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Size Distributions of Maternal and Fetal DNA in Maternal Plasma

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Background: **The discovery of fetal DNA in maternal plasma has opened up an approach for noninvasive prenatal diagnosis. Despite the rapid expansion in clinical applications, the molecular characteristics of plasma DNA in pregnant women remain unclear.**

Methods: **We investigated the size distribution of plasma DNA in 34 nonpregnant women and 31 pregnant women, using a panel of quantitative PCR assays with different amplicon sizes targeting the *leptin* gene. We also determined the size distribution of fetal DNA in maternal plasma by targeting the *SRY* gene.**

Results: **The median percentages of plasma DNA with size >201 bp were 57% and 14% for pregnant and nonpregnant women, respectively ($P < 0.001$, Mann–Whitney test). The median percentages of fetal-derived DNA with sizes >193 bp and >313 bp were 20% and 0%, respectively, in maternal plasma.**

Conclusion: **Plasma DNA molecules are mainly short DNA fragments. The DNA fragments in the plasma of pregnant women are significantly longer than those in the plasma of nonpregnant women, and the maternal-derived DNA molecules are longer than the fetal-derived ones.**

Prenatal diagnosis is part of established obstetric care in most developed countries. Currently, methods for obtaining fetal tissues for prenatal diagnosis include chorionic villus sampling and amniocentesis. These methods, however, are invasive and carry a risk of fetal loss.

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In 1997, we reported the existence of fetal-derived DNA in maternal plasma by demonstrating the amplifiability of Y-chromosome-specific sequences in women carrying male fetuses *(1)*. Later we showed that fetal DNA comprised a mean of 3.4% and 6.2% of the total DNA in maternal plasma in early and late gestation, respectively *(2)*. These findings have opened up the possibility of noninvasive prenatal diagnosis by plasma DNA analysis. Clinical applications of this biological phenomenon include fetal RhD genotyping *(3, 4)*, fetal aneuploidy detection *(5, 6)*, and prenatal diagnosis of several genetic diseases, including myotonic dystrophy, congenital adrenal hyperplasia, and β-thalassemia *(7–10)*.

Many fundamental questions concerning the characteristics of the circulating DNA in maternal plasma remain unanswered, including the size distribution of plasma DNA fragments. In this report, we describe the size distribution of fetal and maternal DNA present in maternal plasma.

To study the fragment-length distribution of plasma DNA, we developed two panels of quantitative PCR with a range of amplicon sizes targeting the *leptin* and *SRY* genes. The *leptin* gene is located on chromosome 7 and is present in all human cells, whereas the *SRY* gene is located on the Y chromosome and is present only in male cells. Because fetal DNA constitutes a mean of only 6.2% of maternal plasma DNA in the third trimester *(2)*, the size distribution of the *leptin* gene in maternal plasma would predominantly represent the size distribution of maternal DNA. On the other hand, because the *SRY* gene is present only in the fetal cells in women bearing a male fetus, the size distribution of the *SRY* gene should represent the size distribution of fetal-derived DNA in maternal plasma.

Better characterization of maternal plasma DNA could lead to a more thorough understanding of the mechanisms of plasma DNA release and elimination in pregnant women. These data are not only of academic interest but also of practical importance for the future design of molecular markers for prenatal diagnostic purposes.

Materials and Methods

PARTICIPANTS

Healthy pregnant women without pregnancy-associated complications attending the Department of Obstetrics and Gynaecology at the Prince of Wales Hospital, Hong Kong, were recruited with informed consent. Ethics approval was obtained from the Clinical Research Ethical Committee of The Chinese University of Hong Kong. We also recruited 35 healthy nonpregnant female and 20 male volunteers as controls.

BLOOD COLLECTION AND DNA EXTRACTION

Antecubital venous blood (10–15 mL from each patient) was collected in EDTA-containing tubes. Plasma was obtained from blood samples after centrifugation at 1600*g* for 10 min and microcentrifugation at 16 000*g* for 10 min as described previously *(11)*. DNA was extracted with a QIAamp Blood Kit (Qiagen) according to the manufacturer's recommendations listed in the blood and body fluid protocol. DNA was extracted from 2 mL of plasma and eluted in 300 μL of H_2O.

