Cell-Free DNA in Maternal Plasma

Is It All a Question of Size?

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ABSTRACT: **Fetal cell-free DNA (cf-DNA) represents only a small fraction of the total cf-DNA in maternal plasma. This feature has rendered it difficult to reliably distinguish fetal alleles which are not very disparate from maternal ones, such as those involving point mutations, by conventional polymerase chain reaction (PCR)-based approaches. It has recently been shown that cell-free fetal DNA molecules have a smaller size than comparable cf-DNA molecules of maternal origin, and that this feature can be exploited for the selective enrichment of fetal DNA sequences, thereby permitting the detection of otherwise masked fetal genetic traits. By the use of this approach, we have shown that it is possible to detect fetal genetic loci for microsatellite markers, as well as point mutations involved in disorders such as achondroplasia and β-thalassemia.**

KEYWORDS: **circulatory DNA; prenatal diagnosis; electrophoresis; size fractionation**

INTRODUCTION

The discovery of fetal cell-free DNA (cf-DNA) in maternal plasma and serum in 1997 opened up a new avenue for the noninvasive analysis of fetal genetic traits, especially for those fetal loci completely absent from the maternal genome, such as Y-chromosome-specific sequences, or the fetal RHD gene in rhesus-D-negative pregnant women.[1–3] These analyses have proven to be so reliable that they are already implemented clinically in several European centers.[4,5]

The analysis of fetal genetic loci less disparate from maternal ones (e.g., point mutations) has been less successful, and has been limited to a small

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number of case reports.[6] This is largely due to the preponderance of cf-DNA sequences of maternal origin in the maternal circulation, rendering the detection of more subtle fetal differences difficult when using conventional or real-time polymerase chain reaction (PCR)-based approaches.

To date few studies have attempted to examine the biophysical properties of cell-free fetal DNA, but rather have focused on the immediate clinical applications. In order to address this issue, we decided to examine this aspect in greater detail, in the hope that this would lead to the discovery of the differences between fetal and maternal cf-DNA molecules, which could be used to distinguish between them. Previous examinations using quantitative real-time PCR had shown that cell-free fetal DNA was more abundant than that of circulating fetal cells by a factor of almost 1,000.[7] It was also demonstrated that it is unlikely that cell-free fetal DNA stems from the demise of fetal cells that have crossed the placental barrier, such as erythroblasts, in that elevations in cell-free fetal DNA were found to occur in the absence of any elevated transplacental fetal cell trafficking.[8] Furthermore, cell-free fetal DNA was shown to have a very short half-life (<15 min), disappearing very rapidly following delivery, conditions under which fetal cells can persist for months or even decades.[7]

These various findings have led to the suggestion that cell-free fetal DNA may be of placental origin.[9] This suspicion has been confirmed by three lines of separate evidence:

(i) The absence of cell-free fetal DNA sequences in cases of placental mosaicism.
(ii) The presence of cell-free fetal DNA in instances where part of the placenta was retained post partum.
(iii) The quantitative detection of cell-free fetal DNA sequences with placenta-specific methylation patterns.

On the other hand, the vast majority of normal cf-DNA in plasma has been proposed to be derived from the hemopoietic system, presumably from the red-cell lineage as erythroblasts enucleate to mature into erythrocytes.[3] Although this process usually involves a tight interaction with macrophages which engulf and degrade the erythroblast nuclei, enucleation can occur in the absence of such interactions. The release of cf-DNA by the placenta is likely to involve apoptotic shedding of syncytiotrophoblast debris as this monolayer is constantly replenished.[9] Hence, because fundamental differences may exist between cf-DNA released into the circulation by these two different mechanisms, it is possible that physical differences may exist between fetal and maternal cf-DNA species that may aid in distinguishing between them.

Fetal cf-DNA is Smaller than Maternal cf-DNA

In our approach to look for biophysical differences between fetal and maternal cf-DNA, we were curious to determine whether these possessed any

apoptotic characteristics.[10] For this reason we separated cf-DNA isolated from maternal plasma by standard gel electrophoresis and examined it by Southern blotting using high-copy Alu and DNA Y-chromosome segment (DYS) 14 sequences. Although the Alu sequence probe was able to pick up characteristic apoptotic fragments, differing in size by approximately 180 bp (base pairs), we were not able to detect any male fetal DNA with the DYS 14 probe, indicating that the level of cell-free fetal DNA was too low to be detectable by such rather archaic approaches. Of interest is that the Alu probe indicated that cf-DNA sequences were present having a size in excess of 24 kb. Because our recent analysis of nuclear DNA in terminal differentiating erythroblasts indicates that this is cleaved into megabase fragments (>40 Mb), it is possible that the large molecular weight cf-DNA we have detected is derived from enucleating erythroblasts (Hristoskova *et al*., unpublished observations).

