Arch Gynecol Obstet (2006) 273: 221–226
DOI 10.1007/s00404-005-0068-0

ORIGINAL ARTICLE

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Cell-free foetal DNA in maternal plasma does not appear to be derived from the rich pool of cell-free foetal DNA in amniotic fluid

Received: 20 July 2005 / Accepted: 27 July 2005 / Published online: 28 September 2005

Abstract *Background*: Large quantities of cell-free foetal DNA have been detected in amniotic fluid, and it has been proposed that this material may contribute to the pool of cell-free foetal DNA in maternal plasma. *Methods*: Twelve maternal blood samples were obtained from pregnant women about to undergo an amniocentesis. Cell-free DNA was extracted from the maternal plasma samples and the matched amniotic fluid samples. The amount of cell-free foetal DNA was quantified by real-time PCR assays for the SRY and RHD genes. *Results*: Amniotic fluid was found to contain vast quantities of cell-free DNA (median concentration = 3,978 copies/ml amniotic fluid). The concentration of cell-free foetal DNA in maternal plasma was much lower (median concentration = 96.6 copies/ml maternal plasma). No significant correlation could, however, be determined between these two pools of cell-free foetal DNA. *Conclusions*: Our data confirm that amniotic fluid contains prodigious quantities of cell-free foetal DNA, but as no relationship exists between this material and that in the maternal circulation, it is unlikely that the amnion contributes to the presence of cell-free foetal DNA in maternal plasma.

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Introduction

The analysis of cell-free foetal DNA in maternal plasma or serum is rapidly gaining in importance as a new method for the prenatal diagnosis of foetal genetic loci, particularly for the determination of the foetal Rhesus status and sex, in pregnancies at risk for an X-linked disorder [1, 2]. Consequently, commercial services are already being offered in Europe for the latter two applications [3, 4]. Furthermore, quantitative analysis of cell-free foetal DNA concentrations in maternal plasma have indicated that these are elevated in pregnancies at risk or affected by pre-eclampsia [5, 6], those with preterm labour [7], as well as those with aneuploid foetuses, particularly those with trisomy 21 [8, 9]. These findings have suggested that cell-free foetal DNA may serve as a potential marker to screen for such pregnancies [2, 10].

A current dilemma, especially with regard to the latter application, is that it is currently not clear what the origin of cell-free foetal DNA in the maternal circulation is [11]. This knowledge is of considerable importance, especially when dealing with disorders having an underlying placental aetiology such as pre-eclampsia, in that it would be best if the new marker studied would somehow reflect upon the causal pathology, rather than just be a secondary consequence of it, as the latter would reduce the specificity of the new test.

Although the most likely source of the pool of cell-free foetal DNA in maternal plasma is the placenta, this remains to be conclusively proven. Data supporting the placental origin of this foetal material include the rapid disappearance of cell-free foetal DNA from the maternal circulation following delivery and removal of the placenta [12], as well as the association of cell-free foetal DNA molecules with placentally derived microparticles [13]. Furthermore, cases of placental mosaicism have been shown to be associated with the lack of particular foetal genetic loci in the cell-free fraction of foetal DNA in maternal plasma [14].

On the other hand, it has been suggested that this cell-free foetal material could be derived from the apoptotic demise of foetal cells which have entered the maternal circulation [15]. This scenario does not appear to be very likely, as the quantity of cell-free foetal DNA in maternal plasma is at least 100-fold higher than the number of foetal cells present in the maternal circulation [16]. Furthermore, even short-lived foetal cells such as erythroblasts are present in the maternal circulation for several weeks post partum, during which period no traces of cell-free foetal DNA exist anymore. In addition, we have previously shown that elevations in cell-free foetal DNA can occur under conditions, such as preterm labour, which are not associated with an elevated trafficking of foetal cells [17]. This latter finding strongly suggests that the two entities, circulating foetal cells and cell-free foetal DNA, are not casually related [18].

Recently, however, a very intriguing other possibility has been suggested, namely amniotic fluid, which was found to contain copious amounts of cell-free foetal DNA [19]. In order to test this pertinent hypothesis, we studied cell-free foetal DNA levels in 12 individually matched amniotic fluid and maternal blood samples.

