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Cell-Free DNA in Maternal Plasma: Has the Size-Distribution Puzzle Been Solved?

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The detection of cell-free fetal DNA from maternal plasma and serum by Dennis Lo and colleagues at Oxford University, UK, opened an exciting route for the noninvasive determination of fetal genetic traits *(1)*. It has proved very effective for identifying fetal genetic loci completely absent from the maternal genome, such as those on the Y chromosome or the *RHD*[3] (Rh blood group, D antigen) gene *(2)*. Consequently, this approach is already being used in clinical settings for the determination of fetal sex in pregnancies at risk for X chromosome–linked disorders or fetal Rh D status in pregnancies at risk for hemolytic disease of the newborn. To date, several thousand analyses have been performed in several European centers. Commercial services are in the process of being launched in the US.

The detection of fetal genetic loci less disparate from the maternal genome, such as point mutations or those involved in fetal aneuploidy, is more complex because of the preponderance of maternal cell-free DNA sequences (>90% of total cell-free DNA) *(3)*. To overcome this problem, studies have undertaken to determine whether physical differences exist between maternal and fetal cell-free DNA fragments, specifically whether the fragments exhibit different size distributions.

In the first of these studies, Chan and colleagues *(4)* used real-time PCR amplicons of different sizes, from 107 bp to 524 bp for the *SRY* (sex determining region Y) locus on the Y chromosome and from 105 bp to 798 bp for the *LEP* (leptin) gene locus, which is common to both maternal and fetal genomes. By performing a series of real-time PCR analyses, these investigators were able to measure the relative concentrations of the various different-sized fetal and maternal cell-free DNA fragments. Their study indicated that most of the fetal cell-free DNA fragments were shorter than 193 bp (or, rather, not detected with a 193-bp assay), and few if any fragments were larger than 313 bp. On the other hand, 57% of the maternal cell-free DNA fragments were larger than 201 bp.

In the study by Li and colleagues, use was made of a more archaic method, in which cell-free DNA was fractionated by conventional agarose gel electrophoresis and the proportions of fetal and maternal fragments in a set of discrete fragments were then determined by real-time PCR analysis *(5)*. This study demonstrated that fetal cell-free DNA fragments were typically smaller than 300 bp, whereas maternal fragments were appreciably larger. By exploiting this difference, Li and colleagues showed that this approach could be used for the selective enrichment of fetal cell-free DNA fragments (to approximately 30%–50% of total DNA), thereby permitting the detection of otherwise masked fetal genetic loci, such as microsatellite sequences, point mutations, or single-nucleotide polymorphisms *(2)*.

In a new study, Fan and colleagues have now used cutting-edge "next-generation sequencing" technology to address this issue *(6)*. The aim of their study was to apply paired-end sequencing to all the cell-free DNA fragments present in maternal plasma and then use bioinformatics to determine the size distribution from the millions of reads obtained. Their data indicate that the majority of cell-free DNA fragments were approximately 162 bp in length and were rarely larger than 340 bp. The majority of cell-free fetal DNA fragments, as determined via the use of Y chromosome–specific sequencing, had lengths of approximately 130–150 bp but were rarely longer than 250 bp. In general, very few fragments larger than 1 kb were sequenced. On the basis of this assessment, the authors concluded that although size fractionation may assist in the enrichment of fetal cell-free DNA fragments, it may not be particularly useful for applications that rely on length measurement in situ, such as the use of "deep sequencing" for the noninvasive detection of fetal aneuploidy *(7, 8)*.

Although these new data largely agree with the size assessments made by Chan et al. *(4)* and Li et al. *(5)*, there are some discrepancies that may be worth further pursuit in depth. They include the inability of the presented next-generation sequencing approach to detect cell-free DNA fragments >1 kb, whereas fragments of >23 kb can readily be detected with "postgeneration"

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[3] Human genes: *RHD*, Rh blood group, D antigen; *SRY*, sex determining region Y; *LEP*, leptin.

tools, such as Southern blotting, findings that are readily evident in the work by Li and colleagues.

The question therefore arises whether the next-generation sequencing approach, which relies on the ligation of cell-free DNA fragments to specific linkers before amplification and sequencing, may display a bias against large or very small fragments. If this were the case, the actual sizes of fetal and maternal cell-free DNA fragments would remain unclear. Therefore, perhaps it may be necessary to use 2 or 3 technologies simultaneously to unequivocally address this complex issue.

In this context, an appreciation of the underlying biology that leads to the apparent difference in size between fetal and maternal cell-free DNA fragments could be useful. Accordingly, an understanding of syncytialization and trophoblast deportation may provide insight into the process whereby fetal cell-free fragments are generated *(9)*, and investigations of erythroblast maturation and enucleation may shed new light on the nature of cell-free DNA in general *(10)*. Such research may assist in unraveling the puzzle of cell-free DNA fragment size.

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