Quantitative Abnormalities of Fetal DNA in Maternal Serum in Preeclampsia

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Background: There is much recent interest in the biologic and diagnostic implication of cell-free non-host DNA in the plasma and serum of human subjects. To determine if quantitative abnormalities of circulating non-host DNA may be associated with certain pathologic processes, we used circulating fetal DNA in preeclampsia as a model system.

Methods: We studied 20 preeclamptic women and 20 control subjects of comparable gestational age (means, 32 and 33 weeks, respectively). Male fetal DNA in maternal serum was measured using real-time quantitative PCR for the SRY gene on the Y chromosome.

Results: The imprecision (CV) of the assay was 2.7%. The median circulating fetal DNA was increased five-fold in 20 preeclamptic women compared with 20 control pregnant women (381 vs 76 genome-equivalents/mL, \( P < 0.001 \)).

Conclusions: These observations suggest that preeclampsia is associated with disturbances in the liberation and/or clearance mechanisms of circulating DNA. These results also raise the possibility that measurement of circulating DNA may prove useful as a marker for the diagnosis and/or monitoring of preeclampsia.

Circulating cell-free DNA has been found in the plasma of human subjects (1, 2). The recent application of molecular biologic techniques has allowed the molecular characterization of circulating DNA in certain pathologic and physiologic conditions. Thus, tumor-associated genetic changes have been found in the plasma and serum of patients suffering from a number of cancers (3–5). After solid organ transplantation, donor-derived DNA has been found in the plasma of the recipients (6). Physiologically, fetal-derived DNA has been found in the plasma and serum of pregnant women (7). Taken together, these studies indicate that circulating non-host DNA is a common phenomenon in many clinical and physiologic scenarios. However, little is known regarding the pathophysiologic conditions affecting the concentrations of circulating non-host DNA.

We have recently described a real-time quantitative PCR assay for measuring the concentration of circulating fetal DNA in maternal plasma and serum (8). This assay exploits the 5’ to 3’ exonuclease activity of the Taq DNA polymerase, which leads to the liberation of a fluorescent reporter during DNA amplification (9–11). The monitoring of the increase in the fluorescence signal during PCR allows accurate quantification of the template copy number before amplification. Our results showed that fetal DNA constitutes 3.4% and 6.2% of the total plasma DNA in maternal blood in early and late pregnancy, respectively (8).

In this study, we investigated the concentration of circulating fetal DNA in preeclamptic pregnancies in an effort to understand the pathologic processes that may influence the concentration of fetal DNA in maternal plasma. We decided to study preeclampsia because previous authors reported increased transfer of fetal-derived cells, such as trophoblasts (12) and fetal erythroblasts (13), into the maternal circulation. Our present study should reveal whether, in addition to disturbed cellular transfer, nucleic acid traffic would also be affected in preeclampsia.

Materials and Methods

Patients

Pregnant women attending the Department of Obstetrics and Gynecology at the Prince of Wales Hospital, Shatin, Hong Kong and the Nuffield Department of Obstetrics and Gynecology, Oxford, UK were recruited with informed consent. Approval was obtained from the Research Ethics Committee of The Chinese University of Hong Kong and the Central Oxfordshire Research Ethics Committee. Preeclampsia was defined as a sustained rise in diastolic blood pressure to 90 mmHg or higher from
previously lower values, with new and sustained proteinuria in the absence of urinary tract infection. The control pregnant women were not on medication and had no hypertension or proteinuria (defined as more than a trace on dipstick urine analysis). The preeclamptic and control subjects were matched for gestational age. The mean gestational ages of the preeclamptic and control subjects were 32 (range, 27–41 weeks) and 33 weeks (range, 28–40 weeks), respectively. Each participating center contributed a matched number of preeclamptic and control subjects. Maternal antecubital venous blood (5–10 mL) was collected into plain tubes for the isolation of serum. Ten pregnant women carrying female fetuses (gestational age, 37–43 weeks) were also recruited as negative controls. The investigators carrying out the molecular analysis of the samples were unaware of the clinical status of the patients from whom the samples were obtained.

PROCESSING OF BLOOD SAMPLES
Blood samples were centrifuged at 3000 g, and serum samples were carefully removed from blood collection tubes and transferred into plain polypropylene tubes. Great care was taken to ensure that the blood clot was undisturbed when serum samples were removed. The samples were stored at −70 or −20 °C until further processing.

