvasive prenatal testing for other sex chromosome aneuploidies and fetal sex.[18,19]

Although data are available regarding the results of clinical trials and noninvasive prenatal testing laboratory performance for autosomal aneuploidy, less is known regarding noninvasive prenatal testing laboratory performance when testing for fetal sex chromosomes and sex chromosome aneuploidies. According to a recent meta-analysis,[20] as compared with the autosomal aneuploidies, the published validation studies on sex chromosome aneuploidies have generally involved smaller numbers of cases. Furthermore, prenatal testing is complicated by the fact that there are no precedents in clinical practice with regard to specifically screening for sex chromosome aneuploidies. Noninvasive determination of fetal sex also poses ethical problems that arise from social sex selection. Notably, although the obstetric and genetic professional societies have made recommendations regarding noninvasive prenatal testing for autosomal aneuploidy,[21–24] they have remained relatively silent regarding screening for sex chromosome aneuploidies despite the fact that these tests have been clinically available for more than 2 years.

We present a summary of initial clinical experience for sex chromosome testing using the verifi® prenatal test. Additionally, we describe some of the underlying biological reasons for discordant sex chromosome results and discuss management considerations for these cases.

MATERIALS AND METHODS

The data presented here were collected or generated as part of cell-free DNA testing for fetal aneuploidy in the Illumina (formerly Verinata Health, Redwood City, CA) noninvasive prenatal testing laboratory that is accredited by the College of American Pathology and certified by the Clinical Laboratory Improvements Amendments Act. Sex chromosome aneuploidy testing is offered as an additional option for women undergoing noninvasive prenatal testing for fetal autosomal aneuploidy (chromosomes 13, 18, and 21). The inclusion criteria for this observational study were: patients undergoing noninvasive prenatal testing for autosomal aneuploidy who also selected the fetal sex test option and had a result for sex chromosome status reported in the laboratory information management system database used for this query.

All testing was performed on maternal whole blood samples in cell-free DNA BCT™ tubes received at Illumina within 5 days of the blood draw and accessioned with a complete test requisition form authorized by an ordering health care provider. The clinical indications for testing were determined by the ordering physician.

Genome-wide massively parallel sequencing of cell-free DNA isolated from maternal plasma was performed as per previously validated laboratory procedures using methods for sample preparation, sequencing, and analysis that were similar to those reported by Futch et al.[18] Sex chromosome results were classified into one of six discrete categories: XX, XY, monosomy X, XXX, XXY, and XYY based on the normalized chromosome values obtained for both X and Y. The verifi® test does not distinctly identify each cell line if sex chromosome mosaicism is present. For those women who opted for sex chromosome aneuploidy testing, the clinical test results reported sex chromosome aneuploidy status as "detected" or "not detected." If no sex chromosome aneuploidy was detected, the fetal sex was reported as XY or XX.

Demographic information was obtained from the test requisition form. All cases of sex chromosome aneuploidy detected were phoned to the ordering health care provider by a certified genetic counselor employed by Illumina. Clinical outcome information for cases in which a sex chromosome aneuploidy was detected was requested during this initial phone call. If no outcome information was obtained during the pregnancy, an outcome request form was faxed to the health care provider approximately 2 weeks after the estimated date of confinement. Additionally, health care providers are regularly encouraged to provide information on all discordant results (false-negatives and false-positives) to the noninvasive prenatal testing laboratory. Any information available on fetal ultrasound examination, prenatal or postnatal karyotype studies, or pregnancy outcome was recorded in the noninvasive prenatal testing laboratory follow-up database by the genetic counselor.

For the purposes of this study and internal documentation, sex chromosome aneuploidy cases were categorized into one of the following groups: 1) "concordant" if the noninvasive prenatal testing sex chromosome aneuploidy result matched the fetal or neonatal karyotype, 2) "discordant" if the noninvasive prenatal testing sex chromosome aneuploidy result did not match the fetal or neonatal karyotype, or 3) "no follow-up available" if no follow-up information was reported back to the noninvasive prenatal testing laboratory.

