ORIGINAL ARTICLE

Clinical application of fetal sex determination using cell-free fetal DNA in pregnant carriers of X-linked genetic disorders

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As the first step in prenatal diagnosis of X-linked genetic disorders, chorionic villus sampling (CVS) for fetal sex determination is generally performed at 11–13 weeks of gestation. However, as the procedure-related miscarriage rate of CVS is 0.5–1.0%, non-invasive methods such as PCR of cell-free fetal DNA (cff-DNA) in maternal plasma are preferable. Here, we determined fetal sex at 9–12 weeks of gestation using PCR of cff-DNA in three pregnant carriers of Duchenne muscular dystrophy. The fetal sex was accurately determined in all three cases, as confirmed by ultrasound and amniocentesis at 16 weeks (for the two female fetuses) and CVS at 12 weeks (for the one male fetus). This procedure could avoid unnecessary CVS in female fetuses.

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INTRODUCTION

As a first step in the prenatal diagnosis of X-linked genetic disorders, chorionic villus sampling (CVS) for fetal sex determination is generally available to pregnant carriers of Duchenne muscular dystrophy (DMD) at 11–13 weeks of gestation. If the fetus is female, she must have a wild-type genotype or a heterozygous DMD mutation, the same as her mother. If the fetus is male, further genetic analysis is necessary, because a male fetus is either wild type or affected by DMD. However, the procedure-related miscarriage rate of CVS is 0.5–1.0%.¹ For CVS, the miscarriage rate was increased in departments performing <136 procedures per year compared with departments performing more than 136 procedures per year, while for amniocentesis the miscarriage rate was only increased in those departments performing <45 procedures per year.² In addition, to reach the plateau of learning curve, 400 procedures are needed for CVS, while 30 procedures are needed for amniocentesis.^{3,4} CVS is more difficult to master than amniocentesis and the sampling failure rate of CVS is at least three times that of amniocentesis.^{2–5} Therefore, to avoid the risks of CVS in pregnant female DMD carriers, a non-invasive procedure for fetal sex determination in early pregnancy is desirable.

In 1997, cell-free fetal DNA (cff-DNA) was found in the maternal plasma, and this discovery offered exciting new avenues for non-

invasive prenatal diagnosis.⁶ The cff-DNA in maternal plasma is detectable after 7 weeks of gestation and is undetectable 2 h after delivery.^{7,8} Fetal DNA in maternal plasma includes placental DNA, and comprises around 3–6% of the total cell-free DNA in maternal plasma during early and late pregnancy, respectively.^{8,9} PCR of Y chromosome-specific sequences on cff-DNA from maternal plasma is one such method for non-invasive fetal sex determination until 9 weeks of gestation.^{10,11} Compared with CVS, the advantages using cff-DNA in maternal plasma is that the sampling method is non-invasive and carries no risk for the mother or her baby, and that fetal sex can be diagnosed before 11–13 weeks of gestation, which is the best sampling period of CVS. Using this method as the first step, we have outlined a schema for prenatal diagnosis in carriers of DMD as follows (Figure 1): determine fetal sex between 9 and 12 weeks of gestation by nested PCR of cff-DNA in maternal plasma. If the fetus is male, follow-up with CVS at 12 weeks of gestation. If the fetus is female, CVS can be avoided; fetal sex is confirmed later in the pregnancy by ultrasound and amniocentesis at 16 weeks.

Here, we report our experiences regarding the clinical application of using cff-DNA from maternal plasma for the prenatal diagnosis of DMD.

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Figure 1 Outline of schema for prenatal diagnosis in pregnant carriers of Duchenne muscular dystrophy.

MATERIALS AND METHODS

All study protocols were approved by the Committee for Ethical Issues on Human Genome and Gene Analysis of Nagasaki University. All the pregnant women and their partners gave written informed consent and received genetic counseling.

Cases

Case 1, a 29-year-old woman, gravida 2, para 2, was referred to our clinic, because she planned to obtain a prenatal diagnosis of DMD at 9 weeks of gestation. Because her first son had DMD, she underwent a genetic test and was diagnosed as a DMD carrier (Figure 2a; subjects II-1 and I-1). During a previous pregnancy, CVS was performed for prenatal diagnosis of DMD at 12 weeks of gestation, and genetic analysis concluded that fetus was male and had no DMD mutation (Figure 2a; subject II-2).

Case 2, a 23-year-old woman, gravida 3, para 1, was referred to our clinic at 7 weeks of gestation. Because her first son had DMD, she underwent a genetic test and was diagnosed as a DMD carrier (Figure 2b; subjects II-1 and I-1).