DNA EXTRACTION FROM SERUM SAMPLES
DNA from serum samples was extracted using a QIAamp Blood Kit (Qiagen) using the “blood and body fluid protocol” as recommended by the manufacturer (14). A 400–800 μL serum sample was used for DNA extraction per column. The exact amount used was documented to enable the calculation of the target DNA concentration (8).

REAL-TIME QUANTITATIVE PCR
The theoretical and practical aspects of real-time quantitative PCR have been described in detail elsewhere (8, 10, 11). Real-time quantitative PCR analysis was performed using a Perkin-Elmer Applied Biosystems 7700 Sequence Detector. The amplification and product reporting system used is based on the 5′ nuclease assay (9) (marketed by Perkin-Elmer as the TaqMan assay) in which the liberation of a fluorescent reporter is coupled to the amplification reaction. Amplification primers and fluorescent probes, designed to detect the SRY gene on the Y chromosome and the β-globin gene on chromosome 11, were as described previously (8).

TaqMan amplification reactions were set up as described previously (8), using 5 μL of the extracted serum DNA. Thermal cycling conditions and the use of uracil N-glycosylase were as described previously (8). Each sample was analyzed in duplicate. A calibration curve was run in parallel and in duplicate with each analysis.

Amplification data collected by the 7700 Sequence Detector and stored in the Macintosh computer were then analyzed using the Sequence Detection System software developed by Perkin-Elmer Applied Biosystems. The detection threshold was set at 10 SD above the mean baseline fluorescence, calculated from cycles 1–15 (10). An amplification reaction in which the fluorescence intensity increased above the threshold during the course of thermal cycling was defined as a positive reaction. The cycle number at which the fluorescence increased above the threshold was designated as the threshold cycle (C_T). The C_T value was used to quantify the starting template number as described previously (8). The quantification results were expressed as genome-equivalents/mL. One genome-equivalent was defined as the quantity of a particular DNA sequence present in one diploid male cell.

ANTICONTAMINATION MEASURES
Strict precautions against PCR contamination were used (15). In addition, the TaqMan assay also included an additional anticontamination measure in the form of preamplification treatment using uracil N-glycosylase, which destroyed uracil-containing PCR products (16). Multiple negative water blanks were included in every analysis.

Results
The performance of real-time quantitative SRY PCR on a serial dilution of male DNA produced a series of amplification curves. The curves gradually shifted toward the right as fewer and fewer starting template molecules were present (Fig. 1A). The system was sensitive enough to detect the DNA equivalent from a single target cell. A linear relationship was observed when the C_T was plotted against the input target quantity, with the latter plotted on a common logarithmic scale (Fig. 1B). The linearity of the plot (Fig. 1B) indicates that the C_T value could be used to quantify the starting copy number of unknown samples over a wide dynamic range.

The analytical intraassay CV of C_T values obtained using the real-time TaqMan SRY assay was 1.5% (mean ± SD, 33.6 ± 0.5), as determined by 20 replicate quantitative PCR assays of DNA extracted from the serum of a woman carrying a male fetus in the third trimester of pregnancy. The total CV of C_T values obtained using the system starting from DNA extraction followed by quantitative PCR was determined to be 2.7% (mean ± SD, 33.4 ± 0.9) by 20 replicate extractions of serum samples from the same pregnant subject. The CV of the DNA extraction system was calculated to be 2.2%. For the analysis of the clinical samples, preeclamptic and matched non-preeclamptic samples were always extracted and analyzed in the same assay.

Fig. 1C shows the amplification plots from serum DNA from a number of preeclamptic and control pregnant women. The amplification plots from preeclamptic women were located to the left of control subjects (Fig. 1C), indicating that higher concentrations of circulating fetal DNA were present in these two preeclamptic compared with the two control individuals.
Comparison of these amplification plots with a calibration curve of serially diluted male DNA allowed the conversion of CT values into fetal DNA concentrations in genome-equivalents/mL. Fig. 2 shows the ranges of fetal DNA concentrations observed in 20 preeclamptic and 20 control subjects. The median fetal DNA concentrations in preeclamptic and control pregnancies were 381 genome-equivalents/mL (interquartile range, 194–788 genome-equivalents/mL) and 76 genome-equivalents/mL (interquartile range, 54–163 genome-equivalents/mL), respectively. Fetal DNA concentrations were higher in preeclamptic than control pregnancies (Mann–Whitney rank-sum test, \( P < 0.001 \)). Removal of the apparent outlier in the preeclamptic group showing the highest circulating fetal DNA concentration (2375 genome-equivalents/mL) (Fig. 2) did not significantly affect the median fetal DNA concentration in the preeclamptic group (325 genome-equivalents/mL) nor the significant difference between the preeclamptic and control group (Mann–Whitney rank-sum test, \( P < 0.001 \)). None of the serum samples from the 10 women carrying female fetuses had any SRY signal.

