Massively Parallel Sequencing of Maternal Plasma DNA in 113 Cases of Fetal Nuchal Cystic Hygroma

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OBJECTIVE: To estimate the accuracy and potential clinical effect of using massively parallel sequencing of maternal plasma DNA to detect fetal aneuploidy in a cohort of pregnant women carrying fetuses with nuchal cystic hygroma.

METHODS: The MatErnal BLood IS Source to Accurately diagnose fetal aneuploidy (MELISSA) study database was queried to identify eligible patients carrying fetuses with cystic hygroma (n=113) based on clinical ultrasonographic examination reports near enrollment. Archived plasma samples were newly sequenced and normalized chromosome values were determined. Aneuploidy classifications for chromosomes 21, 18, 13, and X were made using the massively parallel sequencing data by laboratory personnel blinded to fetal karyotype and compared for analysis.

RESULTS: Sixty-nine of 113 (61%) patients had fetuses with abnormal karyotypes, including trisomy 21 (n=30), monosomy X (n=21), trisomy 18 (n=10), trisomy 13 (n=4), and other (n=4). There were 44 euploid cases; none was called positive for aneuploidy. The massively parallel sequencing detection rates were as follows: T21: 30 of 30, T18: 10 of 10, T13: three of four, and monosomy X: 20 of 21, including two complex mosaic cases. Overall, using massively parallel sequencing results of the four studied chromosomes, 107 of 113 (95%, 95% confidence interval [CI] 88.8–98.0) cases were accurately called by massively parallel sequencing, including 63 of 65 (97%, 95% CI 89.3–99.6) of cases of whole chromosome aneuploidy.

CONCLUSION: Massively parallel sequencing provides an accurate way of detecting the most prevalent aneuploidies associated with cystic hygroma. Massively parallel sequencing could advance prenatal care by providing alternative point-of-care noninvasive testing for pregnant women who either decline or do not have access to an invasive procedure.

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With widespread incorporation of first-trimester prenatal screening, the fetal neck is routinely examined sonographically. Measurement of the nuchal translucency thickness is an important component of

**For a list of other members of the MELISSA Study Group, see the Appendix online at http://links.lww.com/AOG/A368.*

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the first-trimester scan, and cystic hygroma, when present, is commonly visible. A nuchal cystic hygroma is described as an enlarged hypoechoic space visible at the posterior neck of the fetus in which septations may also develop. Its pathophysiology is thought to be related to abnormalities in formation of major lymphatic channels.[1] The prevalence of first-trimester cystic hygroma is one in 285, and it is strongly associated with fetal chromosomal aneuploidy and death.[1,2] Among the most common associated aneuploidies in the first trimester are trisomy 21, monosomy X (45,X), trisomy 18, and trisomy 13.[1,2] Cystic hygroma can also be diagnosed during the second trimester[3] and in some cases resolves before birth.[1,2]

Given the known association between cystic hygroma and fetal chromosome abnormalities, it is currently standard of care to offer chorionic villus sampling (CVS) or amniocentesis to determine fetal karyotype. Invasive procedures, however, carry risk of miscarriage, and patient access to CVS providers is sometimes limited. Massively parallel sequencing of maternal plasma DNA provides a potential alternative to detect fetal chromosome aneuploidy in this high-risk population.[4] We hypothesized that noninvasive prenatal testing in the setting of cystic hygroma could potentially provide fetal karyotype information and thus help in pregnancy management. We tested this using plasma samples and clinical data from the MatErnal BLood IS Source to Accurately diagnose fetal aneuploidy (MELISSA) study.[4]

MATERIALS AND METHODS

The MELISSA study (Clinicaltrials.gov NCT01122524) was conducted at 60 U.S. medical centers under approval by local institutional review boards. Enrollment occurred from June 2010 through August 2011. Written informed consents were obtained from all participants. Eligibility criteria for the study have been previously described and included women at high risk for fetal aneuploidy undergoing invasive prenatal procedures to determine fetal karyotype.[4] For the present study, the electronic clinical database of the MELISSA study was searched to identify archived plasma samples from pregnant women carrying fetuses with sonographically diagnosed cystic hygroma at the participating study sites.