Case 3, a 29-year-old woman, gravida 3, para 1, was referred to our clinic at 9 weeks of gestation. Because her younger brother had DMD, she underwent a genetic test and was diagnosed as a carrier of DMD, which was caused by deletion of exon 47 in the *DMD* gene (Figure 2c; subjects II-4 and II-2). Her first and third pregnancies were artificial terminations during the early first trimester (Figure 2c; subjects III-1 and III-3). Her first son was diagnosed with DMD, which was caused by the deletion of exon 47 (Figure 2c; subject III-2).

Sampling and extraction of cff-DNA in maternal plasma

Serial EDTA-blood samples (7 ml) were taken from 9 to 12 weeks of gestation. EDTA-blood samples were also taken from 50 women pregnant with a male fetus and 50 women pregnant with a female fetus. The blood samples were centrifuged twice at 3000g to obtain truly cell-free plasma. Cell-free DNA was extracted from 1.6 ml of maternal plasma using a QIAamp blood mini kit (Qiagen, Hilden, Germany) as described previously.⁹ DNA was eluted into 30 µl of sterile and DNase-free water.

Sex determination using nested PCR of cff-DNA

Sex determination was performed using a multiplex, nested PCR to amplify the X-specific *FMRI* gene and the Y-specific *SRY* gene. PCR primers and conditions have been described previously.¹² The *FMRI*-specific sequence and *SRY*-specific sequence were co-amplified first by two sets of PCR primer pairs, *FMRI*.1 (5'-CCCTGATGAAGAAGCTTGTATCTC-3'), *FMRI*.2 (5'-GAAATTA-CACACATAGGTGGCACT-3'), *SRY*.1 (5'-CTAGACCGCAGAGGCGCCCAT-3')

Figure 2 Pedigrees of pregnant carriers of Duchenne muscular dystrophy. (a) Case 1; (b) Case 2; (c) Case 3. ETC indicates ectopic pregnancy. Arrows indicate the cases. Filled squares indicate males affected with Duchenne muscular dystrophy; filled inner circles indicate female carriers. P indicates the current pregnancy.

and *SRY*.2 (5'-TAGTACCCACGCCTGCTCCGG-3'). PCR reaction was performed in a 20 µl mixture containing 5 µl cff-DNA, 10 µM of each primers, 250 µM dNTP, 0.5 U Ampli Taq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), and 2 µl of 10× PCR buffer (Applied Biosystems), using GeneAmp PCR System 9700 (Applied Biosystems). The nested PCR with primers *FMRI*.3 (5'-TCGCCTTTCTCAAATCCAAG-3'), *FMRI*.2, *SRY*.3 (5'-CATCCAGAGCGTCCCTGGCTT-3') and *SRY*.4 (5'-CTTTCCACAGCCAC ATTTGTC-3') was performed in a total volume of 20 µl using 1 µl of the first PCR product as a template. The first and the nested PCR were cycled 40 times at 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s. A volume of 10 µl of the nested PCR products were analyzed on 2.0% agarose gels. PCR products of cff-DNA had only the 261-bp fragment from X chromosome—the fetuses were female—while those had both a 261-bp from X chromosome and a 198-bp fragment from Y chromosome and the fetus was, therefore, male. The results of the PCR-based sex determination were confirmed after delivery.

RESULTS

Accuracy of fetal sex determination using cff-DNA in maternal plasma

Gradient dilution experiments (male DNA was gradually diluted in female DNA background) showed the minimum sensitivity of the nested PCR assay to detect male-specific allele in female DNA background was 1:100 ~ 1:1000 male:female DNA pool (Figure 3). Moreover, fetal gender discrimination was accurate at 9–12 weeks of gestation in 100% of cases. Both X- and Y-specific PCR products were detected in all 50 plasma samples from women pregnant with a male fetus, while only X-specific PCR products were detected in all 50 plasma samples from women pregnant with a female fetus.

Figure 3 Gradient dilution experiments of the nested PCR assay. Male DNA was gradually diluted in female DNA background. Lanes labeled MF8 and MF7 indicate cff-DNA from a woman pregnant with a male fetus at 8 weeks of gestation and 7 weeks of gestation, respectively; D1, D2, D3 and D4 indicate 1:1, 1:10, 1:100 and 1:1000 male:female DNA pool, respectively; F and M indicate female and male DNA, respectively; W, distilled water; S, size marker (pIBI31/*Msp* I). * and ** show a 261 bp PCR product from X chromosome and a 198 bp PCR product from Y chromosome, respectively.

