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Increased Fetal DNA Concentrations in the Plasma of Pregnant Women Carrying Fetuses with Trisomy 21

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Background: **The recent discovery of the presence of circulating cell-free fetal DNA in maternal plasma opens up new prenatal diagnostic applications and provides new avenues for clinical investigation. It is of research and potential diagnostic interest to determine whether fetal trisomy 21 may be associated with quantitative abnormalities of circulating fetal DNA in maternal plasma.**

Methods: **Maternal plasma samples were prospectively collected from two centers situated in Hong Kong and Boston. Samples collected from Boston consisted of 7 women carrying male trisomy 21 fetuses, 19 carrying euploid male fetuses, and 13 carrying female fetuses. Samples collected from Hong Kong consisted of 6 women carrying male trisomy 21 fetuses, 18 carrying euploid male fetuses, and 10 carrying female fetuses. Male fetal DNA in maternal plasma was measured using real-time quantitative Y-chromosomal PCR.**

Results: **For patients recruited from Boston, the median circulating fetal DNA concentrations in women carrying trisomy 21 and euploid male fetuses were 46.0 genome-equivalents/mL and 23.3 genome-equivalents/mL, respectively ($P = 0.028$). For patients recruited from Hong Kong, the median circulating fetal DNA concentrations in women carrying trisomy 21 and euploid male fetuses were 48.2 genome-equivalents/mL and 16.3 genome-equivalents/mL, respectively ($P = 0.026$). None of the samples from women carrying female fetuses had detectable Y-chromosomal signals.**

Conclusions: **Abnormally high concentrations of circulating fetal DNA are found in a proportion of women carrying fetuses with trisomy 21. The robustness and reproducibility of real-time PCR analysis of maternal plasma makes it a valuable tool for cross-institutional collaboration involving centers located in different parts of the world.**

The transfer of nucleated fetal cells into the circulation of pregnant women is a well-recognized phenomenon *(1, 2)*. Promising data have been reported that suggest that the analysis of fetal nucleated cells isolated from maternal blood may be useful for the prenatal diagnosis of fetal chromosomal aneuploidies *(3–6)*. It has also been demonstrated that the number of circulating fetal nucleated cells is increased when the fetus is affected by trisomy 21 *(7)*.

Recent data indicate that in addition to fetal nucleated cells, circulating cell-free fetal DNA is also present in maternal blood *(8)*. Cell-free fetal DNA offers a new alternative source of fetal genetic material for noninvasive prenatal diagnosis *(9, 10)*. The development of quantitative assays for circulating fetal DNA has provided powerful tools for studying the variations in fetal DNA concentrations in different physiological *(11, 12)* and pathological *(13, 14)* conditions.

In this study we investigated the concentrations of circulating cell-free fetal DNA in the plasma of pregnant women carrying fetuses affected by trisomy 21. These data may improve our understanding of the fetal characteristics affecting the liberation of fetal DNA into maternal blood and may provide results for assessing the potential of circulating fetal DNA analysis as a screening tool for trisomy 21.

Materials and Methods

PATIENTS

Pregnant women attending the Prenatal Diagnosis Unit at the Department of Obstetrics and Gynecology, Prince of Wales Hospital, Hong Kong and pregnant subjects under investigation for fetal chromosomal aneuploidies by the New England Medical Center, Boston, MA were recruited with informed consent. Approval was obtained from the corresponding institutional review boards.

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For the Boston arm of the study, plasma samples from women carrying male trisomy 21 fetuses recruited between February 1998 and December 1998 were entered into the study. Plasma samples from women carrying euploid male fetuses and female fetuses were selected at random from subjects recruited during the same period and matched for gestational age to the subjects carrying male trisomy 21 fetuses. In total, 7 women carrying male trisomy 21 fetuses, 19 carrying euploid male fetuses, and 13 carrying female fetuses were recruited from Boston. The mean gestational ages of the subjects carrying trisomy 21 and euploid male fetuses were 17.3 weeks (12–21 weeks) and 17.1 weeks (12–21 weeks), respectively. Ten Boston cases were recruited after amniocentesis for indications including abnormal maternal serum screening results, abnormal ultrasound scan results, and advanced maternal age. Information regarding fetal gender and aneuploidy status was obtained by karyotyping of amniotic fluid culture.