Materials and methods

Sample recruitment and processing

Approval for the study was granted by our institutional review board and written informed consent was obtained in all instances. All maternal blood samples (15 ml EDTA) were obtained immediately prior to amniocentesis. The median gestational age was 15.1 weeks. Amniotic fluid samples were collected from the washing steps conducted prior to the amniotic cells being placed into culture at the Department of Medical Genetics, University Clinics, Basel, Switzerland.

Both the plasma samples and amniotic fluid were cleared by high-speed centrifugation in a microcentrifuge (12,000 *g* for 10′) and the supernatant was carefully removed, to ensure that no cellular debris was carried over. DNA was prepared from these samples using commercial column technology (Qiagen, Basel, Switzerland) according to a protocol well established in our laboratory [9]. For the analysis of maternal plasma, 800 µl was extracted and eluted into 50 µl elution buffer, 5 µl was used per PCR reaction. For the analysis of amniotic fluid, 400 µl was extracted and eluted into 50 µl elution buffer, 5 µl was used per PCR reaction.

Real-time PCR analysis

The concentration of cell-free foetal DNA in these amniotic fluid or plasma samples using real-time PCR assays specific for the SRY gene on the Y chromosome, as well as the foetal Rhesus D gene in Rhesus D negative pregnant women, using protocols well established in our laboratory [20]. The dual labeled-probe and primer sequences were as follows:

FAM is 6-carboxyfluorescein, TAMRA is 6-carboxytetramethylrhodamine and VIC is a registered trademark of Applied Biosystems.

SRY:	
Forward:	5′ TCC TCA AAA GAA ACC GTG CAT 3′
Reverse:	5′ AGA TTA ATG GTT GCT AAG GAC TGG AT 3′
Probe:	5′ (FAM) CAC CAG CAG TAA CTC CCC ACA ACC TCT TT (TAMRA) 3′
RhD:	
Forward:	5′ CCT CTC ACT GTT GCC TGC ATT 3′
Reverse:	5′ AGT GCC TGC GCG AAC ATT 3′
Probe:	5′ (VIC) TAC GTG AGA AAC GCT CAT GAC AGC AAA GTC T (TAMRA) 3′

For the TaqMan PCR analysis, we used 25 µL reaction volumes containing 5 µL of the extracted DNA, 300 nM of each amplification primer and 100 nM of the dual-labelled TaqMan probe. The reaction mixture containing the necessary components provided in the TaqMan PCR Core reaction Kit (Perkin Elmer, Branchburg, NJ), contained 2.5 µL of 10× Buffer A, 3.5 mM $MgCl_2$, 100 µM dNTPS, 0.025 U/µL AmpliTaq Gold and 0.01 U/µL Amp Erase. The amplification runs were carried out using a 2 min incubation at 50°C, to permit Amp Erase activity, followed by an initial denaturation step at 95°C for 10 min, which facilitates activation of the AmpliTaq Gold polymerase activity, followed by 40 cycles of 1 min at 60°C and 15 s at 95°C.

Statistical analysis

To determine whether any significant correlation between the levels of cell-free foetal DNA in maternal plasma and the amount of cell-free foetal DNA in the corresponding sample of amniotic fluid this was tested using Spearmańs coefficient of rank correlation. All calculations were performed using the statistical package for social sciences (SPSS for Windows 10.0).

Table 1 Levels of cell-free cell-free foetal DNA levels in amniotic fluid and matched maternal plasma samples

ID	Concentration of cell-free foetal DNA in amniotic fluid		Concentration of cell-free foetal DNA in maternal plasma	
	SRY	RhD	SRY	RhD
1	6878	0[a]	124.5	0[a]
2	26018	23706	149	130
3	0	0	0	0
4	0	43429	0	178
5	0	44600	0	112
6	1078	1636.5	286	246
7	347	229	31	39
8	821942	811078	259	254
9	0	425404	0	161
10	255	0	69	0
11	0	829	0	600
12	7302	8176.5	13	0

Concentration of foetal DNA is given in copies/ml maternal plasma

[a]No clinical outcome for the foetal RhD status could be obtained for this sample

Results

Our study indicated that Y specific sequences could be detected in both the amniotic fluid and plasma samples in 7 of the cases examined. This correlated very well with the foetal sex, as only 7 of the 12 cases were male. No false positive results were recorded in those 5 cases with female foetuses. Our analysis confirmed that very high levels of cell-free foetal DNA were indeed present in the amniotic fluid (Table 1). In one instance, we noted an exceptionally high concentration of cell-free foetal DNA of 821,994 copies/ml amniotic fluid. The exclusion or inclusion of this apparent outlier did not significantly alter any of the subsequent statistical analysis. The analysis of the remaining samples indicated that the median concentration of cell-free foetal DNA in the amniotic fluid was 3,978 copies/ml amniotic fluid (range = 255–26,019 copies/ml). In the corresponding maternal plasma samples, we determined a median cell-free foetal DNA concentration of 96.9 copies/ml maternal plasma (range = 13–286 copies/ml maternal plasma).