If noninvasive prenatal testing did not identify a sex chromosome aneuploidy, the fetal sex was reported as XY or XX. Fetal sex chromosome results were categorized into one of the following groups: 1) "concordant" if the noninvasive prenatal testing sex result matched the fetal or neonatal karyotype or phenotype, 2) "discordant by karyotype" if the noninvasive prenatal testing sex result did not match the fetal or neonatal karyotype, 3) "discordant by ultrasound" if the noninvasive prenatal testing sex result did not match ultrasound or postmortem examination of sex; or 4) "no follow-up available" if no follow-up information was reported back to the noninvasive prenatal testing laboratory.

All identified "discordant" results underwent a review of sample batch records and other pertinent information by the laboratory directors to rule out possible technical or laboratory-associated explanations for the discordance.

Summary data were reported as frequencies, percentages, means, and standard deviations. Comparison of mean gestational age between "no sex chromosome aneuploidy detected" and "sex chromosome aneuploidy detected" groups was performed using analysis of variance for a single factor. The same analysis was used to compare mean maternal ages between the false-positive monosomy X or XXX cases and the "concordant" and "no follow-up available" cases in which monosomy X or XXX was detected.

RESULTS

Noninvasive prenatal testing results were available for 18,161 samples tested for sex chromosome classification during the study period (Fig. 1). Follow-up information was available for 194 cases. Demographic characteristics of this population are shown in Table 1.

For the 17,957 out of 18,161 cases (98.9%) in which no sex chromosome aneuploidies were detected, the sex chromosome results are shown in Figure 1. The XY to XX ratio was 1.06.

For the 63 of 9,236 results of XY by noninvasive prenatal testing in which we have clinical follow-up information available, there were 14 total cases of discordance reported by either ultrasonography (n=10) or karyotype (n=4). None of the documented cases of XY discordance were explained by laboratory error. For the 10 cases of discordance by ultrasonography, the noninvasive prenatal testing result was reported as XY, but the ultrasound examination was suggestive of female genitalia. Two additional cases of discordance by ultrasound (female genitalia) were initially reported to the laboratory. However, on repeat ultrasound examination, the fetal genitalia were confirmed to be male and thus concordant with the noninvasive prenatal testing result. In three of the four cases of discordance by karyotype, the fetal karyotype was XX; in two of these, a co-twin demise of unknown sex was documented. In one noninvasive prenatal testing result of XY, the fetal karyotype was XXY (false-negative).

There are two additional noteworthy cases in which the clinical laboratory was contacted because discordance was suspected after a noninvasive prenatal testing result of XY. In the first case, the initial ultrasound examination was suggestive of female genitalia. Internal review of the noninvasive prenatal testing results remained consistent with an XY sex chromosome result. Further discussion with the ordering provider led to a repeat ultrasound examination; this resulted in a diagnosis of ambiguous genitalia. Additional ultrasonographic findings included short long bones, ventriculomegaly, a flattened facial profile, and a possible heart defect. This combination of fetal anomalies suggested a clinical diagnosis of campomelic dysplasia. An intrauterine fetal death occurred at 38 weeks of gestation, and no autopsy was performed. The fetal karyotype was 46, XY with normal microarray and *sex-determining region Y* testing. In the second case, a repeat ultrasound examination remained consistent with female genitalia. Detailed review of the maternal history was significant for receiving a kidney transplant from a male donor. In both of these cases, the noninvasive prenatal testing result correctly detected Y chromosome sequences and the discordance prompted further investigation.

For the 70 of 8,721 results of XX by noninvasive prenatal testing in which we have clinical follow-up information available, there were 18 total cases of discordance reported by either ultrasonography (n=10) or karyotype (n=8). None of the documented cases of XX discordance were explained by laboratory error. All 10 cases of discordance by ultrasound examination were suggestive of male genitalia. For the eight cases of discordance by karyotype, the karyotype was XY in four cases, monosomy X in three cases, and mosaic monosomy X in one case (four false-negative sex chromosome aneuploidy cases).

Sex chromosome aneuploidies were detected in 204 cases (1.1%). The most frequently detected was monosomy X (n=148) followed by XXX (n=38), XXY (n=12), and XYY (n=6) (Table 1; Fig. 1). Clinical follow-up information was available for 61 of the 204 cases.