Figure 4 Fetal sex determination using PCR of cell-free fetal DNA (cff-DNA) in maternal plasma. (a) Case 1; (b) Case 2; (c) Case 3. Lanes labeled 9, 10, 11 and 12 indicate cff-DNA at the respective week of gestation; FF and MF indicate cff-DNA from a woman pregnant with a female or male fetus, respectively; F and M indicate female and male DNA, respectively; W, distilled water; S, size marker (pIBI31/*Msp* I). * and ** show a 261 bp PCR product from X chromosome and a 198 bp PCR product from Y chromosome, respectively.

Prenatal fetal sex determination using maternal plasma samples

For sex determination of the fetuses of the above three Cases, nested PCR of cff-DNA in maternal plasma was performed at 9–12 weeks of gestation. PCR products from female DNA showed only a 261-bp fragment, while those from male DNA had both a 261-bp and a 198-bp fragment (Figures 4a–c). PCR products of cff-DNA from Cases 1

and 2 had only the 261-bp fragment—the fetuses were female (Figures 4a and b)—while those from Case 3 had both fragments (Figure 4c) and the fetus was, therefore, male. According to our protocol, ultrasound examination and amniocentesis were provided to Cases 1 and 2 at 16 weeks of gestation: both fetuses showed normal female genitalia and a normal female karyotype (46, XX). In Case 3, CVS was performed at 12 weeks of gestation and the fetal sex was confirmed as male. Exon 47 of *DMD* was detected in DNA from a cultured villus sample and from his parents, but not in DNA from his affected elder brother (data not shown).

DISCUSSION

Compared with the conventional techniques of prenatal diagnosis, including CVS and amniocentesis, the main advantage using cff-DNA in maternal plasma is that the sampling method is non-invasive and carries no risk for the mother or her baby. Another advantage is its reliability in determining fetal sex very early, from 7 weeks of gestation.^{10,11} Amniocentesis is only available after 15 weeks of gestation, because before then it carries a risk of talipes equinovarus. Similarly, CVS is only performed between 11 and 13 weeks of gestation, because before 10 weeks it is possibly related to an increased risk of limb reduction defects.^{13,14} Early prenatal diagnosis is desirable because, in the case of a positive diagnosis, termination of pregnancy is more stressful during the second trimester than during the first. Although CVS is a first trimester option for the prenatal diagnosis of X-linked genetic disorders,¹ early non-invasive fetal sex determination could avoid unnecessary CVS of female fetuses, and also give more time for parents to consider the invasive procedure in the case of male fetuses. This technique could be applied to the detection or exclusion of genetic sequences that are not present in mother, that is, fetal rhesus D status in D-negative mothers, or genetic conditions inherited from the father or arising *de novo*.^{15,16}

False positive and false negative results in sex determination using cff-DNA are still a concern. False positives can result from technical issues such as contamination, or clinical abnormalities such as the presence of a non-identical vanishing twin or a confined placental mosaicism/chimerism.^{9,16} False negative results due to failure to detect the Y-chromosome sequence may still be problematic. To reduce these problems, maternal plasma analysis can be paired with ultrasound examination and amniocentesis; ultrasonic identification of fetal sex is possible within the first 12 weeks of gestation.¹⁷ Even if ultrasonography reveals a misdiagnosis, prenatal diagnosis is still feasible through amniocentesis. Sample processing protocols clearly affect the quality of cell-free DNA in the maternal plasma. When only a single centrifugation step is performed, cff-DNA is detected in non-pregnant women, reflecting contamination by residual circulating cells from previous pregnancies.^{18,19} A second microcentrifugation step is essential to obtain truly cell-free plasma.²⁰ To find the best procedure for non-invasive sex determination using cff-DNA, the use of multicopy sequence as target sequence on Y chromosome is one of the options, because the assay using multicopy *DYS 14* sequence demonstrated a 10-fold better detection rate and quantification limits for fetal DNA compared with the assay using single copy *SRY* gene.²¹

In conclusion, fetal sex determination using cff-DNA in maternal plasma could avoid unnecessary CVS of female fetuses, decreasing parental anxiety, the cost and the attached risk. As such, genetic counselors and obstetricians should be aware of the potential for earlier and safer prenatal diagnosis, although it should be stressed that the technique is still in development and has potential to be used more widely for the purposes of family balancing or for preference of a particular sex.^{2,23} Because of the ethical, social and legal issues

surrounding fetal sex determination,^{22,23} we recommend that the clinical use of this procedure should be reserved for those fetuses at risk of a gender-specific inherited disorder.

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