Relationship between Severity of Hyperemesis Gravidarum and Fetal DNA Concentration in Maternal Plasma, Yumi Sugito,1 Akihiko Sekizawa,1* Antonio Farina,1,2 Yasuo Yukimoto,3 Hiroshi Saito,1 Mariko Iwasaki,3 Nicola Rizzo,2 and Takashi Okai1 (1 Department of Obstetrics and Gynecology, Showa University School of Medicine, 1-5-8, Hatanodai, Shinagawa-ku, Tokyo 142-8666, Japan, 2 Institute of Embryology, Obstetrics and Gynecology, University of Bologna, Bologna 40138, Italy; * author for correspondence: fax 81-3-3782-2984, e-mail sekitazawa@med.showa-u.ac.jp)

Cell-free fetal DNA is present in maternal plasma (1), where its concentration increases during some abnormal conditions that can occur during pregnancy, including preterm delivery (2), preeclampsia (3–5), invasive placentia (6), and fetal trisomy 21 (7, 8). The origin of fetal DNA is not clear, but a body of evidence in the literature suggests that it comes mainly from the destruction of villous trophoblasts that border the intervillous space filled with maternal blood (5, 9). Although the pathogenesis of hyperemesis gravidarum (HG) is obscure, activation of natural killer and cytotoxic T cells is higher in the blood and uterine decidua of women with HG with respect to healthy pregnant women (10). Thus, if HG and DNA concentrations were correlated, one possible common pathway that could explain these conditions might be an overactivated maternal immune system that destroys trophoblasts, causing both HG and higher concentrations of cell-free fetal DNA. The aim of this study was to investigate a possible correlation between HG and fetal DNA concentrations. We also evaluated the relationship between the cell-free fetal DNA concentration and the severity of HG.

A total of 202 pregnant women (gestational age, 6–16 weeks) bearing a single male fetus, who presented at Showa University Hospital between July 2000 and July 2002 were enrolled in this study. Forty-five consecutive pregnant women with HG were classified into three groups based on the severity of the condition and matched for gestational age with 157 controls. All samples were analyzed blindly without knowledge of case-control status.

The three HG groups were generated according to the following criteria: mild HG (nausea and vomiting but no need for admission), moderate HG (admission for HG with dehydration that needed infusion therapy but lacking all of the criteria that define severe HG), and severe HG (admission for HG with ketonuria >3+, based on urine dipstick, and weight loss >3 kg). Maternal blood samples were obtained from 45 patients with HG carrying a single male fetus between 6 and 14 weeks of gestation (median gestational age, 10 weeks) at the time of admission. Seventeen patients were classified as having mild HG (median gestational age, 11.0 weeks; range, 8–14 weeks), 11 had moderate HG (median gestational age 9.5 weeks; range, 6–13 weeks), and 17 had severe HG (median gestational age, 10.5 weeks; range, 7–14 weeks). The available data are summarized in Table 1. All of the patients provided written informed consent, and the Ethics Committee of the University approved the study.

Maternal blood samples (7 mL) collected into tubes containing EDTA were separated by centrifugation at 3000g within 3 h after collection. After additional centrifugation, the supernatant was collected into fresh tubes and stored at −20°C until further processing.

DNA was extracted from 1.5 mL of the plasma samples by use of the QIAamp Blood Mini Kit (Qiagen) and was eluted from columns with 50 μL of water. The Y-chromosome-specific DYS14 sequence was subsequently quantified by PCR on a LightCycler (Roche Diagnostics) as described previously (11). Briefly, we used primers DYS14-713F (5′-CAT CCA GAG CGT CCC TGG-3′) and

![Table 1. Characteristics of the data set stratified according to HG group.](image)

a GA, gestational age.

b Because the distribution was gaussian, median and mean are considered as equivalent.
DYS14-880R (5'-TTC CCC TTT GTT CCC CAA A-3') and the dual-labeled fluorescent probe DYS14-883T (5'-FAM-CGA AGC CGA GCT GCC CAT CA-TAMRA-3', where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxymethylrhodamine). Fluorogenic PCRs were performed according to the manufacturer's instructions in a reaction volume of 20 μL, with all components except the fluorescent probe and amplification primers obtained from a reagent set (LightCycler-Fast Start DNA Master Hybridization Probes; Roche Diagnostics). A total of 13.1 μL of the extracted plasma DNA was the template for each reaction. Thermal cycling for DYS14 PCR consisted of denaturation at 95 °C for 10 min, 40 cycles of denaturation at 95 °C for 10 s, and then annealing and extension at 60 °C for 20 s. The number of genome-equivalents of male DNA present in the plasma sample was determined by comparison with a calibration curve constructed with dilutions of male genomic DNA. The genome-equivalent used for conversion was 6.6 pg as described previously (12). LightCycler software (Roche Diagnostics) was used to analyze amplification data and to calculate the concentrations of DYS14.

Strict precautions were taken against contamination, and several negative-control water blanks were included in all analyses. A female staff member performed all procedures, including sample preparation, DNA extraction, and PCR amplification.

We used multiple linear regression to plot log10 DNA values vs the available covariates (gestational age at the time of blood drawing and HG). The presence of HG was added as “dummy variable” and had values of 0 (absent), 1 (mild), 2 (moderate), or 3 (severe). Median fetal DNA concentrations were calculated as a function of increasing gestational age by use of weighted log-linear regression for the 157 control samples, and all fetal DNA amounts in the HG groups and controls are expressed as multiples of the median (MoM). The normality of the fetal DNA distribution was assessed by a Kolmogorov–Smirnov test and a probability plot of log10 fetal DNA MoM values (on a log scale) vs the predicted gaussian percentile values. Comparisons among and between groups were performed by ANOVA and the Bonferroni post hoc test, respectively. In the statistical model, the effect of gestational age was considered constant across groups; we thus explored the effect on fetal DNA values of the severity of HG.