SIZE DISTRIBUTION ANALYSIS OF TOTAL PLASMA DNA IN PREGNANT WOMEN

To determine the size distribution of DNA fragments in maternal plasma, we developed nine real-time PCR assays to amplify different-sized amplicons targeting the *leptin* gene. These PCR assays used nine different reverse primers, one common forward primer, and one common TaqMan minor-groove-binding probe *(12)*. The sequences of the primers and the corresponding amplicon sizes are listed in Table 1. PCRs were set up in a reaction volume of 50 μL and included components (except the TaqMan probe and amplification primers) supplied in a TaqMan PCR Core Reagent Kit (Applied Biosystems). Each reaction contained 5 μL of 10× buffer A; 4 mM $MgCl_2$; 200 μM each of dATP, dCTP, and dGTP; 400 μM dUTP; 1 μM each of the forward and reverse primers; 500 nM TaqMan probe; 2 U of AmpliTaq Gold polymerase; 0.5 U of AmpErase uracil N-glycosylase; and 50 nL/μL dimethyl sulfoxide; 5 μL of plasma DNA was used as template. Each real-time quantitative PCR was performed in duplicate in an Applied Biosystems 7700 Sequence Detector. Amplification data were collected and analyzed by the Sequence Detection System Software (Ver. 1.9; Applied Biosystems).

Two thermal profiles were used for the nine PCR assays. After initial incubation at 50 °C for 2 min and 95 °C for 10 min, 50 cycles of thermal cycling at 95 °C for 30 s, 58 °C (for amplicon sizes from 105 to 356 bp) or 60 °C (for amplicon sizes ≥449 bp) for 1 min, and 72 °C for 1 min were carried out. A calibration curve constructed with serial 10-fold dilutions of buffy coat DNA from 10^5 to 10^{-1} copies was run for each assay and in duplicate. Multiple negative water blanks were included in every analysis. The plasma concentrations of the different-sized DNA fragments were expressed as copies/mL of plasma.

SIZE DISTRIBUTION ANALYSIS OF FETAL DNA IN MATERNAL PLASMA

For quantitative analysis of the size spectrum of fetal DNA in maternal plasma, we designed a panel of six PCR

Table 1. Primers, probes, and their corresponding annealing temperatures for both conventional and real-time quantitative PCR.

Gene	Sequence	Forward/reverse/probe	Amplicon size, bp	Annealing temperature, °C
leptin	5′-CAGTCTCCTCCAAACAGAAAGTCA-3′	Forward		–[a]
	5′-GTCCATCTTGGATAAGGTCAGGA-3′	Reverse	105	58
	5′-GATATTTGGATCACGTTTCTGG-3′	Reverse	145	58
	5′-CAGCTCTTAGAGAAGGCCAGCA-3′	Reverse	201	58
	5′-CTCTGTGGAGTAGCCTGAAGCTT-3′	Reverse	280	58
	5′-CTCAGCACCCAGGGCTGAG-3′	Reverse	356	58
	5′-GTGTCCATGCAATGCTCTTCA-3′	Reverse	449	60
	5′-CCTTCCTGGTGAGAATAGGATCC-3′	Reverse	576	60
	5′-GGTCACATTGAGGCATTCAGTGAG-3′	Reverse	697	60
	5′-GGATGTGGTCTTCATGGAACTCCT-3′	Reverse	798	60
	5′-(FAM)[b]CGGTTTGGACTTC(MGBNFQ)-3′	Probe		–[a]
SRY	5′-AAAGGCAACGTCCAGGATAGAG-3′	Forward		–[a]
	5′-TGAGTTTCGCATTCTGGGATT-3′	Reverse	107	58
	5′-CCACTGGTATCCCAGCTGCT-3′	Reverse	137	58
	5′-TGTAATTTCTGTGCCTCCTGGA-3′	Reverse	193	58
	5′-ACTTCGCTGCAGAGTACCGAA-3′	Reverse	313	58
	5′-TAAGTGGCCTAGCTGGTGCTC-3′	Reverse	392	58
	5′-ATGTTACCCGATTGTCCTACAGC-3′	Reverse	524	58
	5′-(FAM)AAGCGACCCATGAA(MGBNFQ)-3′	Probe		–[a]

[a] The annealing temperatures of the forward primers and probes correspond to the annealing temperatures of the reverse primers used.
[b] FAM, 6-carboxyfluorescein; MGBNFQ, minor groove binding nonfluorogenic quencher.

assays with different-sized amplicons targeting the *SRY* gene. The PCR systems consisted of six different reverse primers, one common forward primer, and one common TaqMan minor-groove-binding probe. The sequences of the primers and probe are listed in Table 1. Apart from the primers, the probe, and the annealing temperature, the components and the setup of the real-time PCR assays were identical to those described above. An identical thermal profile was used for all PCR reactions. After initial incubation at 50 °C for 2 min and 95 °C for 10 min, 50 cycles of thermal cycling at 95 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min were carried out.