During the MELISSA study, the results of one fetal ultrasonographic examination for each enrolled patient were recorded based on the examination performed closest to the date of enrollment (during either the first or second trimester). Because the focus of the MELISSA study was on fetal chromosome abnormalities and not sonographic findings, there were no specific definitions for sonographic abnormalities, which were collected on a "fetal ultrasound page." The information entered into the data field was monitored against the original source documentation (eg, the ultrasound report) at each clinical site. For the cystic hygroma study, all patients with an eligible blood sample that had a singleton pregnancy and a karyotype result, and with selection of the field "cystic hygroma" on the fetal ultrasound page or "other, please specify" with the words "cystic hygroma" entered in the comments field, were identified. From this search, a list with 113 patients was generated. Of these 113, 74 patients had plasma samples that were previously sequenced during the MELISSA trial. In this study, all 113 residual frozen plasma samples were newly resequenced at the Verinata Health research laboratory using the most recent version of the sequencing chemistry (Illumina TruSeq 3.0) and normalized chromosome values were determined using methods as previously described.[4,5] Laboratory personnel performing sequencing and aneuploidy classifications were blinded to the fetal karyotype.

Each sample was classified for four independent categories: aneuploidy status for chromosomes 21, 18, and 13 as well as the presence or absence of monosomy X. For the autosomes (chromosomes 21, 18, and 13), the sequencing laboratory classified the sample in one of three ways: "aneuploidy detected," "no aneuploidy detected," or "aneuploidy suspected (borderline value)." For monosomy X, the classification was either "detected" or "not detected."

The sequencing classification zones for fetal aneuploidy in the current study differed slightly from the ones that we previously reported.[4] Classification of autosomal "aneuploidy detected" required a normalized chromosome value of greater than 4.0, and a normalized chromosome value of less than 3.0 was used for "no aneuploidy detected." Samples with normal chromosome values between 3.0 and 4.0 for the autosomes were called "aneuploidy suspected (borderline value)." To classify the status for monosomy X, normalized chromosome values for both X and Y were used. Monosomy X "detected" required a normalized chromosome value X less than -3.0 and an normalized chromosome value Y less than 3.0. When normalized chromosome value values were outside this range (eg, normalized chromosome value X greater than -3.0 and normalized chromosome value Y greater than 3.0), then monosomy X was "not detected."

At the time of analysis, sequencing classifications were compared with cytogenetic results from CVS or amniocentesis as the reference standards. For chromosome 21, 18, and 13 performance calculations, both "aneuploidy detected" and "aneuploidy suspected (borderline value)" results were considered

"positive." Sensitivities, specificities, and exact 95% confidence intervals (CIs) were computed using the Clopper-Pearson method.

RESULTS

The demographic characteristics of the 113 eligible patients are shown in Table 1. In addition to nuchal cystic hygroma, 43 of the 113 patients in this cohort also showed other fetal abnormalities by sonography. Of the 113 patients, 88 (78%) had the fetal karyotype determined by CVS, 23 (20%) by amniocentesis, and two were obtained from products of conception. Sixty-nine of the 113 (61%) patients had fetuses with abnormal karyotypes, including 30 cases of trisomy 21, 21 cases of monosomy X, 10 cases of trisomy 18 (one was mosaic), four cases of trisomy 13, and four others (Table 2). Two of the 21 participants included in the monosomy X category had complex mosaic karyotypes (Table 2), including one patient whose fetus was diagnosed as having three cell lines by CVS (45,X,inv(9)(p12q13)[3]/46,XX,inv(9)(p12q13)[7]/46,XX[30]). The possibility of maternal cell contamination was suggested in the cytogenetic report and an amniocentesis was recommended. At amniocentesis, the fetal karyotype was 46, XX. There was no further follow-up as part of the MELISSA study. We also included in the "euploid" category two cases of pericentric inversion of chromosome 9, inv(9)(p12q13), which is a common chromosome variant, and generally considered to be benign.