Both gestational age (weeks + days) at the time of blood drawing and HG classification, plotted vs log10 fetal DNA, were associated with a direct proportional increase. The fetal DNA concentration in the plasma of pregnant women in the control group gradually increased as the pregnancy progressed. The overall intercept attributable to the presence of HG was 0.261 (P <0.001). The coefficient of the slope was 0.013 (P <0.001). When we stratified the intercept generated by the presence of HG for the four HG classifications (absent, mild, moderate, and severe), we obtained a value of 0.401 for severe HG (P <0.001) and intercepts of 0.062 and 0.179 for mild and moderate HG, respectively (not significant). The group with severe HG had a very different pattern than all of the others, with an estimated DNA value ~3 times higher than controls at 13–15 weeks of gestation.

The probability plot profile of fetal DNA MoM values in the four groups is shown in Fig. 1. As shown, the severity of HG is associated with a progressively higher mean fetal DNA concentration. It is visually evident how the data followed a log-gaussian profile, at least between the 2.5th (~2.0 SD) and 97.5th (~2.0 SD) percentiles of unaffected patients, for women without HG, as judged by the probability plots. The Kolmogorov–Smirnov test also confirmed the gaussian pattern of the data set stratified for groups (P >0.05). Again, severe HG showed a very distinctive pattern compared with all of the others. The MoM distributions of the control and three HG groups are shown on the right in Fig. 1. The median (SD) MoM values for the control, mild, moderate, and severe HG groups were 1.00 (0.82), 1.26 (0.82), 1.61 (0.81), and 2.41 (2.75), respectively, and increased with the severity of the condition. Because the distributions were gaussian, we used a parametric test to compare groups and assumed that medians and means had very similar values. We used MoM values because they represent the standard analysis in prenatal diagnosis as widely reported by Wald et al. (13). ANOVA yielded a P value <0.001, and the Bonferroni post hoc test showed that only the severe HG group significantly differed from all of the others (P <0.05).

We previously reported that the cell-free fetal DNA concentration increased in the maternal plasma of 16 pregnant women with HG compared with unaffected pregnant women (14); in the present study, we confirmed, by parametric statistical analysis, this hypothesis by measuring the cell-free fetal DNA concentration in 202 preg-
nant women stratified into four groups. We also found that the clinical severity of HG is directly associated with the increase in fetal DNA. This finding indicates that the pathogenesis of HG may be related to the pathway that produces the increase in fetal DNA concentration.

It has been reported that fetal DNA increases in the maternal plasma of preeclamptic women (3–5) and women with invasive placenta (6). The explanation of such an increase in preeclampsia is that the insufficient damage to villous trophoblasts, which subsequently increases the amount of fetal DNA in maternal plasma (5).

In cases of invasive placenta, trophoblasts invading uterine muscle are attacked by the maternal immune system, which increases the amount of fetal DNA in maternal plasma (6). Activated natural killer and cytotoxic T cells in the uterine decidua protect the mother from invasion by trophoblasts (15–17). If the maternal immune system completely tolerates the fetus, the myometrium might be invaded by growing trophoblasts, but in the presence of abnormalities of the immune interaction between the mother and the fetus, invasion of trophoblasts into the myometrium would lead to increased concentrations of fetal DNA in maternal plasma. In HG, a similar situation can occur: even if the pathogenesis of HG remains obscure, a possible explanation has been described by Minagawa et al. (10), who reported that functional activation of natural killer and cytotoxic T cells is more prominent in the blood and uterine decidua of women with HG than in women with uncomplicated pregnancy. Thus, we speculate that hyperactivation of the maternal immune system may be responsible for the onset of HG, probably while maternal immune tolerance to the semiallograft is being established. This observation could explain why fetal DNA and HG are related and proportionally correlated. Because the majority of cell-free fetal DNA likely originates from placental trophoblasts (5,9), trophoblasts might be more damaged in severe HG than in uncomplicated pregnancies or in cases of mild HG occurring during formation of the placenta.

In conclusion, in the present study we found that increased concentrations of cell-free fetal DNA are correlated with the severity of HG. We speculate that such an increase in the plasma of patients with HG may be derived from trophoblasts destroyed by the hyperactivated maternal immune system.

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture of Japan (Grants 15591163 and 14770870) and by the TAKEDA Science Foundation.

**References**


**Single-Nucleotide Polymorphism Genotyping by Melting Analysis of Dual-Labeled Probes: Examples Using Factor V Leiden and Prothrombin 20210A Mutations**, Hakim El Housni, Pierre Heimann, Jasmine Parma, and Gilbert Vassart (Department of Molecular Genetics, Erasme Hospital, Batiment C niveau 5, 808 Route de Lennik, 1070 Brussels, Belgium; *author for correspondence: fax 32-2-555-4527, e-mail helhousni@ulb.ac.be)

The emergence of fluorescence techniques in 96- or 384-well formats has led to high-throughput single-nucleotide polymorphism (SNP) detection methods. Among these, allele discrimination based on real-time PCR technologies has been developed along two main methodologic approaches. In the first approach, a temperature-dependent fluorescent signal is generated by hybridization of a probe at the end of each PCR cycle. The monitoring of fluorescence emission is followed during (1, 2) or at the end of (3, 4) the PCR process. In the latter case, allele detection is achieved by carrying out of a melting curve analysis.

In the second approach, exploiting the classic 5’ nuclease assay (TaqMan® technology, a temperature-indepen-