Correlation of Fetal DNA and Human Chorionic Gonadotropin Concentrations in Second-Trimester Maternal Serum, Yoko Ohashi, Norio Miharu, Hiroshi Honda, Osamu Samura, and Koso Ohama (Hiroshima University School of Medicine, Department of Obstetrics and Gynecology, 1-2-3 Kasumi Minami-ku, Hiroshima 734-8551, Japan; * author for correspondence: fax 81-82-257-5264, e-mail nmiharu@hiroshima-u.ac.jp)

The discovery of cell-free fetal DNA in maternal serum and plasma has opened a new avenue for noninvasive prenatal diagnosis and provided a useful marker of complicated pregnancy (1–7). In recent years, the analysis of fetal DNA in maternal serum or plasma has afforded successful prenatal diagnosis of fetal rhesus D status (2) and single-gene disorders (3), as well as fetal gender (4). In addition, an increased fetal DNA concentration in maternal serum or plasma has been demonstrated as a useful marker in preeclampsia (5,6) and preterm labor (7). However, the origin of the fetal DNA and the biologic significance of its increase in complicated pregnancies remain unclear. Speculation as to the possible origin of fetal DNA has included the leakage of nucleated fetal cells into maternal circulation across the placenta or the direct release of trophoblastic DNA into maternal circulation. Human chorionic gonadotropin (hCG), which is produced by trophoblasts and excreted directly into maternal circulation, is also reportedly increased in preeclampsia and preterm labor (8–10). The present study examined the association between fetal DNA and hCG concentrations in maternal serum by simultaneously measuring fetal DNA and hCG concentrations in healthy pregnant women.

Sixty-three healthy pregnant women (gestational age, 15–17 weeks) attending the Department of Obstetrics and Gynecology at Hiroshima University Hospital for amniocentesis were recruited. By the time samples were obtained, none of the women had manifested such pregnancy-related complications as maternal hypertension or threatened abortion. After informed consent was obtained under a protocol approved by the Research Ethics Committee of Hiroshima University, 12 mL of antecubital venous blood was drawn for serum separation just before amniocentesis. A 6-mL aliquot of each blood sample was immediately centrifuged at 800g for 10 min, and the serum was removed. Intact hCG concentrations in 0.5 mL of the serum were measured by time-resolved fluoroimmunoassay. After the remaining 6 mL of each blood sample was centrifuged at 3000g for 10 min, the serum was carefully removed and transferred into plain polypropylene tubes. The serum samples were recently centrifuged at 3000g for 10 min, and the supernatants were collected into fresh polypropylene tubes. The samples were stored at −20°C until used for DNA extraction and PCR assay.

After the karyotype of each fetus was ascertained from the cytogenetic results of amniocentesis, we selected 31 women who were confirmed to be carrying a single male fetus. The fetal karyotypes were as follows: 28 fetuses with 46,XY karyotype, 1 fetus with 47,XY,+21 karyotype, 1 fetus with 46,XY,dup(1), and 1 fetus with 46,XY,dup(4). The fetus with 46,XY,dup(1) showed cystic hygroma on ultrasonography.

DNA was extracted from 1.2 mL of each stored serum sample by a QIAamp DNA Blood Mini Kit (Qiagen) according to the “blood and body fluid spin protocol” with minor modifications. The extracted DNA was concentrated in 70 μL of water, of which 15 μL was used as a template for each reaction.

We performed real-time quantitative PCR assays on an ABI PRISM 7700 Sequence Detector (Applied Biosystems) according to the method described by Lo et al. (11) with minor modifications. The SRY gene in male fetuses was used as a molecular marker for fetal DNA in maternal serum. We used amplification primers SRY-109F (5′-TGG CGA TTA AGT CAA ATT CGC-3′) and SRY-245R (5′-CCC CCT AGT ACC CTG ACA ATG TAT T-3′) and a dual-labeled fluorescent TaqMan probe [SRY-142T; 5′-(6-carboxytetramethylrhodamine)-3′-AGT AGA GCA GTC AGG GAG GCA GA (6-carboxytetramethylrhodamine)-3′] to detect the SRY sequence.