Table 1. Patient Demographics

Total N	113
Maternal age (y)	
Mean±SD	32.2±5.8
Median	32.9
Minimum–maximum	18–44
Race	
White	83
African American	11
Asian	10
Multiracial	9
Gestational age (wk)	
Mean±SD	13.2±2.0
Median	12.6
Minimum–maximum	10–21
Trimester	
1st	87
2nd	26
Karyotype source	
CVS	88
Amniocentesis	23
Product of conception	2

SD, standard deviation; CVS, chorionic villus sampling.
Data are n unless otherwise specified.

Figure 1 shows the massively parallel sequencing results according to normalized chromosome values after analyzing for aneuploidy of chromosomes 21, 18, 13, and X (shown for female fetuses only). The data were as follows: trisomy 21 sensitivity 100% (95% CI 88.4–100.0) with 29 of 30 samples in the "aneuploidy detected" zone and one of 30 in the "aneuploidy suspected (borderline value)" zone. Chromosome 21 specificity was 98.8% (95% CI 93.5–100.0) with one false-positive in the "aneuploidy suspected" zone. Ten of 10 fetuses with trisomy 18 were correctly detected for chromosome 18; sensitivity was 100% (95% CI 69.2–100.0) and trisomy 18 specificity was also 100% (95% CI 96.5–100.0). For trisomy 13, the sensitivity was 75% (95% CI 19.4–99.4) with two of four in the "aneuploidy detected" zone and one of four in the "aneuploidy

Table 2. Karyotypes of 113 Patients With Fetal Cystic Hygroma

Category	Karyotype
Monosomy X (n=21)	
	45,X (n=18)
	45X,inv(9)(p12q13) (n=1)
	45,X[9]/47,XXX[11] (n=1)
	45,X,inv(9)(p12q13)[3]/46,XX,inv(9)(p12q13)[7]/46,XX[30]*
Trisomy 21 (n=30)	
	47,XX,+21 (n=15)
	47,XY,+21 (n=14)
	47,XY, +21[21]/48,XY,+21+mar[4] (n=1)
Trisomy 18 (n=10)	
	47,XX,+18 (n=4)
	47,XY,+18 (n=5)
	47,XX,+18[42]/46,XX[8] (n=1)
Trisomy 13 (n=4)	
	47, XX+13 (n=1)
	47, XY+13 (n=1)
	46,XX,+13,der(13; 13)(q10; q10) (n=1)
	47,XY, inv (9) (p12q13)x2, +13 (n=1)
Other (n=4)	
	47,XY,+der(14 or 22)[10]/46,XY[10] (n=1)
	46,XX,add (X) (p22.1) (n=1)
	47,XY,+22 (n=1)
	47,XXY (n=1)
Normal (n=44)	
	46,XX (n=16)
	46,XY (n=26)
	46,XX,inv(9) (p12q13) (n=1)
	46,XY,inv(9) (p12q13) (n=1)

* From chorionic villus sampling at 12-3/7 weeks gestation in patient who subsequently showed normal karyotype (46,XX) by amniocentesis at 15-5/7 weeks.

