Early report

Presence of fetal DNA in maternal plasma and serum

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Summary

Background The potential use of plasma and serum for molecular diagnosis has generated interest. Tumour DNA has been found in the plasma and serum of cancer patients, and molecular analysis has been done on this material. We investigated the equivalent condition in pregnancy—that is, whether fetal DNA is present in maternal plasma and serum.

Methods We used a rapid-boiling method to extract DNA from plasma and serum. DNA from plasma, serum, and nucleated blood cells from 43 pregnant women underwent a sensitive Y-PCR assay to detect circulating male fetal DNA from women bearing male fetuses.

Findings Fetus-derived Y sequences were detected in 24 (80%) of the 30 maternal plasma samples, and in 21 (70%) of the 30 maternal serum samples, from women bearing male fetuses. These results were obtained with only 10 μL of the samples. When DNA from nucleated blood cells extracted from a similar volume of blood was used, only five (17%) of the 30 samples gave a positive Y signal. None of the 13 women bearing female fetuses, and none of the ten non-pregnant control women, had positive results for plasma, serum or nucleated blood cells.

Interpretation Our finding of circulating fetal DNA in maternal plasma may have implications for non-invasive prenatal diagnosis, and for improving our understanding of the fetomaternal relationship.

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Introduction

The passage of nucleated cells between mother and fetus is well recognised.[1,2] One important clinical application is the use of fetal cells in maternal blood for non-invasive prenatal diagnosis.[3] This approach avoids the risks associated with conventional invasive techniques, such as amniocentesis and chorionic-villus sampling. Substantial advances have been made in the enrichment and isolation of fetal cells for analysis.[3,4] However, most techniques are time-consuming or require expensive equipment.

There has been much interest in the use of DNA derived from plasma or serum for molecular diagnosis.[5] In particular, there have been reports that tumour DNA can be detected by molecular techniques in the plasma or serum of cancer patients.[6–8] Such reports prompted us to investigate whether fetal DNA can be detected in maternal plasma and serum.

Methods

Patients

Pregnant women attending the John Radcliffe Hospital (Oxford, UK) were recruited before amniocentesis or delivery. We obtained approval of the project from the Central Oxfordshire Research Ethics Committee. Informed consent was obtained in each case. 5–10 mL maternal peripheral blood was collected into one tube containing edetic acid and one plain tube. For women undergoing amniocentesis, maternal blood was always taken before the procedure, and 10 mL amniotic fluid was also collected for fetal sex determination. For women recruited just before delivery, fetal sex was noted at the time of delivery. Control blood samples were also taken from ten non-pregnant women, and the samples were processed in the same way as those obtained from the pregnant women.

Sample preparation

Maternal blood samples were processed 1–3 h after venesection. Blood samples were centrifuged at 3000 *g*, and plasma and serum were carefully removed from the edetic-acid-containing and plain tubes, respectively, and transferred into plain polypropylene tubes. Great care was taken to ensure that the buffy coat or the blood clot was undisturbed when plasma or serum samples, respectively, were removed. After removal of the plasma samples, the red-cell pellet and buffy coat were saved for DNA extraction with a Nucleon DNA extraction kit (Scotlabs, Strathclyde, Scotland, UK). The plasma and serum samples then underwent a second centrifugation at 3000 *g*, and the recentrifuged plasma and serum samples were collected into fresh polypropylene tubes. The samples were stored at −20°C until further processing.

DNA extraction from plasma and serum samples

Plasma and serum samples were processed for PCR by a modified version of Emanuel and Peska's method.[9] 200 μL plasma or serum in a 0·5 mL eppendorf tube was heated at 99°C for 5 min on a heat block. The heated sample was then centrifuged at maximum speed in a microcentrifuge, after which the clear supernatant was collected and 10 μL used for PCR.

DNA extraction from amniotic fluid

The amniotic-fluid samples were processed for PCR by the method of Rebello and colleagues.[10] 100 μL amniotic fluid was transferred into a 0·5 mL eppendorf tube, and mixed with an equal volume of 10% Chelex-100 (Bio-Rad). After addition of 20 μL mineral oil to prevent evaporation, the tube was incubated at 56°C for 30 min on a heat block. The tube was then vortexed briefly and incubated at 99°C for 20 min. The treated amniotic fluid was stored at 4°C until PCR, and 10 μL was used in a 100 μL reaction.