SIZE DISTRIBUTION ANALYSIS OF PLASMA DNA IN HEALTHY MALES

The size distribution of DNA extracted from the plasma of 16 healthy male controls was determined in assays targeting both the *leptin* and *SRY* genes as described above.

Results

QUANTITATIVE PCR FOR FRAGMENT SIZE ANALYSIS

The real-time PCR assays were able to detect one copy of the *leptin* gene or the *SRY* gene. Because the DNA concentrations in different individuals differed widely, the relative concentration for each amplicon size was calculated such that the size distribution of DNA in each individual could be compared and summarized. The relative concentration was calculated by dividing the absolute concentration of DNA determined by a PCR system for a particular amplicon size by the absolute concentration of DNA determined in the PCR system with the shortest amplicon used (105 bp for the *leptin* gene and 107 bp for the *SRY* gene).

SIZE DISTRIBUTION OF TOTAL PLASMA DNA IN PREGNANT AND NONPREGNANT WOMEN

Plasma DNA from 31 pregnant women and 34 nonpregnant women was analyzed. The median concentrations of plasma DNA obtained with primers producing the 105-bp product were 200 and 186 copies/mL for pregnant and nonpregnant women, respectively. There was no statistically significant difference between the plasma DNA concentrations for the two groups ($P = 0.851$, Mann–Whitney rank-sum test). The size distributions of plasma DNA of the two groups are shown in Fig. 1A. Similarly, there was no significant difference between the relative concentrations of the two groups with an amplicon length of 145 bp. In contrast, at amplicon sizes ≥201 bp, there were significant differences between the relative concentrations in pregnant and nonpregnant women.

SIZE DISTRIBUTION OF FETAL-DERIVED DNA IN MATERNAL PLASMA

Plasma DNA samples from 21 pregnant women who were carrying male fetuses were analyzed for the size distribution of DNA fragments encoding the *SRY* gene.

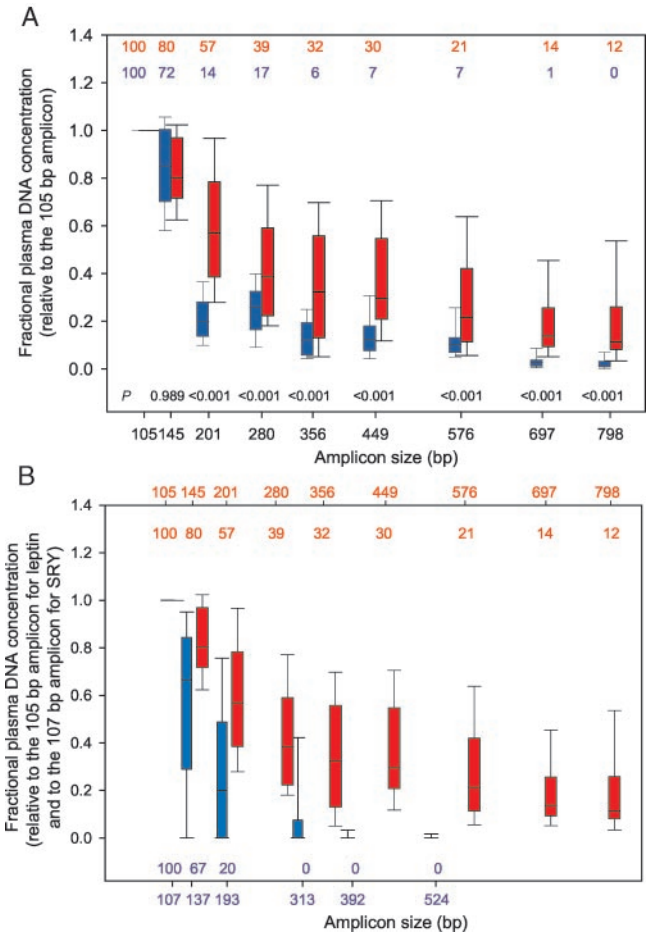

Fig. 1. Plots of fractional concentrations of plasma DNA in pregnant and nonpregnant women (*A*) and fetal-derived and maternal-derived DNA in maternal plasma (*B*).

