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SHORT COMMUNICATION

Replicate real-time PCR testing of DNA in maternal plasma increases the sensitivity of non-invasive fetal sex determination

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Background We determined fetal sex in pregnancies referred for invasive prenatal diagnosis procedures by analysis of DNA in maternal plasma.

Methods Twelve pregnancies at risk of X-linked haemophilia and 32 pregnancies at risk of chromosomal aneuploidies at a gestational age ranging from 10 to 18 weeks recruited before chorionic villus sampling or amniocentesis were involved in the study. Male fetal DNA in maternal plasma was detected by using real-time polymerase chain reaction with the *SRY* gene as a marker.

Results The specificity of the system reached 100% (no Y signal was detected in 17 women pregnant with a female fetus) and the sensitivity reached 100% (*SRY* amplification in 27 examined samples).

Conclusions Amplification of free fetal DNA in maternal plasma is a valid and rapid technique for predicting fetal sex in first- and second-trimester pregnancies and could allow the restriction of invasive sampling procedures to male fetuses at risk of X-linked disorders. Copyright © 2003 John Wiley & Sons, Ltd.

KEY WORDS: fetal DNA; fetal gender; maternal plasma; real-time PCR; *SRY* gene

BACKGROUND

Current experimental non-invasive methods for the prenatal diagnosis of fetal gender use free extracellular fetal DNA (Lo *et al*., 1997; Poon and Lo, 2001) and fetal cells (Bianchi, 1998; Hahn *et al*., 1998; Steele *et al*., 1996) isolated from maternal peripheral blood. However, the rarity of circulating fetal cells in maternal blood and technical intensiveness during fetal cell–maternal blood enrichment have limited the use of this approach in clinical routine. On the other hand, free extracellular fetal DNA in the serum or plasma of pregnant women seems to be a promising non-invasive alternative for at least fetal gender and RhD status determination (Faas *et al*., 1998; Lo *et al*., 1998a; Hahn *et al*., 2000; Zhong *et al*., 2000).

A novel approach uses the real-time quantitative PCR (RQ-PCR) to detect and quantify male fetal and total DNA levels in maternal peripheral blood (Lo *et al*., 1998b; Zhong *et al*., 2001).

In this prospective study, we determined fetal gender in first- and second-trimester pregnancies at risk of X-linked haemophilia and chromosomal aneuploidies by the analysis of DNA in maternal plasma using real-time PCR and the *SRY* gene as a marker for the detection of the fetal Y chromosome.

METHODS

Patients

Twelve consecutive pregnant women (seven of them first-time pregnant) at risk of X-linked haemophilia in the fetus (11 haemophilia A and 1 haemophilia B) at a gestational age ranging from 10 to 14 (Φ 12,1) weeks were recruited for the study. To verify the specificity and sensitivity of the assay on a larger cohort, another 32 pregnant women between 10 and 18 (Φ 16) weeks of gestation referred for chorionic villus sampling or amniocentesis because of advanced maternal age, altered serum screening or fetal nuchal translucency were included into the study. In all cases, maternal blood was obtained before the invasive prenatal diagnostic procedure. The Local Ethics Committee approval and informed consent was obtained for all patients in the study.

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DNA extraction from plasma samples

Five millilitres of maternal peripheral blood from pregnant women was collected into EDTA-containing tubes and processed within a few hours (maximally 24 h). In detail, blood samples were centrifuged firstly at 1200 g for 10 min., then plasma samples were recentrifuged and the supernatants were collected and stored at −80 °C until futher processing. DNA was extracted from 400 μL of plasma using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To minimise the risk of contamination, DNA was isolated in a laminar air flow using aerosol-resistant tips. DNA was eluted in 50 μL of Buffer AE and 5.0 μL of DNA was used as a template for the PCR reaction.

Real-time PCR analysis

Real-time PCR analysis was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystem, Branchburg, New Jersey, USA). The SRY TaqMan system consisted of an SRY-109 (forward) primer, 5′-TGG CGA TTA AGT CAA ATT CGC-3′; an SRY-245 (reverse) primer, 5′-CCC CCT AGT ACC CTG ACA ATG TAT T-3′ and a probe SRY-142T, 5′(FAM) AGC AGT AGA GCA GTC AGG GAG GCA GA (TAMRA)-3′ (Lo *et al*., 1998b). Sequence data were obtained from the GeneBank sequence database L08063 (*SRY* gene) as previously described by Lo *et al*. (1998b).

TaqMan amplification reactions were set up in a reaction volume of 25 μL using the TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, New Jersey, USA). DNA amplifications were carried out in 8-well reaction optical tubes/stripes (Applied Biosystem, Branchburg, New Jersey, USA). The TaqMan PCR conditions were followed as described in TaqMan guidelines: 50 cycles at 95 °C for 15 s and at 60 °C for 1 min with 2-min preincubation at 50 °C required for optimal AmpErase uracil-N-glycosylase (UNG) activity and 10-min preincubation at 95 °C required for the activation of AmpliTaq Gold DNA polymerase. Each sample was analysed twice in six replicates. A patient's specimen was considered positive if one or more of the six individual replicates were positive (threshold cycle <40).