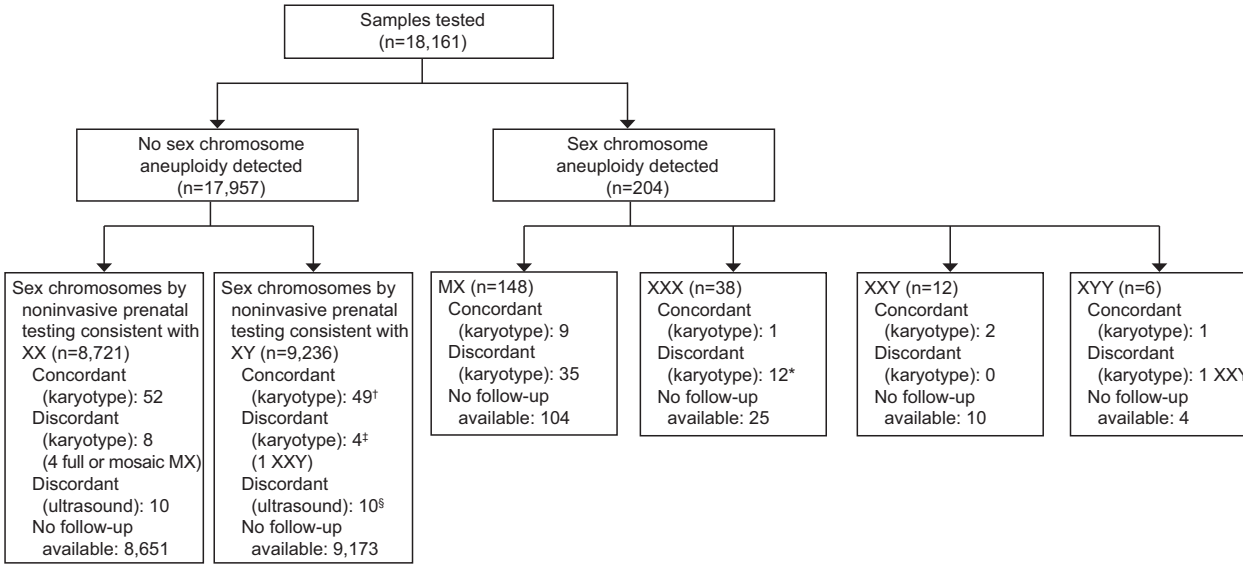

Fig. 1. Overall laboratory data set showing numbers of cases represented in this report with numbers of concordant and discordant cases as well as cases with no follow-up information available. *Maternal karyotype subsequently consistent with XXX cell line in two cases. †In two cases initially reported as discordant, repeat ultrasonography confirmed concordance (male genitalia). In another case, discordance was initially reported, but subsequent ultrasonography revealed ambiguous genitalia and karyotype on the demised fetus was concordant (46,XY). ‡Co-twin demise documented in two cases. §In one case, the mother was status post kidney transplant from male donor. MX, monosomy X.

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For the 44 of 148 results of monosomy X by noninvasive prenatal testing on which we have clinical follow-up information available, there were 35 false-positive results. Among these 35, 34 were reported as XX and one as XY on fetal karyotype. The mean maternal age in the false-positive cases for monosomy X was 36.7 compared with 31.7 years for nondiscordant cases ($P<.001$).

Table 1. Patient Demographics on Cohort Tested for Sex Chromosomes (n=18,161)

Demographic	Value
Maternal age (y)	35.7±4.9 (14–53)
Gestational age (wk)	14.0±4.0* (10–36)
Gestational age groups (wk)	
1st trimester (up to 13)	11,759 (65.1)
2nd trimester (14–27)	6,126 (33.9)
3rd trimester (28–40)	191 (1.06)
Aneuploidy detected	
Monosomy X	148 (0.81)
XXX	38 (0.21)
XXY	12 (0.07)
XYY	6 (0.03)

Data are mean±standard deviation (minimum–maximum) or n (%).
* Mean gestational ages between patients with sex chromosome aneuploidy detected compared with no sex chromosome aneuploidy detected were not statistically different (14.4 and 14.0, respectively; $P=.141$).