For the Hong Kong arm of the study, plasma samples from women carrying male trisomy 21 fetuses recruited between June 1997 and November 1998 were entered into the study. Plasma samples from women carrying euploid male fetuses and female fetuses were selected at random from subjects recruited during the same period and matched for gestational age to the subjects carrying male trisomy 21 fetuses. In total, 6 women carrying male trisomy 21 fetuses, 18 carrying euploid male fetuses, and 10 carrying female fetuses were recruited from the Hong Kong center. The mean gestational ages of the subjects carrying trisomy 21 and euploid fetuses were 18.0 weeks (16–21 weeks) and 17.2 weeks (16–21 weeks), respectively. In all cases, maternal peripheral blood samples were collected before any invasive procedures such as amniocentesis. Information regarding fetal gender and aneuploidy status was obtained by karyotyping of amniotic fluid culture.

For subjects recruited from Boston, 16–24 mL of maternal antecubital venous blood was collected into tubes containing EDTA and processed as described previously by a protocol designed originally for the isolation of fetal nucleated red cells from maternal blood *(15)*, except that the plasma was harvested, frozen at −80 °C, and then sent to the co-investigators in Hong Kong for fetal DNA detection. Samples collected from Boston were batched and sent frozen on dry ice by a courier service to Hong Kong. The investigators in Hong Kong were "blinded" to the fetal gender and aneuploidy status of the cases. The code was broken only after the plasma PCR results had been sent back to Boston. For subjects recruited from Hong Kong, 5–10 mL of maternal antecubital venous blood was collected into EDTA tubes. Plasma was harvested as described previously *(11)*.

DNA extraction from plasma samples

DNA from plasma samples was extracted using a QIAamp Blood Kit (Qiagen), using the "blood and body fluid protocol" as recommended by the manufacturer *(11)*; 400–800 μL of plasma sample was used for DNA extraction per column. The exact amount used was documented to enable the calculation of target DNA concentrations *(11)*.

Real-time quantitative PCR

Y-chromosomal sequences in male fetuses were used as molecular markers for fetal DNA in maternal plasma. Fetal-derived Y-chromosomal sequences were detected using real-time quantitative PCR *(11, 13)*. The theoretical and practical aspects of real-time quantitative PCR for fetal-derived Y-chromosomal sequences in maternal plasma DNA have been described in detail elsewhere *(11, 13)*. An additional real-time PCR assay for the *β-globin* gene was used as a positive control for the amplifiability of the plasma samples. The analytical performance of these assays have been reported previously *(11, 13)*.

The calculation of fetal DNA concentration as genome-equivalents per milliliter of maternal plasma has been described previously *(11, 13)*. One genome-equivalent was defined as the quantity of a particular DNA sequence present in one diploid male cell.

Strict precautions against PCR contamination *(16)* and the use of uracil-*N*-glycosylase as an additional anticontamination measure were as described previously *(11)*.

Results

The concentrations of fetal-derived Y-chromosomal sequences in the plasma of the women recruited from Boston are shown in Fig. 1. The median maternal plasma fetal-derived Y-chromosomal concentrations in women carrying trisomy 21 and euploid male fetuses are 46.0

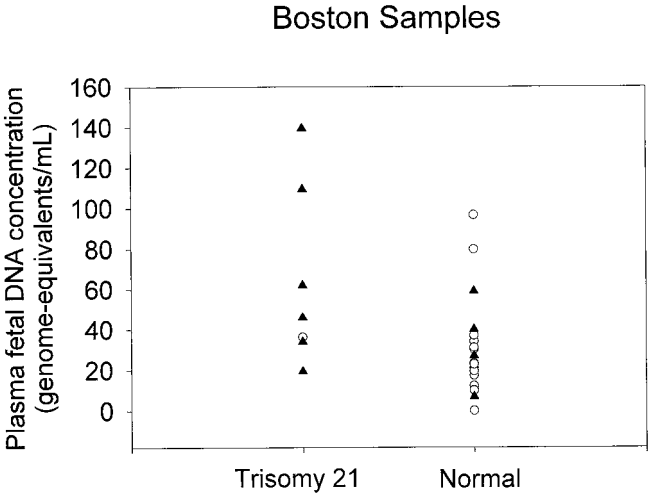

Fig. 1. Fetal DNA concentrations in maternal plasma in women recruited from the Boston center who were carrying trisomy 21 and euploid male fetuses.

The trisomy 21 and control (*Normal*) groups are as indicated on the *x-axis*. Fetal DNA concentrations in maternal plasma are expressed in genome-equivalents/mL and are plotted on the *y-axis*. ○ and ▲ denote samples that were obtained before and after amniocentesis, respectively.

genome-equivalents/mL and 23.3 genome-equivalents/mL, respectively. The difference between the two groups was statistically significant ($P = 0.028$, Mann–Whitney rank-sum test). None of the 13 women carrying female fetuses showed Y-chromosomal signals in maternal plasma DNA. As a quality control, real-time quantitative PCR for the *β-globin* gene was performed, which demonstrated the existence of amplifiable DNA in all samples.