The β-globin TaqMan system consisting of two primers β-globin-354 (forward), 5′-GTG CAC CTG ACT CCT GAG GAG A-3′; β-globin-455 (reverse), 5′-CCT TGA TAC CAA CCT GCC CAG- 3′ and a dual-labelled fluorescent TaqMan probe β-globin-402T, 5′(FAM) AAG GTG AAC GTG GAT GAA GTT GGT GG (TAMRA)- 3′ served as a control and differentiated between a true-negative and a false-negative result coming from a deficient DNA extraction process or PCR inhibitors in the eluted DNA (Lo *et al*., 1998b). Amplicons for the β-globin control gene were detected in all analysed samples.

RESULTS

SRY real-time PCR analysis of maternal plasma was in complete concordance with fetal sex in all 12 women at risk of haemophilia (7 males and 5 females) (Table 1) and all 32 pregnant women at risk of fetal aneuploidy (20 males and 12 females) (Table 2).

Overall, in our setting, the sensitivity of the real-time PCR system was 100% as SRY-specific PCR amplicons were detected in 27 out of 27 of the studied plasma samples from women bearing a male fetus. The specificity approached 100% since no Y-chromosome-positive signals were detected in 17 women currently pregnant with a female fetus. The sensitivity of the system was considerably increased by performing 6 to 10 replicates of each sample in duplicate real-time PCR analyses using DNA samples isolated from maternal plasma. PCR tests done without a sufficient number of replication may miss a proportion of positive specimens, particularly those with small amounts of target SRY DNA present in maternal plasma at early pregnancies. In a three-replicate single assay, the specificity of the system remained 100%, however, the sensitivity reached

Table 1—Fetal gender determination in pregnancies at risk of X-linked haemophilia

No.	UPN	Week of gestation	Karyotype	*SRY* amplification	Risk
1	316	12	46, XX	−	Hemophilia A
2	336	11	46, XX	−	Hemophilia A
3	348	12	46, XX	−	Hemophilia A
4	358	12	46, XX	−	Hemophilia A
5	370	12	46, XY	+	Hemophilia A
6	404	13	46, XY	+	Hemophilia B
7	419	13	46, XX	−	Hemophilia A
8	454	10	46, XY	+	Hemophilia A
9	455	10	46, XY	+	Hemophilia A
10	459	13	46, XY	+	Hemophilia A
11	478	13	46, XY	+	Hemophilia A
12	483	14	46, XY	+	Hemophilia A
	Range	10–14			
	Mean	12,1			

UPN; unique patient's number.

Table 2—Fetal gender determination in pregnancies at risk of chromosomal aneuploidies

No.	UPN	Week of gestation	Karyotype	*SRY* amplification	Risk
1	274	17	46, XX	−	Maternal age
2	284	18	46, XY	+	Screening
3	295	17	46, XY	+	Screening
4	303	17	46, XY	+	Screening
5	309	18	46, XY	+	Maternal age
6	318	16	46, XY	+	Screening
7	319	17	46, XY	+	Maternal age
8	320	17	46, XY	+	Maternal age
9	321	18	46, XX	−	Screening
10	322	17	46, XY	+	Maternal age
11	324	15	46, XX	−	Maternal age
12	325	12	46, XX	−	Maternal age
13	326	12	46, XY	+	Maternal age
14	328	17	46, XX	−	Screening
15	332	13	46, XX	−	Maternal age
16	333	17	46, XY	+	Maternal age
17	338	17	46, XX	−	Screening
18	340	15	46, XY	+	Maternal age
19	342	19	46, XY	+	Maternal age
20	346	16	46, XX	−	Maternal age
21	347	17	46, XX	−	Maternal age
22	350	18	46, XX	−	Maternal age
23	352	16	46, XY	+	Maternal age
24	354	18	46, XY	+	Screening
25	360	17	46, XX	−	Maternal age
26	365	17	46, XY	+	Maternal age
27	369	16	46, XY	+	Maternal age
28	376	18	46, XY	+	Screening
29	385	17	46, XY	+	Maternal age
30	417	11	46, XY	+	NT
31	467	10	46, XY	+	Frequent abortions
32	485	13	46, XX	−	NT
	Range	10–18			
	Mean	16			

92.6% as we would be able to detect *SRY*-gene positivity only in 25 out of 27 male fetuses. These two false-negative results were found in pregnant women at the 10th week of gestation. A follow-up six-replicate reanalysis revealed *SRY*-gene positivity in male fetuses, which was missed in the three-replicate setting. In other cases, the reanalysis confirmed previous positive or negative *SRY* amplification results.

The quantitative analysis done in a three-replicate single setting showed that the median fetal DNA level in maternal plasma obtained from women carrying a singleton fetus with a normal male karyotype was 24.5 genome-equivalents/mL (range 0–47.5).