As these samples had been obtained from Rhd pregnant women, we also examined the levels of cell-free foetal DNA using a real-time assay for the RhD gene. This analysis indicated that 9 of the 12 foetuses had an RhD genotype, which was in good agreement with the analysis from the clinical laboratory. In one case, which was scored by our examination of both maternal plasma and amniotic fluid as an Rhd negative foetus, no clinical outcome regarding the foetal Rhesus genotype was available (Table 1). In our analysis of the other cases, the median concentration of cell-free foetal DNA in the amniotic fluid was 23,707 copies/ml amniotic fluid (range = 229–811078 copies/ml). In the corresponding maternal plasma samples, we determined a median cell-free foetal DNA concentration of 161 copies/ml maternal plasma (range = 39–600 copies/ml maternal plasma).

Our analysis indicated a very good degree of correlation between the levels of SRY-positive and RhD-positive cell-free foetal DNA in maternal plasma ($r = 0.2$; $P = 0.67$; $n = 5$; Fig. 1a), as well as in amniotic fluid ($r = 0.2$; $P = 0.67$; $n = 5$; Fig. 1b).

We next examined whether a correlation existed between the amount of cell-free foetal DNA in the amniotic fluid to the matched plasma samples by Spearman rank analysis, which indicated that no significant correlation was present for SRY-positive cell-free foetal DNA ($r = 0.2$; $P = 0.67$; $n = 7$) or RhD-positive cell-free foetal DNA ($r = 0.02$; $P = 0.95$; $n = 8$) (Fig. 2a, b).This feature was true even when a combined analysis of both foetal loci was performed ($r = 0.2$; $P = 0.67$; $n = 15$).

Discussion

Our data confirm that amniotic fluid contains copious amounts of cell-free foetal DNA [19], and that this is true for multiple foetal loci, such as the SRY and RhD genes. In our analysis we have examined whether this rich pool of cell-free foetal DNA contributes to that in the maternal circulation, using these two foetal loci. Our data, obtained for 7 cases analysed by a Taqman real-time PCR assay for the SRY gene and 8 cases examined by a Taqman real-time PCR for the SRY gene, suggests that no relationship exists between the pool of cell-free DNA in the amniotic fluid and that in the maternal circulation. Therefore, it appears unlikely that the amnion contributes to the amount of cell-free foetal DNA in the maternal circulation.

Although it might be argued that an asynchronous relationship might exist by the intermittent perfusion of cell-free DNA from the amnion across the placenta into the periphery. However, as it has previously been shown that the cell-free foetal DNA in the maternal peripheral circulation has a very short half life in the order of 15 min [12], this latter scenario appears to be rather unlikely.

Since it also appears unlikely that trafficking foetal cells contribute significantly to the level of cell-free foetal DNA in the maternal circulation, in that cell-free foetal