(*A*), *red*, plasma DNA in pregnant women; *blue*, plasma DNA in pregnant women nonpregnant women. *Y axis*, fractional concentrations (with respect to absolute concentration using primers for the 105-bp amplicon); *x axis*, size of amplicon (bp). *Numbers above the plots* represent the median fractional concentrations of amplicon sizes 105, 145, 201, 280, 356, 449, 576, 697, and 798 bp for pregnant women (*top row*; *red*) and nonpregnant women (*bottom row*; *blue*). *Numbers below the plots* represent the *P* values of the comparison of fractional concentrations of plasma DNA in pregnant and nonpregnant women obtained with the Mann–Whitney rank-sum test for the respective sizes. (*B*), *blue*, fetal-derived DNA; *red*, maternal-derived DNA. *Y axis*, fractional concentrations (with respect to the absolute concentrations determined by the shortest amplicon used for each PCR system); *x axis above the plot* (*red*), size of maternal-derived DNA determined by measuring the *leptin* gene (bp); *x axis below the plot* (*blue*), size of fetal-derived DNA determined by measuring the *SRY* gene. *Numbers above the plot* represent the median concentrations (in percentages) of *leptin* gene according to the size listed above (*red*). *Numbers below the plot* represent the median concentrations (in percentages) of the *SRY* gene according to the size listed below (*blue*). The *top*, *middle*, and *bottom lines* in each *box* represent the 75th, 50th, and 25th percentiles of the fractional concentrations. The *error bars* represent the 95th and 5th percentiles.

The results are shown in Fig. 1B. The median relative concentration of the *SRY* gene determined with use of primers producing amplicons longer than 313 bp were <1%. In contrast, the median relative concentration of the *leptin* gene determined with primers producing an amplicon of 392 bp was 32%. As shown in Fig. 1B, fetal DNA molecules were obviously shorter than maternal DNA molecules.

SIZE DISTRIBUTION OF PLASMA DNA IN HEALTHY MALES

In men, the size distributions of plasma DNA was estimated by assays targeting the *leptin* and *SRY* genes (Fig. 2). The median fractional concentrations of plasma DNA estimated by assays targeting the *SRY* gene were 20%, 17%, and 11% for amplicon sizes of 313, 392, and 524 bp, respectively.

Discussion

Although the presence of extracellular DNA in plasma was first described more than 50 years ago *(13)*, the exact mechanism of its release is still unclear. Previous studies have demonstrated a correlation between plasma DNA concentrations and apoptosis of tumor cells in cancer patients *(14, 15)*, and several studies have also shown that the lengths of plasma DNA molecules in cancer patients are very short and are in multiples of nucleosomal DNA *(14, 16)*. However, the molecular features of plasma DNA in pregnant women, as well as in healthy individuals, have not been adequately explored. We believe that this information is of paramount importance to the understanding of the biological events governing the release of circulating DNA in pregnant women and, thus, the future development of markers for prenatal diagnosis and fetal monitoring.

In this study, we showed that the circulating DNA molecules in nonpregnant women are relatively short and that a median of 86% of these DNA molecules are <201 bp. Furthermore, we demonstrated a marked decrease in the measured DNA concentrations when the amplicon size was increased from 145 bp to 201 bp. This suggests that most of the circulating DNA molecules were in the range of 145–201 bp. This observation is consistent with the findings in cancer patients *(14, 15)* and suggests that apoptosis may be an important mechanism for the release of plasma DNA in healthy individuals. In contrast, we observed that the measured concentrations of plasma DNA in pregnant women decreased gradually when the amplicon size was increased and that no major gap was observed between 145 and 201 bp. Furthermore, we observed a significant difference in the size distribution of plasma DNA between pregnant and nonpregnant women when the amplicon size was increased beyond 200 bp. These findings imply that the circulating DNA molecules are longer in pregnant women than in nonpregnant women. In addition, as shown in Fig. 1B, the fetal-derived DNA molecules in the maternal circulation were much shorter than the maternal-derived DNA molecules, and a median of >99% of the fetal-derived DNA molecules was <313 bp in length. Because the apparent differences in the size distribution of maternal and fetal-derived DNA may have been the result of use of different gene targets, i.e., the *leptin* and *SRY* genes, we compared the size distributions of plasma DNA in 16 healthy male controls, targeting the *leptin* and *SRY* genes. We have shown that the size distributions estimated by PCRs for these two gene targets are similar. We therefore conclude that there is indeed a real difference between the sizes of maternal- and fetal-derived plasma.