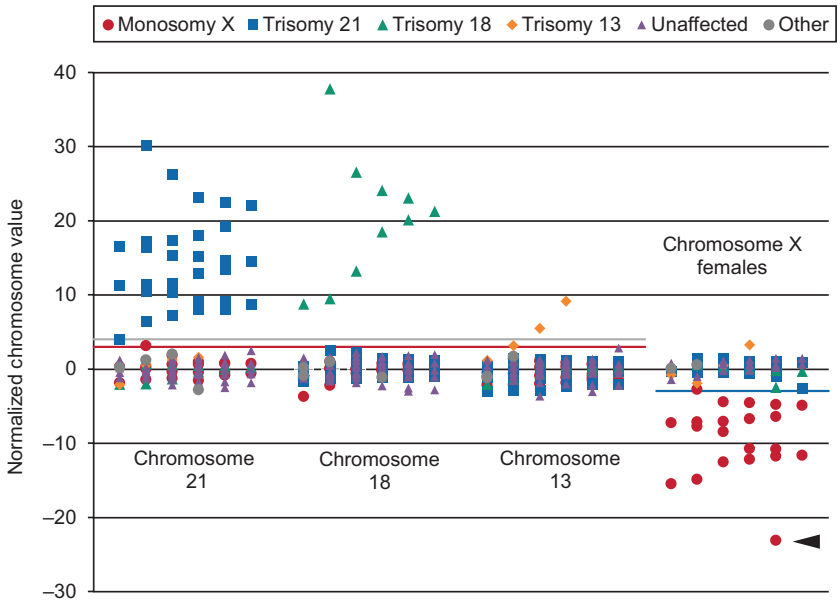

Fig. 1. Normalized chromosome values by massively parallel sequencing for the plasma DNA samples from women carrying fetuses with cystic hygroma. The normalized chromosome values for chromosomes 21, 18, and 13 are shown in clusters from left to right for all 113 samples. The gray line at normalized chromosome values=4 and the red line at normalized chromosome=3 demarcate the zones for aneuploidy classification as described in the "Methods." The normalized chromosome values for chromosome X are shown in the cluster at the far right for the 61 female samples with an normalized chromosome values Y less than 3. The blue line at normalized chromosome value=–3 demarcates the zone below which monosomy X is detected. The *arrowhead* points to the sample with the complex mosaic karyotype by chorionic villus sampling (CVS) (45,X,inv(9)(p12q13)[3]/46,XX,inv(9)(p12q13)[7]/46,XX[30]).

Bianchi. Maternal Plasma DNA Sequencing in Cystic Hygroma. Obstet Gynecol 2013.

suspected" zone. Chromosome 13 specificity was 100% (95% CI 96.7–100.0).

For monosomy X, 19 of the 21 total participants had a karyotype of 45,X and two had complex mosaicism. One of the two with complex mosaicism showed 45,X[9]/47,XXX[11], and the other showed the unusual mosaic karyotype described at CVS that was not seen at amniocentesis (Fig. 1, arrow). Both of these were detected. Overall, there were 20 of 21 samples in the "detected" range for monosomy X (sensitivity: 95.2% [95% CI 76.2–99.9]; specificity: 100% [95% CI 96.1–100.0]). The case that was not detected had 45,X.

None of the 44 patients with euploid fetal karyotypes were classified as aneuploid for chromosomes 13, 18, or 21 or detected for monosomy X. Overall, using massively parallel sequencing results of the four studied chromosomes, 107 of 113 (95%, 95% CI 88.8–98.0) cases were accurately called, including 63 of 65 (97%, 95% CI 89.3–99.6) cases of whole chromosome aneuploidy.

DISCUSSION

This study demonstrates the high sensitivity and specificity of massively parallel sequencing of maternal plasma DNA to detect fetal aneuploidy in a cohort of pregnant women with fetal cystic hygroma. Overall, the findings support a potential role for prenatal testing by maternal plasma DNA sequencing as a point-of-care option in the subsequent management of pregnant women carrying fetuses with cystic hygroma. The advantages of this approach are its immediate accessibility by peripheral blood draw (especially in patients who are risk-adverse and do not want any invasive testing, locations with limited access to CVS, or in cases posing challenges or contraindications to a successful procedure) and its ability to detect the most common aneuploidies associated with cystic hygroma (95% in this study). In addition, as a result of the high rate of in utero fetal demise with cystic hygroma, there may be an advantage to starting the diagnostic workup as soon as possible, particularly when fetal viability has been confirmed sonographically. Should there be a demise, there would be at least some genetic information available that may help with counseling regarding etiology and recurrence risk.

Many pregnant women whose fetuses have cystic hygroma will choose to have a CVS or amniocentesis if readily available. Massively parallel sequencing is, however, an additional option for cases in which pregnancy termination is not being considered by the patient and in patients who do not want an invasive prenatal test. In these patients, knowledge of the underlying fetal condition may improve expectant management, and in cases in which aneuploidy is not detected, they can then be followed for possible spontaneous resolution. A prior report comparing women whose fetuses were prenatally diagnosed with aneuploidy and chose to continue their pregnancies compared with women whose neonates were diagnosed at birth demonstrated that early knowledge of fetal

aneuploidy was beneficial.[6] Specific benefits included better perception of the physical experience of pregnancy and emotional experience of birth, the ability to prepare for a newborn with special needs by parental education and meeting with specialists, and planning for delivery at a tertiary medical center equipped to treat neonates with special needs, thus preventing separation of mother and newborn.