For the 13 of 38 results of XXX by noninvasive prenatal testing on which we have clinical follow-up information available, there were 12 false-positive results. In 2 of these 12 cases, a maternal karyotype was performed and revealed an XXX cell line. Mean maternal age in the false-positive cases for XXX was 31.6 compared with 37.8 years for nondiscordant cases ($P=.008$).

There was clinical follow-up information available on two of 12 reported cases of XXY by noninvasive prenatal testing. Among these, there were no false-positive cases reported. There was follow-up information available on two of six cases of XYY on noninvasive prenatal testing. Among these, there was one case of a known difference in the fetal karyotype. The fetus was XXY, whereas the noninvasive prenatal testing result was XYY.

DISCUSSION

Noninvasive prenatal testing for sex chromosome aneuploidies and fetal sex determination is more complicated than for autosomal aneuploidy and can be confounded by a variety of different maternal and fetal biological phenomena. As such, patients should be counseled about the benefits and limitations before undergoing testing for fetal sex chromosomes. In the setting of discordance between noninvasive prenatal

testing results and karyotype or ultrasonography for fetal sex, the clinician should consider further testing, because there may be a clinically relevant underlying explanation such as a maternal sex chromosome abnormality. Furthermore, given the complexity of noninvasive sex chromosome testing, professional societies should consider drafting management recommendations.

In this noninvasive prenatal testing cohort, there were 13 cases of sex chromosome aneuploidy confirmed by fetal karyotype, 48 cases determined to be false-positive, and 143 cases with no follow-up information available (Fig. 1). Based on the incomplete clinical follow-up information in this cohort, test performance cannot be calculated. Furthermore, ascertainment bias was potentially introduced as a result of clinician follow-up with the laboratory on cases with discordant as opposed to concordant results. However, a range of possible false-positive frequencies can be determined. For example, if the 48 known discordant cases with sex chromosome aneuploidy by noninvasive prenatal testing were the only false-positive cases, the overall percentage of false-positive results would be 48 of 18,161 (0.26%). Alternatively, if all 143 cases with no follow-up available were also false-positive, the overall percentage of false-positive results would be 191 of 18,161 (1.05%). Thus, the true false-positive rate for sex chromosome aneuploidy in this cohort ranges between 0.26% and 1.05%.

All but one of the discordant sex chromosome aneuploidy results involved the X chromosome. As we have shown, the discordant X chromosome results could be maternal or fetal (which is really placental DNA) in origin (Fig. 2). There was more discordance for monosomy X than for any other sex chromosome aneuploidy. Interestingly, the maternal age for the