Because our approach using Southern blotting was not sensitive enough to detect cell-free fetal DNA, we next looked for the presence of such DNA molecules using real-time PCR. For this analysis, total cf-DNA from maternal plasma was again subjected to gel electrophoresis, following which each lane of the gel-containing DNA was carefully sliced into discrete fragments, having sizes of less than 0.3 kb, 0.3–0.5 kb, 0.5–1.0 kb, 1.0–1.5 kb, 1.5–23 kb, and larger than 23 kb. The cf-DNA in each agarose fragment was eluted and the quantity of total cf-DNA (indicative of that of maternal origin) was quantified by an assay for the GAPDH gene, whereas the amount of fetal cf-DNA was determined using an assay specific for the SRY locus on the Y-chromosome. This examination indicated that the vast proportion of fetal cf-DNA (approximately 70%) had a molecular size of less than 300 bp, whereas most of the maternal cf-DNA (approximately 75%) was greater than 300 bp.

Of interest is that our data obtained using gel electrophoresis is remarkably similar to those obtained independently by Dennis Lo and his coworkers, who used PCR amplicons of different sizes for their analyses.[11] These new facets concerning the biophysical properties of fetal and maternal cf-DNA implied that fetal cf-DNA molecules in maternal plasma can be enriched for on the basis of a smaller size than comparable maternal cf-DNA molecules.

Detection of Highly Polymorphic Microsatellite Sequences

To test this hypothesis we chose to examine a series of highly polymorphic microsatellite loci, also termed STRs (short tandem repeats).[10] The reason for choosing these markers is that they can be used to readily distinguish between fetus and mother in a gender-independent manner. Once we had genotyped both maternal and fetal DNA samples for informative markers which could aid in distinguishing fetal and maternal cf-DNA, we examined a series of maternal plasma samples taken early and late in pregnancy. The analysis of total cf-DNA in maternal plasma indicated that only the maternal STR alleles could

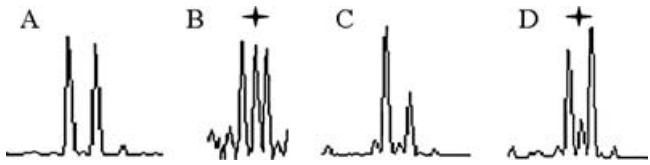

FIGURE 1. Size fraction of cf-DNA in maternal plasma permits the detection of a paternally inherited STR allele (indicated by a *star*) in a fetus with trisomy 21. (**A**) Maternal genomic DNA. (**B**) Fetal genomic DNA, with presence of an additional allele (*star*). (**C**) Total circulatory DNA: inability to detect additional fetal allele. (**D**) Size-fractionated circulatory DNA: ready detection of additional fetal allele (*star*).

be reliably detected, whereas in most cases the paternally inherited fetal STR allele is totally absent (FIG. 1). This is not unexpected, as the same sequences are used to PCR amplify both the maternal and fetal STR alleles. As there is a greater abundance of the maternal STR alleles, the fetal ones are effectively competed out of the PCR reaction. However, when the cf-DNA fraction, which had a size of less than 300 bp was taken, the paternally inherited fetal STR allele was readily detected in all instances (FIG. 1). Indeed, in many cases it appeared as if the maternally inherited fetal STR allele was present in greater abundance than the noninherited maternal allele (FIG. 1).

Therefore, this experiment conducted on a total of 7 samples using a number of different STR markers on chromosome 21 indicated that the size fractionation of cf-DNA from maternal plasma did permit the ready detection of fetal genetic loci which were not apparent in the analysis of the total cf-DNA sample.[10]

Detection of Fetal Point Mutations: Achrondroplasia and β-Thalassemia

Confident that our approach may permit the detection of even more subtle differences between the maternal and fetal genome, we next set out to determine whether we could detect paternally inherited fetal point mutations. In the first instance we examined a sample obtained from a pregnancy at risk for achondroplasia. In this case the father had a dominant mutation in the FGFR3 (fibroblast growth factor receptor 3) gene, which can be detected by Scf1 restriction analysis of the PCR amplification product. Our analysis of total cf-DNA indicated that the paternal mutant allele could be faintly detected.[12] The presence of this mutant allele could, however, be much more robustly demonstrated in the cf-DNA fraction having a size of less than 300 bp.[12]

For our next examination we focused our attention on the hemoglobinopathies, probably the largest kind of hereditary genetic disorder in man, with several hundred thousand lethally affected pregnancies occurring yearly.[13]

We have a long-standing interest in the prenatal diagnosis of β-thalassemia, having previously collaborated with investigators at the University in Bari, Italy, on this topic.[14] Because a large number of mutations are involved in this disorder, pregnancies which are not at risk of being affected can be excluded from further invasive prenatal diagnostic analysis if the paternal mutant allele is not inherited by the fetus. For this reason we chose a study cohort of pregnancies at risk for β-thalassemia where the parents do not share the same β-globin mutation.[15]