In this pilot arm of the project, six samples in the trisomy 21 group and four samples in the euploid group were obtained after amniocentesis, with blood sampling occurring from immediately after amniocentesis to 18 days after the procedure. The two samples exhibiting the highest concentration of fetal DNA in maternal plasma (Fig. 1) were collected at 6 and 8 days after amniocentesis. The single case in the trisomy 21 group that was collected before amniocentesis had a fetal DNA concentration of 36.2 genome-equivalents/mL. A postamniocentesis sample from this individual was taken 7 days after the procedure, and the fetal DNA concentration was 24.0 genome-equivalent/mL. For the euploid group, the median fetal DNA concentrations in the 4 cases recruited after amniocentesis and the 15 cases recruited before the procedure were 33.5 genome-equivalents/mL and 22.7 genome-equivalents/mL, respectively, and were not statistically significant from one another ($P = 0.582$, Mann–Whitney rank-sum test).

To confirm the pilot Boston data, a parallel study was conducted using samples collected from the Hong Kong center. Special precautions were taken to ensure that all samples were collected before any invasive procedure such as amniocentesis. The concentrations of fetal Y-chromosomal sequences in the plasma of women recruited by the Hong Kong center and who were carrying male trisomy 21 and euploid male fetuses are shown in Fig. 2. The median fetal-derived Y-chromosomal concentrations in the plasma of women carrying trisomy 21 and euploid male fetuses were 48.2 genome-equivalents/mL and 16.3 genome-equivalents/mL, respectively. The difference between the two groups was statistically significant ($P = 0.026$, Mann–Whitney rank-sum test). As a control, no Y-chromosomal signal was detectable in the plasma of 10 women carrying female fetuses. As a quality control, real-time quantitative PCR for the *β-globin* gene was performed, which demonstrated the existence of amplifiable DNA in all samples.

No significant correlation between the plasma Y-chromosomal and *β-globin* sequence concentrations was observed among the 50 samples obtained from pregnant women bearing male fetuses recruited from the two centers (Spearman rank-order correlation, correlation coefficient = 0.23, $P = 0.1$).

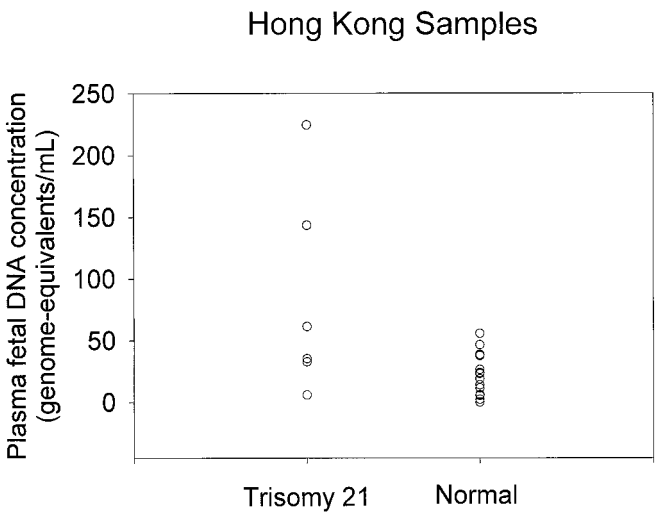

Fig. 2. Fetal DNA concentrations in maternal plasma in women recruited from the Hong Kong center who were carrying trisomy 21 and euploid male fetuses.

The trisomy 21 and control (*Normal*) groups are as indicated on the *x-axis*. Fetal DNA concentrations in maternal plasma are expressed in genome-equivalents/mL and are plotted on the *y-axis*.

Discussion

The recent discovery of the presence of circulating cell-free fetal DNA in maternal plasma has raised many unanswered biological questions and opened up numerous diagnostic applications *(8)*. Prenatal screening for fetal trisomy 21 is now considered part of routine obstetric care in many parts of the world. To gain further understanding of the pathologic conditions that may affect the concentration of circulating cell-free fetal DNA, we measured the concentration of circulating fetal DNA in women carrying fetuses affected by trisomy 21. Our results may also yield information with regard to the diagnostic potential of circulating fetal DNA as a screening tool for trisomy 21.