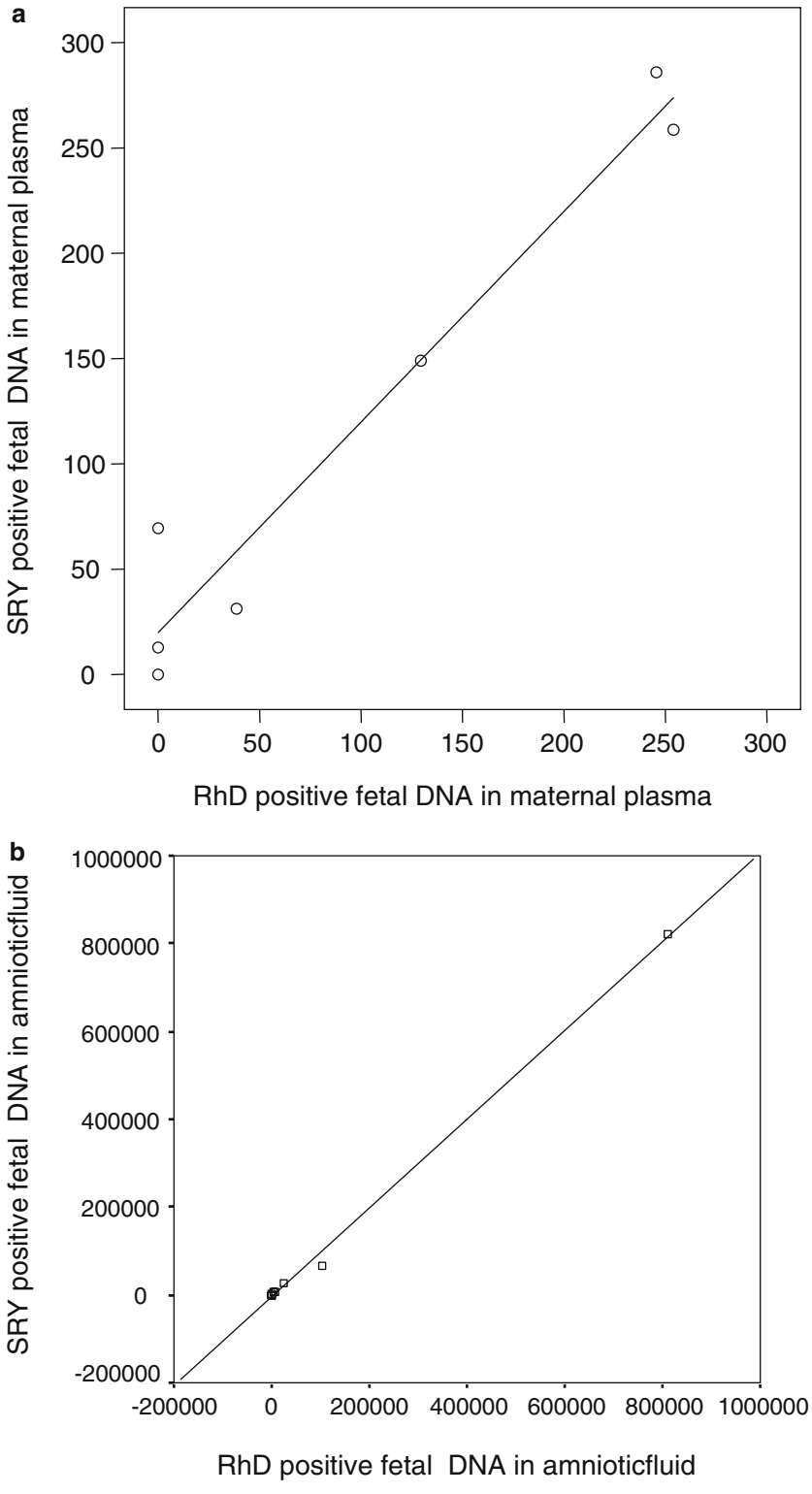

Fig. 1 Significant correlation between SRY and RhD positive cell-free foetal DNA levels in maternal plasma or amniotic fluid. **a** Cell-free foetal DNA levels in maternal plasma. **b** Cell-free foetal DNA levels in amniotic fluid. Concentration of foetal DNA is given in copies/ml maternal plasma

DNA levels can be elevated under conditions, such as preterm labour, where foetal cell traffic is not altered [17, 18], the question arises where this cell-free foetal genetic material stems from.

Although it remains to be conclusively proven, it appears that the placenta is the most likely source [21]. This is supported by evidence that cell-free foetal DNA is rapidly removed from the maternal circulation following delivery [12]. This hypothesis is further supported by reports indicating that placental mosaicism is reflected by absence of the same foetal genetic locus in the cell-free portion of foetal DNA in the