PCR

The PCR was carried out broadly as described elsewhere[11] with reagents from a GeneAmp DNA Amplification Kit (Perkin Elmer, Foster City, CA, USA). The detection of Y-specific fetal sequence from maternal plasma, serum, and cellular DNA was done as described with primers Y1·7 and Y1·8, designed to amplify a single-copy sequence (DYS14).[12] The Y-specific product was 198 bp. 60 cycles of Hot Start PCR with Ampliwax technology were used on 10 μL maternal plasma or serum, or on 100 ng maternal nucleated blood-cell DNA; each cycle consists of a denaturation step at 94°C for 1 min, and a combined reannealing/extension step at 57°C for 1 min. 40 cycles were used for amplification of amniotic fluid. PCR products were analysed by agarose-gel electrophoresis and ethidium-bromide staining. PCR results were scored before fetal sex was revealed to the investigator.

Results

Serial dilutions of male genomic DNA in 1 μg female genomic DNA were carried out and amplified by the Y-PCR system with 60 cycles of amplification. Positive signals were detected up to the 1/100 000 dilution—ie, the approximate equivalent of a single male cell.

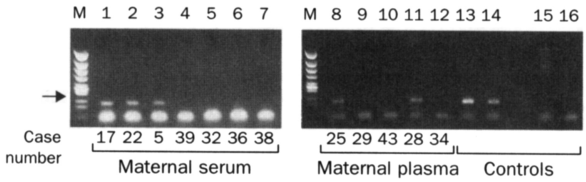
The optimum volume of heated plasma and serum samples for PCR amplification was assessed by subjecting 1 μL, 2 μL, 10 μL, 30 μL, and 50 μL heated plasma or serum samples from male individuals to Y-PCR. The best signal was obtained from 10 μL plasma or serum. Complete inhibition of amplification was reached with 50 μL. Thus, 10 μL heated plasma or serum samples were used for subsequent experiments.

Maternal plasma and serum samples were collected from 43 women who were between 12 and 40 weeks pregnant. There were 30 male and 13 female fetuses. Among the 30 women bearing male fetuses, Y-positive signals were detected in 24 plasma samples and 21 serum samples when 10 μL of the samples was used for PCR (figure and table). When DNA from nucleated blood-cells was used for Y-PCR, positive signals were detected in only five of the 30 cases (table). None of the 13 women bearing female fetuses, and none of the ten non-pregnant control women, had a positive Y signal when plasma, serum, or cellular DNA was amplified.

Discussion

Our results show that fetal DNA is present in maternal plasma and serum. Use of maternal plasma or serum for the detection of fetal DNA for non-invasive prenatal diagnosis may therefore be possible. Ironically, plasma is the material routinely discarded in the early stages of many DNA-extraction protocols, and also after the density-centifugaton step used by many investigators for non-invasive prenatal diagnosis. This is probably one of the reasons why the presence of fetal DNA in maternal plasma has not been explored previously.

Maternal serum is being used by many centres for biochemical screening of chromosomal aneuploidies and neural-tube defects. An approach by which DNA-based diagnosis is done on serum samples could be incorporated into existing screening programmes.



Amplification of fetal Y-chromosomal sequences from maternal plasma and serum

Lanes 13 and 14=Y-PCR on male genomic DNA (positive controls); lane 13 DNA equivalent to ten male cells; lane 14 DNA equivalent of one male cell. Land 15, 1 μg female genomic DNA (negative control). Lane 16, water (negative control). Arrow marks position of 198 bp Y-PCR product. M=molecular weight marker (ϕX174 DNA digested with *Hinc*II).

Our data show that fetal DNA can be detected in as little as 10 μL maternal plasma and serum. The detection rate is much higher than that for DNA from nucleated blood cells extracted from a similar volume of whole blood. This finding suggests a relative enrichment of fetal DNA in maternal plasma and serum, a phenomenon analogous to the relative enrichment of tumour DNA in the plasma and serum of cancer patients.[7,8] The low detection rate of fetal DNA sequences for DNA from nucleated blood cells

Case number	Gestation (weeks)	Y-PCR		
		Plasma	Serum	Blood
Male fetuses				
1	12	−	−	−
2	14	+	−	−
3	15	+	+	+
4	15	+	+	−
5	15	+	+	−
6	15	+	+	−
7	15	+	−	−
8	15	+	−	−
9	15	+	−	−
10	15	+	−	−
11	15	−	−	−
12	15	−	−	−
13	16	+	+	+
14	16	+	+	−
15	16	+	+	−
16	16	+	+	−
17	16	+	+	−
18	16	+	+	−
19	16	+	+	−
20	17	+	+	−
21	17	+	+	−
22	22	−	+	−
23	40	+	+	+
24	40	+	+	+
25	40	+	+	+
26	40	+	+	−
27	40	+	+	−
28	40	+	+	−
29	40	−	+	−
30	40	−	−	−
Female fetuses				
31	15	−	−	−
32	16	−	−	−
33	16	−	−	−
34	16	−	−	−
35	16	−	−	−
36	16	−	−	−
37	17	−	−	−
38	17	−	−	−
39	17	−	−	−
40	18	−	−	−
41	40	−	−	−
42	40	−	−	−
43	40	−	−	−