Our results indicate that very high concentrations of circulating cell-free fetal DNA in maternal plasma were found in a proportion of pregnancies involving trisomy 21 fetuses. Thus, for samples collected from Hong Kong, the median cell-free fetal DNA in women carrying trisomy 21 fetuses was 2.96-fold higher than that of women carrying euploid fetuses. For samples collected from Boston, the corresponding figure was 1.97-fold higher. The highest maternal plasma fetal DNA concentrations observed in the Hong Kong and Boston trisomy 21 cases were 14-fold and 6-fold higher than that of the median concentration of the euploid cases, respectively.

The fact that the results have been replicated in two populations, in Hong Kong and in Boston, confirms the robustness of our conclusions. The design of the Boston arm of the project especially involves different groups of investigators for sample collection and analysis to maximize the objectivity of the results. This latter arm of the project also highlights an advantage of plasma DNA analysis, namely, that the samples can be frozen and sent across the globe with apparently little adverse effects on the analytical results.

The pilot Boston arm of the study involved samples that had been collected after amniocentesis, with the time

of blood sampling from immediately after the procedure to 18 days after amniocentesis. We have carefully considered the possibility that the use of postamniocentesis samples might affect our results. However, on reviewing our data and previous publications, we consider that the effects of amniocentesis were not substantial enough to bias our observations. Thus, published data indicate that previous amniocentesis does not produce significant changes in the number of circulating fetal nucleated cells in maternal blood as measured using quantitative PCR *(7)*. Although a systematic study of the effect of amniocentesis on cell-free fetal DNA in maternal plasma has not been conducted, the rapid clearance of fetal DNA from maternal plasma *(12)* suggests that the effect of previous amniocentesis is likely to be small when maternal blood sampling is performed days after the procedure. This conjecture is supported by our analysis of the maternal blood samples from a case involving a male fetus with trisomy 21 collected both before and at 7 days after amniocentesis, which yielded maternal plasma fetal DNA concentrations of 36.2 genome-equivalents/mL and 24.0 genome-equivalents/mL, respectively. In this case at least, we did not obtain evidence that amniocentesis would produce a significant increase in fetal DNA in maternal plasma. Further support for this conclusion was obtained from a comparison between samples in the euploid groups with and without prior amniocentesis, where no statistically significant difference was observed. Finally, the most powerful validation of our results was obtained by verification of the results of the Boston arm of this study by Hong Kong samples collected prior to any invasive procedures such as amniocentesis.

We used prospectively collected samples specifically processed for the current project to avoid uncertainties associated with archival plasma/serum materials, such as sample integrity and potential sample contamination *(17)*. We believe that proper assurance of sample quality is especially important when clinical specimens collected for other projects are to be analyzed using very sensitive methods such as the PCR.

The main limitation of quantitative analysis of Y-chromosomal sequences in maternal plasma is that this approach can only be used in pregnancies involving male fetuses. The extension of this type of study to pregnancies involving female fetuses would require the development of quantitative assays for autosomal or X-chromosomal polymorphisms. Qualitative versions of these systems have already been developed *(18)*, and it would be possible to convert these assays into a real-time quantitative format.

Our data suggest that circulating fetal DNA may potentially be used as a marker for fetal trisomy 21. However, it should be noted that there is a considerable degree of overlap between the values observed in trisomic and euploid fetuses (Figs. 1 and 2). This implies that a relatively low sensitivity and specificity would result if maternal plasma fetal DNA measurement was used as a sole marker for fetal trisomy 21. Thus, the combination of circulating fetal DNA with other markers of fetal trisomy 21 may be needed before this new marker may be useful clinically. For definitive karyotypic diagnosis, the isolation of circulating nucleated fetal cells still remains the best candidate technology for the development of noninvasive prenatal diagnosis of fetal chromosomal aneuploidies *(6)*.

The mechanism whereby the concentration of circulating cell-free fetal DNA is increased in pregnancies involving fetuses with trisomy 21 is unclear at present. One possible mechanism may involve the numerous histologic abnormalities observed in the placentas of fetuses affected by trisomy 21 *(19–21)*. A second mechanism may be related to the increased trafficking of fetal nucleated cells into maternal circulation when the fetus is affected by trisomy 21 *(7)*. It is possible that a proportion of circulating cell-free fetal DNA is derived from the destruction of fetal nucleated cells that have entered into maternal blood. Further support for the possible relationship between the trafficking of fetal nucleated cells and cell-free fetal DNA into maternal blood can be found in preeclampsia, where parallel increases in circulating nucleated fetal cells *(22, 23)* and cell-free fetal DNA *(13, 24)* have been observed. It is hoped that the further study of fetal nucleated cells and cell-free DNA may improve our understanding of fetomaternal physiology.

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