DISCUSSION

Nucleic acid amplification tests such as PCR enable the detection of low concentrations of organism or rare DNA in clinical specimens. Previous studies of Zhong *et al*. (2001), Sekizawa *et al*. (2001) and Rijnders *et al*. (2001) reported the diagnostic accuracy of fetal gender determination from maternal plasma samples obtained at early pregnancy. Sekizawa *et al*. (2001) demonstrated a sensitivity of 97.2% and a specificity of 100% in a cohort of 302 pregnant women using a LightCycler for RQ-PCR and DYS-14 as a marker for the detection of the fetal Y chromosome. Rijnders *et al*. (2001) correctly predicted fetal sex in 97.8% of the cases that included 45 patients by using an ABI PRISM 7700 Sequence Detector, the *SRY* gene as a marker and a 3-replicate setting. Honda *et al*. (2002) determined fetal gender, in 81 pregnant women during the 5th to 10th week of gestation, with 100% sensitivity using RQ-PCR.

The results obtained in our study on a comparable number of patients support the results of other studies (Lo *et al*., 1998b; Zhong *et al*., 2001; Sekizawa *et al*., 2001; Rijnders *et al*., 2001; Honda *et al*., 2002; Costa *et al*., 2001). We therefore conclude that amplification of free fetal DNA in maternal plasma is a promising approach for valid and rapid fetal sex determination in first- and second-trimester pregnancies. We suggest that performing duplicate RQ-PCR analysis of 6 to 10 replicates of each DNA sample isolated from maternal plasma may considerably increase the sensitivity of *SRY*-gene real-time PCR. Prenatal diagnosis in pregnant women who are carriers of an X-linked genetic disorder might thus allow the restriction of invasive diagnostic procedures to male fetuses only. However, further

studies on a large number of maternal plasma samples are required before including the technique into clinical routine.

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REFERENCES

Bianchi DW. 1998. Current knowledge about fetal blood cells in the maternal circulation. *J Perinat Med* **26**: 175–185.

Costa JM, Benachi A, Gautier E, Jouannic JM, Ernault P, Dumez Y. 2001. First-trimester fetal sex determination in maternal serum using real-time PCR. *Prenat Diagn* **21**: 11 070–11 074.

Faas BH, Beuling EA, Christiaens GC, von dem Borne AE, van der Achoot CE. 1998. Detection of fetal RhD—specific sequences in maternal plasma. *Lancet* **352**: 1196.

Hahn S, Sant R, Holzgreve W. 1998. Fetal cells in maternal blood: current and future perspectives. *Mol Hum Reprod* **4**: 515–521.

Hahn S, Zhong XY, Burk MR, Troeger C, Holzgreve W. 2000. Multiplex and realtime quantitative PCR on fetal DNA in maternal plasma: a comparison with fetal cells isolated from maternal blood: in circulating DNA in plasma. In Stroun M, Anker P (eds). *Ann N Y Acad Sci* **906**: 148–152.

Honda H, Miharu N, Ohashi Y, *et al*. 2002. Fetal gender determination in early pregnancy through qualitative and quantitative analysis of fetal DNA in maternal serum. *Hum Genet* **110**: 75–79.

Lo YMD, Corbetta N, Chamberlain PF, Sargent JL. 1997. Presence of fetal DNA in maternal plasma and serum. *Lancet* **350**: 485–487.

Lo YMD, Hjelm NM, Fidler C, *et al*. 1998a. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* **339**: 1734–1738.

Lo YMD, Tein MSC, Lau TK, *et al*. 1998b. Quantitative analysis of fetal DNA in maternal plasma and serum. Implications for noninvasive prenatal diagnosis. *Am J Hum Genet* **62**: 768–775.

Poon LL, Lo YM. 2001. Circulating fetal DNA in maternal plasma. *Clin Chim Acta* **313**: 151–155.

Rijnders RJ, van der Schoot CE, Bossers B, de Vroede MA, Christiaens GC. 2001. Fetal sex determination from maternal plasma in pregnancies at risk for congenital adrenal hyperplasia. *Obstet Gynecol* **98**: 374–378.

Sekizawa A, Kondo T, Iwasaki M, *et al*. 2001. Accuracy of fetal gender determination by analysis of DNA in maternal plasma. *Clin Chem* **47**: 1856–1858.

Steele CD, Wapner RJ, Smith JB, Haynes MK, Jackson LG. 1996. Prenatal diagnosis using fetal cells isolated from maternal peripheral blood: a review. *Clin Obstet Gynecol* **39**: 801–813.

Zhong XY, Holzgreve W, Hahn S. 2000. Detection of fetal rhesus D and sex using fetal DNA from maternal plasma by multiplex PCR. *Br J Obstet Gynaecol* **107**: 766–769.

Zhong XY, Hahn S, Holzgreve W. 2001. Prenatal identification of fetal genetic traits. *Lancet* **357**: 310, 311.