Amplification of fetal Y-chromosomal sequences from maternal plasma, serum, and blood

resulted from the use of only 100 ng DNA, compared with 1 μg in our previous study.[12] We chose 100 ng because this was the average quantity of DNA extracted from 10 μL whole blood with our genomic DNA-extraction method. This approach allows us to compare the relative detectability of fetal DNA in 10 μL plasma or serum and the cellular component of 10 μL whole blood.

The detection rate of fetal DNA in 10 μL plasma and serum is already high at 80% and 70%, respectively, but these rates can probably be improved. 1 mL maternal blood or serum, for example, will result in a one hundred-fold increase in the absolute amount of fetal genetic material available for analysis. Such a magnitude of increase should lead to a robust and non-invasive system for detection of paternally inherited fetal DNA sequences. However, more work is needed before this can be achieved, since the boiling method we used resulted in a relatively impure DNA extract, which inhibited PCR when volumes much larger than 10 μL were used. We are investigating other DNA-extraction methods for plasma and serum. We believe that concentration methods for plasma and serum DNA could potentially be easier than many fetal-cell isolation methods, such as cell sorting and micromanipulation.

The relative merit of the use of plasma or serum samples requires further investigation. In seven cases—2, 7, 8, 9, 10, 22, and 29—there was discordance in the PCR results obtained from the plasma and serum. A possible explanation for the discrepancies is that the quantity of fetal DNA in these cases may have a limiting effect, and thus sampling errors could be contributing to the observed results. Future research with quantitative PCR assays may elucidate this effect.

In four cases with male fetuses, both plasma and serum were negative for fetal DNA (cases 1, 11, 12, and 30). Three of these women were tested at 15 weeks of pregnancy or earlier (cases 1, 11, and 12). Furthermore, of the seven cases in which there was discordance between plasma and serum samples, all but one were tested before 23 weeks. Taken together, these results suggest that the concentration of fetal DNA increases as gestation progresses, possibly owing to the increase in fetal size. This result is analogous to that of Nawroz and colleagues,[8] who detected in head and neck cancer patients mutant plasma DNA predominantly in those with high tumour load. Future studies should investigate the temporal relation between gestation and the appearance and concentration of fetal DNA in maternal plasma.

As well as sex-linked disorders, techniques for fetal-DNA detection in maternal plasma or serum can also be used to detect many paternally inherited DNA sequences that differ from their maternal counterparts. Clinical examples include fetal rhesus D status assessment[13] and detection of certain haemoglobinopathies.[14] The plasma or serum-based approach might also be applicable to screening for chromosomal aneuploidies (such as Down's syndrome) if there is a quantitative difference in the concentration of fetal DNA in maternal plasma and serum between affected and normal pregnancies; this is a situation analogous to the high concentration of fetal cells detectable in pregnancies that involve aneuploid fetuses.[15] Fetal-cell isolation, however, will nevertheless be necessary for the definitive cytogenetic diagnosis of fetal chromosomal aneuploidies, and for the direct mutational analysis of autosomal recessive disorders caused by a single mutation.

The presence of fetal DNA in maternal plasma and serum has not been previously described. The underlying processes that cause free fetal DNA to be released into the maternal circulation have yet to be explained. Possible mechanisms include cell lysis resulting from physical and immunological damage, and developmentally regulated apoptosis of certain fetal tissues. There are interesting similarities between a growing fetus and a neoplasm: both are immunologically foreign to—and have an extensive vascular interface with—their hosts. Investigation of the variations in fetal-DNA concentrations in conditions in which the placental interface is damaged (eg, pre-eclampsia) would be of clinical and biological interest.

Contributors

Y M Dennis Lo and James S Wainscoat initiated the project. Y M Dennis Lo reviewed literature, supervised the daily practical work and analysis of laboratory results. Noemi Corbetta designed the experimental methods and was the main laboratory worker. Paul F Chamberlain, Vik Rai, Ian L Sargent and Christopher W G Redman designed the clinical module of the project. Paul F Chamberlain and Vik Rai were responsible for the clinical correlation of laboratory results. Ian L Sargent, Christopher W G Redman and James S Wainscoat analysed laboratory results. James S Wainscoat designed the DNA-extraction methods, and supervised the writing of the manuscript. All the authors read and contributed to the writing of the paper.

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