As a control for the ability of serum-extracted DNA to be amplified, all samples were subjected to a TaqMan assay for the \( \beta \)-globin gene. Positive amplification signals were seen in all tested samples, thus confirming the quality of the DNA samples.

### Discussion

Our data indicate that the median concentration of circulating fetal DNA in preeclamptic subjects is fivefold higher than that of non-preeclamptic subjects matched for gestational age. This is the first description of a quantitative abnormality involving circulating non-host DNA.

The mechanisms leading to the increase in maternal serum fetal DNA are unclear at present. Possible pathways include increased liberation of fetal DNA into the maternal circulation and/or reduced clearance of circu-
lating DNA from maternal blood. Because plasma DNA has been postulated to be a marker of cell death (17,18), increased amounts of fetal DNA may be liberated from necrotic (19) or apoptotic (20) areas in the placenta. On the clearance side, the kidneys and liver have been suggested to be the main organs for the removal of circulating DNA (21,22). Because pathologic changes involving the kidneys and liver are well-described in preeclampsia (23), it is likely that these processes might reduce the ability of these organs to remove DNA from the circulation.

The relationship between the entry of fetal nucleated cells into the maternal circulation (24) and cell-free fetal DNA remains to be elucidated. This potential relationship is worth investigating, especially in view of the fact that increased entry of fetal nucleated cells, such as trophoblasts (12) and erythroblasts (13), have been described in preeclampsia. Using an immunocytochemical approach, Chua et al. (12) were able to detect large numbers of trophoblasts in the uterine venous blood of preeclamptic women. However, when they applied the same technique to the analysis of peripheral venous samples from these preeclamptic subjects, they were able to detect trophoblasts in only a minority of cases. These data suggest that a large proportion of trophoblasts was either trapped or destroyed during their passage through the maternal circulation. These observations therefore raise the possibility that fetal DNA is liberated through destruction of circulating fetal nucleated cells, of which the trophoblasts constitute a subset. Circulating fetal DNA in maternal peripheral blood may therefore be a valuable and easily accessible marker of trophoblast trafficking, a process that up to now has been difficult to study in a noninvasive manner. Compared with the analysis of fetal nucleated cells that have entered into the maternal blood, which in many cases requires the use of fetal cell enrichment procedures (25,26), plasma DNA analysis has the advantage of being rapid, reliable, and easily carried out for a large number of samples.

Apart from its biologic implications, the measurement of the fetal DNA concentration in maternal serum may have diagnostic importance in preeclamptic pregnancies. Compared with other markers for preeclampsia, fetal DNA measurement is unique in that it is a genetic marker, whereas other markers, such as activin A and inhibit A (27), are generally hormonal or metabolic markers. By its nature, a genetic marker has the characteristic of being completely fetal-derived. The measurement of fetal DNA concentrations in pregnancies involving a female fetus would require the development of fetal-specific markers outside the Y chromosome. Autosomal genetic markers suitable for this type of analysis have already been described (24).

The potential clinical implication of maternal serum fetal DNA measurement, especially with regard to the prognosis and guidance of clinical management requires future prospective studies. Additional research will also be required to investigate whether abnormal patterns of fetal DNA liberation or clearance may be detectable even before the development of the clinical signs of preeclampsia.

Our data show that real-time quantitative PCR is an accurate and efficient method for the detection and quantification of circulating DNA. With conventional PCR techniques, accurate quantification is difficult once the reaction has reached the plateau phase. One advantage of real-time quantitative PCR is that quantitative information is obtained at the threshold cycle, well before the plateau phase has been reached. The 96-well format allows a large number of samples to be analyzed in 2 h. The homogeneous nature of the assay avoids downstream processing and reduces the chance of carryover contamination. All of these features are advantageous for the potential clinical application of this type of system. Apart from the analysis of fetal DNA in maternal plasma and serum, we also envisage that this type of assay may have applications in other clinical scenarios where non-host DNA is found, such as tumor-derived DNA in oncology patients (5,28) and donor-derived DNA in transplant recipients (6).

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