Of the eluate, 15 μL was used as a template for each reaction. The reactions were carried out in a total volume of 50 μL containing 15 μL of extracted DNA solution, 500 nmol/L each amplification primer, 200 nmol/L the corresponding TaqMan probe, and 25 μL of Universal MasterMix (Applied Biosystems). The MasterMix contains 200 μmol/L each of dATP, dCTP, and dGTP, 400 μmol/L dUTP, 5.0 mmol/L MgCl₂, 80 mL/L glycerol, and 1× TaqMan Buffer A. The thermal cycling was initiated with a 2-min incubation at 50°C, followed by a first denaturation step of 10 min at 95°C and then 50 cycles of 95°C for 30 s and 57°C for 1 min.

To create a calibration curve for quantitative PCR, we extracted DNA from whole blood of a healthy male, measured the DNA concentration, and serially diluted it from 1:10 to 1:100 000. A calibration curve was constructed for each reaction based on these diluted male genomic DNA solutions, which were analyzed in duplicate simultaneously with DNA samples extracted from maternal serum.

Amplification data collected by the 7700 Sequence Detector were analyzed using the Sequence Detection System software developed by Perkin-Elmer Applied Biosystems. The conversion factor of 6.6 pg of DNA per cell (12) was used to express the results as copy numbers. We analyzed all samples in duplicate and determined the concentration of the samples by their means.

Great care was taken to prevent PCR contamination. Aerosol-resistant pipette tips (ART; Molecular Bio-Products) were used for handling all liquids. The extraction of DNA, preparation of amplification reactions, carrying out of amplification reactions, and detection of the PCR products were performed in separate areas. All manipulations except the detection were carried out in a laminar flow hood. Multiple negative water blanks were included in each real-time quantitative PCR analysis. All procedures were carried out by a female staff member.
Amplification signals of the SRY gene were detectable in all serum samples. The mean concentration of the SRY gene was 37.3 copies/mL (range, 2.1–124.7 copies/mL) in 31 cases. The mean hCG concentration was 29.0 IU/mL (range, 8.8–70.0 IU/mL). There was a strong and significant correlation between fetal DNA and hCG concentrations in maternal serum (Pearson correlation coefficient, 0.73; P < 0.0001), as shown in Fig. 1.

In 28 women pregnant with a 46,XY fetus, the mean concentrations of the SRY gene and hCG were 35.1 copies/mL and 27.8 IU/mL, respectively. In the woman pregnant with a 47,XY,+21 fetus, the SRY and hCG concentrations were 47.8 copies/mL and 40.0 IU/mL, respectively. In the woman pregnant with a 46,XY,dup(1) fetus, which showed cystic hygroma, the SRY and hCG concentrations were 38.4 copies/mL and 21.0 IU/mL, respectively. In the woman pregnant with a 46,XY,dup(4) fetus, the SRY and hCG concentrations were 88.3 copies/mL and 58.0 IU/mL, respectively.

The results of the present study reveal a strong correlation between fetal DNA and hCG in second-trimester maternal serum, a finding that is in agreement with those of previous reports. Lo et al. (5) found a fivefold increase in fetal DNA concentration in serum obtained from women affected by preeclampsia compared with those with uncomplicated pregnancies, and Leung et al. (7) reported high concentrations of fetal DNA in maternal serum before preterm labor. On the other hand, several studies have reported that increased hCG in the second trimester is a predictor of adverse obstetric outcome, including preeclampsia and preterm delivery (8–10). Because hCG is secreted from placental trophoblasts and excreted directly into maternal circulation, our finding indicates that there is a strong association between fetal DNA in maternal circulation and the pathologic status of placental trophoblasts.

As for the origin of fetal DNA, two possibilities have been proposed: (a) continuous leakage across the placenta of fetal cells, which are rapidly destroyed by the maternal immune system; or (b) active remodeling of the placenta at the fetomaternal interface with continuous cell lysis and direct release of DNA into the maternal circulation (13). Because we demonstrated a strong correlation between fetal DNA concentrations in maternal serum and trophoblast viability, the origin of fetal DNA in maternal serum is more likely to be the result of direct release from placental trophoblasts. Additionally, we detected a mean fetal DNA concentration of 37.3 copies/mL in maternal serum in pregnancies with a single fetus, whereas Bianchi et al. (14) reported a mean of ~1.2 cells/mL in maternal whole blood during the second trimester. Although varying numbers of fetal cells are considered to be present in maternal blood (15–17), our results show that the amount of cell-free fetal DNA in maternal serum is considerably higher than that of fetal cell DNA-equivalents in a similar volume of maternal blood. Lo et al. (11) also reported that the concentration of cell-free fetal DNA was 24-fold higher in maternal serum than in the cellular fraction of maternal blood. These findings further support the notion that direct release from placental trophoblasts is the origin of fetal DNA in maternal serum.