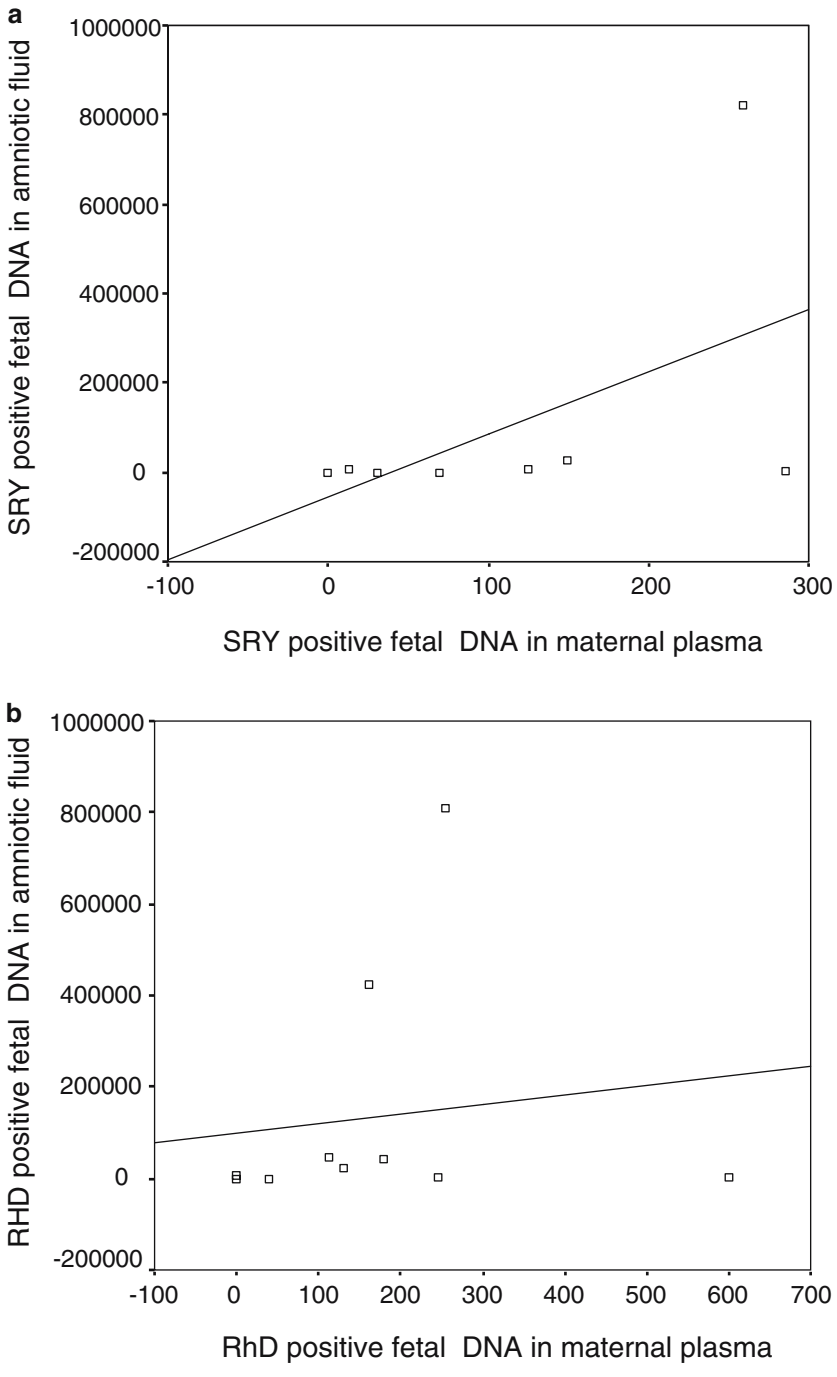

Fig. 2 Lack of correlation between cell-free foetal DNA levels in amniotic fluid and matched maternal plasma samples. **a** Analysis of samples using a real-time PCR assay for the SRY gene. **b** Analysis of samples using a real-time PCR assay for the RhD gene. Concentration of foetal DNA is given in copies/ml maternal plasma

maternal circulation [14]. In addition, cell-free foetal DNA is associated with placental microparticles of syncytiotrophoblast origin [13]. Possibly, the final proof will be obtained by studying foetal genetic loci which are epigenetically modified in the placenta [22].

Once this question has been irrevocably addressed, it will to be elucidated how cell-free foetal DNA is liberated by this organ, and how this process is disturbed in various pregnancy related disorders, particularly hose of pre-eclampsia and preterm labour [21]. Such knowledge may increase our understanding of the underlying aetiologies of these enigmatic disorders, which still present a considerable clinical challenge.

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