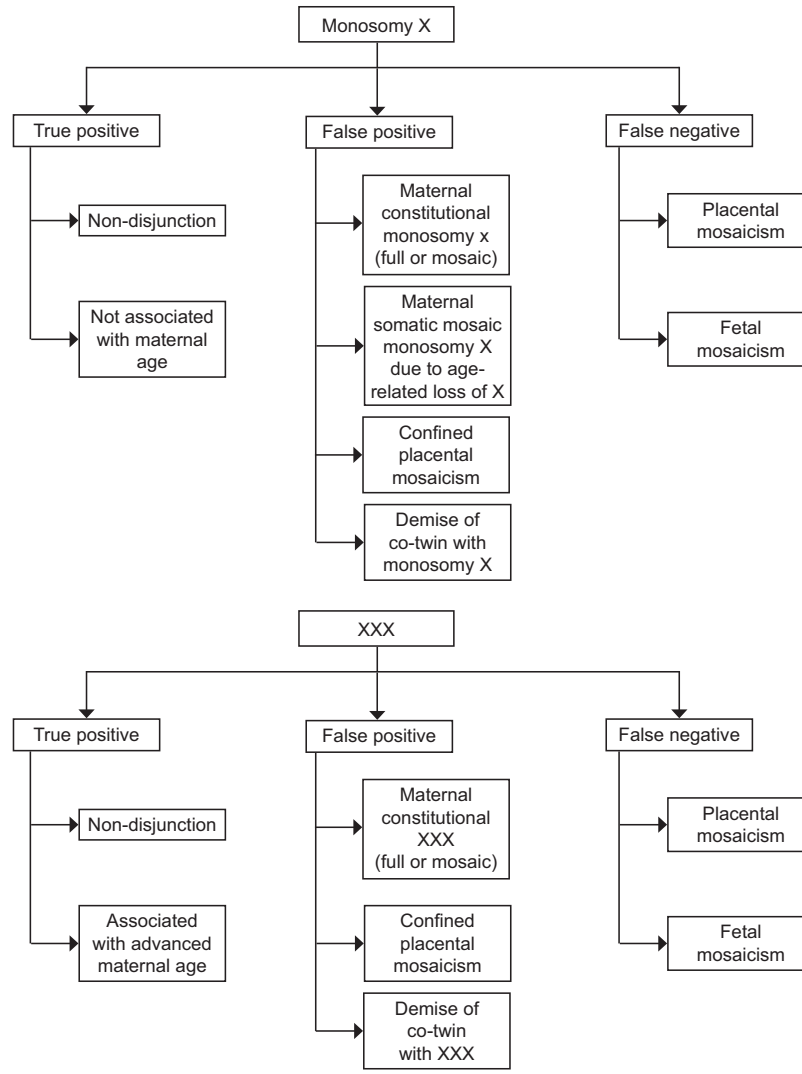

Fig. 2. Possible biological explanations for noninvasive prenatal testing for monosomy X and XXX.

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false-positive cases of monosomy X was higher than the maternal age for the nondiscordant monosomy X cases. An association between X chromosome loss and aging has been previously reported.[25] This could explain some of the false-positive cases, because maternal DNA is being sequenced along with the fetal DNA. With regard to the four false-negative cases of monosomy X (of which one was mosaic), a possible explanation is placental mosaicism (Fig. 2). In fact, Hook and Warburton[16] recently concluded that all fetal cases of monosomy X that survive to the end of gestation are cryptic mosaics with both a 45,X line and a rescue 46, XX line, which is likely to be located in the placenta as a result of the need for expression of *PSF2RA*, a gene that is essential for normal placental function.

Maternal sex chromosome aneuploidies (full or mosaic) are a common biological explanation for sex chromosome aneuploidy discordance that involves the X chromosome (Fig. 2). In a recent study of cases in which noninvasive prenatal testing results suggested a sex chromosome aneuploidy involving a gain or loss of the X chromosome,[26] maternal white blood cell analyses showed that the sex chromosome aneuploidy originated from the mother 8.6% of the time. In our data set, two discordant cases of XXX were documented to be maternal in origin. Furthermore, maternal age was statistically significantly lower in the false-positive cases of XXX, suggesting that these are the result of maternal sex chromosome aneuploidies, whereas the concordant cases are fetal sex