Recently, increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21 was reported (18, 19). These studies showed the possibility of using fetal DNA in maternal plasma for the screening of fetal chromosomal aneuploidies. hCG is one of three established serum biochemical markers, together with α-fetoprotein and unconjugated estriol, for the screening of trisomy 21. High hCG concentrations are predictive of a trisomy 21 fetus. There was one case of pregnancy with 47,XY,+21 in the present study. The SRY and hCG concentrations were 47.8 copies/mL and 40.0 IU/mL, respectively, which represent values slightly higher than the mean fetal DNA and hCG concentrations in pregnancies with 46,XY fetus. These findings suggest that the fetal DNA concentration in maternal serum might be a useful marker for an aneuploid fetus. However, because the concentrations in women pregnant with fetuses with normal karyotypes are widely distributed (20), the association between an increased fetal DNA concentration and pregnancy with a trisomy 21 fetus should be viewed with caution until it can be confirmed by larger case studies. In addition, because the system using the SRY sequence is applicable only to pregnancies with male fetuses, another system that enables the measurement of fetal DNA in female fetuses is required.

In conclusion, this is the first report demonstrating a strong correlation between fetal DNA and hCG concent-
Limitations in second-trimester maternal serum. This finding suggests that the fetal DNA in maternal serum is strongly influenced by the pathologic and biologic status of placent al trophoblasts. Although the origin of fetal DNA in maternal serum has yet to be elucidated, our findings suggest that it is from placent al trophoblasts rather than from nucleated fetal cells circulating in maternal blood. Furthermore, increased fetal DNA and hCG concentrations in maternal serum might be associated with certain pregnancy-related complications.

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References

Indirect Measurement of Bioavailable Testosterone with the Bayer Immuno 1 System, Rachael Davies, 1 Christine Collier, 2 Michael Raymond, 2 Jeremy Heaton, 3 and Albert Clark 1,2 (Departments of 1 Biochemistry, 2 Pathology, and 3 Urology, Queen’s University and Kingston General Hospital, Kingston, Ontario, K7L 3N6 Canada; * address correspondence to this author at: Department of Pathology, Richardson Laboratories, Queen’s University, Kingston, Ontario, K7L 3N6 Canada; fax 613-533-2907, e-mail collier@cliff.path.queensu.ca)

Testosterone is the principal circulating androgen in men. A major fraction of testosterone is specifically bound with high affinity and low capacity to sex-hormone-binding globulin (SHBG), whereas most of the remaining testosterone is bound with low affinity to albumin (ALB), leaving only 1–2% to circulate as “free” testosterone (FT) not bound to protein in serum (1). Because the SHBG-testosterone complex dissociates very slowly in vitro (dissociation half-time, 20 s), SHBG-bound testosterone (SHBG-T) is not considered to be available for biologic action in tissues. According to the original “free hormone hypothesis”, only FT is able to enter target cells. Tait and Burstein (2), however, suggested that because testosterone is only loosely bound to ALB, this fraction is also available for tissue uptake (dissociation half-time, 1 s). The concept of bioavailable testosterone (BAT), also known as “non-SHBG-bound testosterone” (or both FT and ALB-bound testosterone (ALB-T)), was supported by in vitro kinetic calculations (3), but others (4, 5) have challenged the assumptions used for these calculations. Indeed, if SHBG facilitates entry of testosterone into tissues, as reported by Pardridge (6), then SHBG-T would also be “available” for tissue uptake and action. Controversy remains about the best biochemical measurement for testosterone function in humans.

The initial clinical diagnostic evaluation for men with symptoms of hypogonadism involves measurement of the serum total testosterone. In situations such as hyper- and hypothyroidism, obesity, and the use of antiepileptic drugs, the correlation between clinical symptoms and the serum total testosterone concentration has been reported to be poor. This may be attributable in part to the inactivity of the SHBG-T fraction and to variation in the plasma concentration of SHBG in these situations. The fact that plasma SHBG changes during life and in several pathologic situations is well established (7, 8). It is currently common practice to also measure FT or BAT during these investigations (9, 10).

BAT assays are based on the selective precipitation of SHBG-T by ammonium sulfate (AS), leaving ALB-T and FT (i.e., BAT) in the supernatant. The original assays