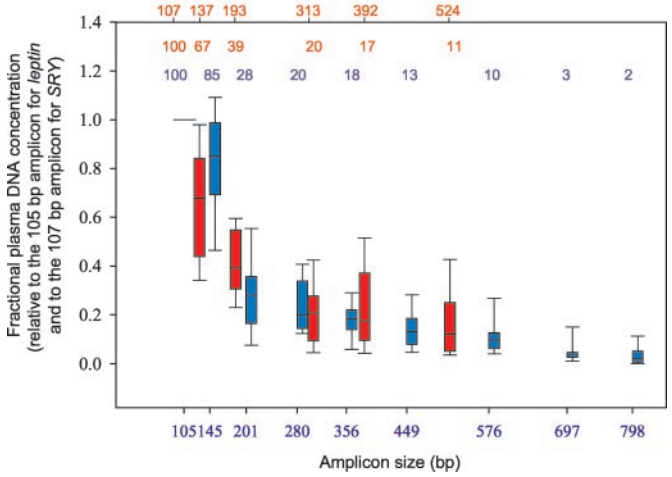

Fig. 2. Plot of fractional concentration of plasma DNA in 16 healthy male controls targeting the *leptin* (*blue*) and *SRY* genes (*red*).

Y axis, fractional concentrations (with respect to the absolute concentrations determined by the shortest amplicon used for each PCR system). *X axis above the plot* (*red*), size of plasma DNA determined by measuring the *SRY* gene (bp); *x axis below the plot* (*blue*), size of plasma DNA determined by measuring the *leptin* gene. *Numbers above the plot* represent the median fractional concentrations (in percentages) of plasma DNA determined by the *SRY* (*top row*; *red*) and the *leptin* genes (*bottom row*; *blue*).

We have previously shown that in clinically stable bone marrow transplantation recipients, most of the circulating DNA species come from the hematopoietic system *(17)*. Because plasma DNA concentrations in such clinically stable bone marrow transplantation recipients and healthy individuals who have not had bone marrow transplantation are similar *(17)*, it is reasonable to propose that the hematopoietic system might also be an important source of plasma DNA in healthy individuals. As demonstrated by the current study, most of these DNA molecules are 145–201 bp in size. This size range is consistent with the hypothesis that apoptosis plays an important role in the generation of circulating DNA.

The data generated from our study indicate that when a woman becomes pregnant, two alterations in the characteristics of plasma DNA are observed: (*a*) the size distribution of total plasma DNA, predominantly maternal in origin, shifts toward DNA fragments of longer lengths; and (*b*) fetal DNA, which has a shorter size distribution than maternal DNA, becomes detectable. Several theoretical possibilities can be postulated to explain the first point. For example, bearing in mind the important role played by the hematopoietic system in the release of plasma DNA *(17)*, it is possible that with hormonal or other physiologic changes during pregnancy *(18, 19)*, enhanced release of DNA of larger molecular sizes by hematopoietic cells may be possible. An alternative explanation is that a second, nonhematopoietic source of plasma DNA may be present during pregnancy. In this regard, one candidate nonhematopoietic source of

plasma DNA is the decidua, which represents the maternal side of the fetomaternal interface. A third possibility is that plasma DNA may be subjected to less severe degradation within the body of a pregnant woman, thus allowing circulating DNA to persist longer as a relatively longer molecule. This could be addressed by comparing the relative DNA clearance rates of pregnant and nonpregnant women in future studies.

The different size distribution of DNA in the plasma of pregnant women may open up a possible way to enrich for fetal DNA by size fractionation of DNA extracted from the plasma of pregnant women. Such enrichment could aid in the prenatal diagnosis of autosomal recessive disorders and chromosomal aneuploidies.

This new information on the molecular characteristics of the different DNA species present in maternal plasma may facilitate understanding of the release and elimination of plasma DNA and the future development of new plasma DNA-based markers.

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