Although we had only analyzed a single case of achondroplasia, this served to illustrate the weakness of conventional PCR-based assays for the accurate detection of fetal point mutations, even when using size-fractionated cf-DNA. Furthermore, as the hemoglobinopathies are a major health concern in many developing countries, we were interested in a detection system that was not overly expensive, and yet was amenable to high throughput analysis. For this reason we chose to use an allele-specific real-time PCR-based approach, which has been shown to be reliable and robust for the analysis of SNPs on pure genomic DNA. Because we were, however, concerned that the quantity of fetal cf-DNA may still be less than that of maternal origin in our size-fractionated cf-DNA sample, we decided to introduce a further precautionary step to enrich for the mutant fetal allele. This was accomplished by first performing a round of PCR using a PNA (peptide nucleic acid) probe specific for the wild-type maternal allele. As this PNA probe has very high affinity for the wild-type maternal allele, its addition to the PCR reaction would effectively inhibit the efficient amplification of this allele, and permit the amplification of the mutant fetal allele. To detect the presence of the mutant fetal allele we used an allele-specific real-time PCR assay with Cybergreen for the detection. In order to ensure that we were truly detecting the mutant fetal allele and not some erroneous amplification of the wild-type allele, we employed a ΔC_T system, whereby the extent of amplification of the wild-type allele was subtracted from that of the mutant allele. In experimental conditions, wherein the mutant allele was diluted into a wild-type background, this system was shown to permit a clear discrimination between the two alleles, and furthermore yield clear cut-off values that could be used for the assessment of their presence. In our analysis of 32 clinical samples taken early in gestation immediately prior to chorionic villus sampling (median gestational age 10.7 weeks), we looked for the presence of the four most common β-globin point mutations in the population group in Bari, southern Italy: IVSI-1, IVSI-6, IVSI-110, and codon 39.[15] One of the samples in our analysis had to be excluded because we were not able to ascertain the fetal genotype from the invasive prenatal diagnosis. A further two samples had to be excluded since the amount of cf-DNA in these samples was very low. It is possible that this facet may have contributed to the one false analysis we recorded. In all other 29 instances, the absence or presence of the paternal mutant allele was correctly determined, yielding a sensitivity of 100% with a concomitant specificity of almost 94%.[15]

Current Drawbacks and Future Directions

The major current bottleneck and technical deficit of the approach we have established is the actual size fractionation of the cf-DNA, as this currently relies on conventional gel electrophoresis, the cutting of a gel slice with the desired molecular weight DNA, and the elution of the cf-DNA from this agarose fragment. These steps are tedious, time-consuming, and prone to contamination. As such, it should be noted that the approach we have developed is currently more an issue of testing a "proof-of-principle," than being a straightforward application that is so secure that it is ready for the clinic.

It is patently clear that an effective method is needed for the efficacious and robust size fractionation of cf-DNA, which is also cost-effective and amenable to high throughput. Unfortunately such a system does not exist at the moment. Currently, we are exploring the use of centrifugal filtration devices (Amicon Milian SA, Geneva, Switzerland), capillary electrophoresis, and microfluidics systems. The latter is being conducted as part of the exploration or "new technologies" in the EU-funded Special Non-Invasive Advances in Fetal and Neonatal Evaluation (SAFE) project.

A further issue is that current analytical approaches do not permit a reliable quantitation of the amount of mutant fetal allele present. Nor is it possible to determine whether the fetus has inherited the mutant allele from the mother as well. Consequently, the assays as they currently stand can only be used for the exclusion of pregnancies at risk, and not for an actual prenatal diagnosis.

CONCLUSIONS

Two independent analyses showed that fetal cf-DNA molecules in maternal plasma are smaller than comparable maternal cf-DNA molecules. This finding suggested that fetal cf-DNA molecules could be enriched on account of a smaller size than maternal ones. In a series of proof-of-principle experiments we showed that this hypothesis was indeed true, and that this approach could be used to detect a number of fetal genetic loci, including STRs and point mutations, which cannot be reliably interrogated on total cf-DNA in maternal plasma.

Despite this exciting development, several hurdles still need to be cleared before this system can be applied to clinical settings, including mechanisms permitting the efficacious size fractionation of cf-DNA and the robust quantitative analysis of the fetal alleles present in this sample. This latter step is necessary if we are to ascertain whether the fetus has inherited both the paternal and maternal mutant alleles, thereby changing the analysis from one of simply excluding pregnancies at risk on the basis of presence or absence of the mutant paternal allele, to one of an actual prenatal diagnosis. This development will also assist in determining whether cf-DNA can be used for the detection of fetal aneuploidies, the long sought-after holy grail of noninvasive prenatal diagnosis.

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