In this study, we demonstrated that a significant proportion (61%) of fetuses with cystic hygroma have abnormal fetal chromosomes. The majority of these were the result of trisomy 21 (43.5%) and monosomy X (30.4%) followed by trisomy 18 (14.5%), trisomy 13 (5.8%), and other karyotypes (5.8%) (Table 3). These percentages generally agree with earlier studies of sonographically detected cystic hygroma, including data from the First and Second Trimester Evaluation of Risk for aneuploidy (FaSTER) trial[1] and a recent 10-year retrospective outcome study.[2] The increased overall prevalence of chromosomal abnormalities in the current study, however, is most likely the result of a combination of the higher risk profile of patients enrolled in the MELISSA study and incomplete karyotype and obstetric outcome data in the other studies.

The connection between cystic hygroma and postnatal Turner syndrome (45,X) was first documented by Singh and Carr in 1966.[7] In the current study, like in prior studies, monosomy X was the second most prevalent fetal karyotype associated with cystic hygroma. Most of the cases were the result of full monosomy X with the exception of the two mosaic cases, both of which were classified as monosomy X by sample sequencing. In one case, the massively parallel sequencing results agreed with the fetal karyotype of 45,X[9]/47,XXX[11]. In the other case involving the three cell lines, massively parallel sequencing agreed with one of the cell lines detected at CVS, but not the amniocentesis or umbilical cord blood results. It is important to note that maternal plasma DNA sequencing is performed on total cell-free DNA; therefore, it may reflect aneuploidy present in the fetus, placenta, or the pregnant woman herself. The possibility of maternal aneuploidy or confined placental mosaicism can generate discordance between results from maternal plasma DNA sequencing and results from conventional cytogenetics (particularly amniocentesis). It is possible that the significantly low normalized chromosome value for chromosome X observed in this case (see Fig. 1, arrow) arose from either maternal mosaicism or confined placental mosaicism, but further follow-up to birth or additional maternal testing was not included in the MELISSA study protocol.

Advances in technology allow continued refinement of methods for massively parallel sequencing. All samples in this study were sequenced using the latest available chemistry from the manufacturer, which provided further improvement in counting statistics per sample and highly reproducible results from previous analyses. Based on the gain in precision, the boundary for the unclassifiable zone for autosomes was redefined between normalized chromosome value 3.0 and 4.0. This change yielded a reduction in the overall number of samples in the "aneuploidy suspected (borderline value)" zone and did not create any new false-negative results. Based on this framework, additional information is provided in that more false-positives may occur in the "aneuploidy suspected" zone than in the detected zone, but both zones are now considered "positive." Like with any cell-free DNA test for fetal aneuploidy, confirmation through an invasive procedure is recommended for such results.

Table 3. Prevalence of Chromosomal Abnormalities in Cystic Hygroma

	FaSTER—General Population	MELISSA—High-Risk Population
Enrolled	38,033	2,625*
Cystic hygroma	134 (1:285)	113 (1:25)
Normal chromosomes	65/132† (49.2)	44/113 (38.9)
Abnormal chromosomes	67/132† (50.8)	69/113 (61.1)
Trisomy 21	25/67 (37.3)	30/69 (43.5)
Monosomy X	19/67 (28.3)	21/69 (30.4)
Trisomy 18	13/67 (19.4)	10/69 (14.5)
Trisomy 13	6/67 (9)	4/69 (5.8)
Other	4/67 (6)	4/69 (5.8)

FaSTER, First and Second Trimester Evaluation of Risk for aneuploidy trial; MELISSA, MatErnal BLood IS Source to Accurately diagnose fetal aneuploidy study.
Data are n (ratio) or n/N (%).
* Singletons with karyotype and eligible samples.[4]
† Two of 134 cystic hygroma cases failed to follow-up.[1]

In summary, massively parallel sequencing provides an accurate way of detecting the most prevalent fetal aneuploidies associated with cystic hygroma. Massively parallel sequencing could advance prenatal care by providing immediate point-of-care noninvasive alternative testing for pregnant women who either decline or do not have access to an invasive procedure.

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