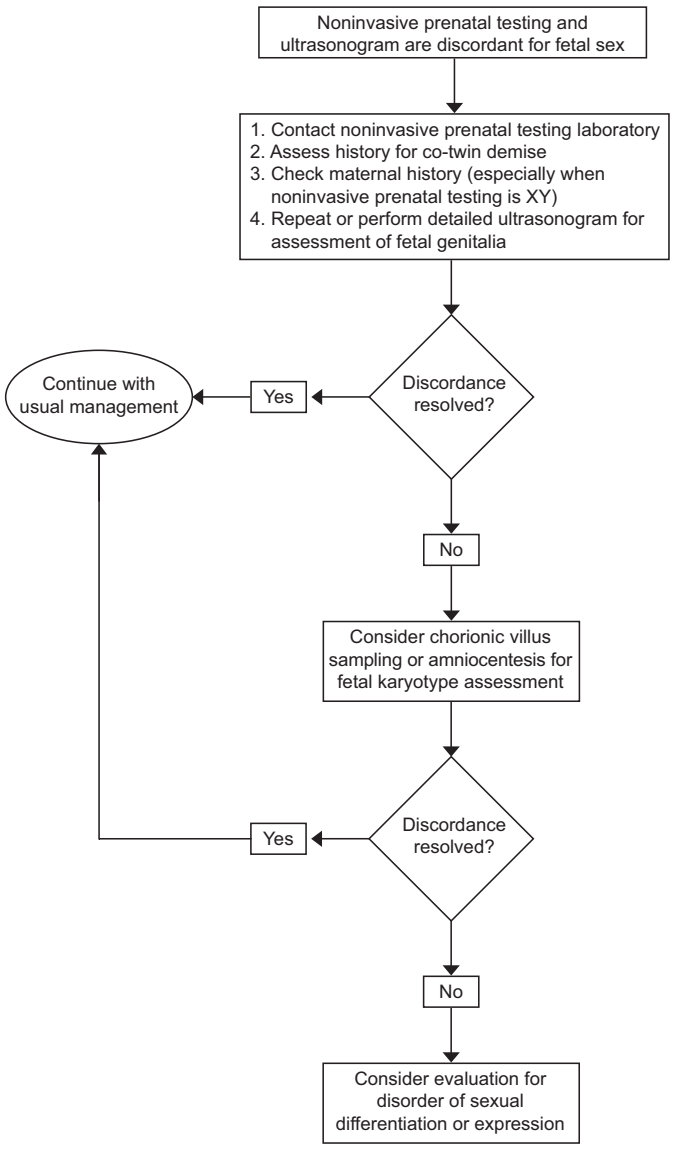

Fig. 3. Suggested clinical management for cases in which there is discordance between noninvasive prenatal testing results and ultrasound examination for fetal sex.

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chromosome aneuploidies resulting from maternal age-associated nondisjunction of the X chromosome. The prevalence of maternal sex chromosome aneuploidies has been underestimated and may indicate a wider phenotypic spectrum for sex chromosome aneuploidies including normal fertility for women with full or mosaic monosomy X.[26,27] Although not observed in this study, another potential biological explanation for sex chromosome aneuploidy discordance is confined placental mosaicism[28] (Fig. 2).

After a sex chromosome aneuploidy by noninvasive prenatal testing is detected, the pregnant woman may desire a diagnostic procedure to confirm the fetal karyotype. In cases in which a noninvasive prenatal testing result and fetal karyotype are discordant, maternal blood chromosome studies should be considered to determine if the mother has a sex chromosome aneuploidy.

Despite having incomplete clinical follow-up data, the ratio of XY to XX samples in this study (1.06) was very close to the U.S. birth statistics (1.05) reported in 2012.[29] Based on detailed follow-up information collected on the small number of known discordant cases, we propose several possible etiologies including: co-twin demise, disorders of sexual differentiation, inaccurate ultrasonographic imaging, or maternal bone marrow or organ transplant. In the latter, circulating Y DNA sequences derive from low-grade rejection of the transplanted organ, which results in cellular turnover.[30] Additionally, endocrine disorders that result in masculinization of a female fetus such as congenital adrenal hyperplasia may confound ultrasound interpretation of fetal sex, although no such cases were reported here.

In clinical practice, health care providers and patients will occasionally encounter cases in which noninvasive prenatal testing results for fetal sex and ultrasonographic findings of fetal genitalia are discordant. In such cases, we propose that clinicians should perform some simple, noninvasive steps (Fig. 3) before performing a diagnostic procedure for fetal sex determination. These include contacting the noninvasive prenatal testing laboratory for review of the X and Y values obtained, assessing the maternal history for prior transplant, querying the pregnancy history for the use of assisted reproductive technology and the possibility of a co-twin demise, and performing detailed ultrasound examination of the fetal genitalia. If these are unrevealing, a diagnostic procedure such as amniocentesis could be considered to resolve the sex discordance.

As a result of the many different potential biological explanations for discordance between noninvasive prenatal testing results and karyotype and clinical findings, considerable pretest and posttest counseling is critical. Although patients can be informed of the generally high sensitivities and specificities for sex chromosome aneuploidy and fetal sex determination with noninvasive prenatal testing,[20] the limitations of the test and the possible need for further evaluation if test results are discordant should also be discussed.

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