

Cell-free fetal DNA and intact fetal cells in maternal blood circulation: implications for first and second trimester non-invasive prenatal diagnosis

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Both intact fetal cells as well as cell-free fetal DNA are present in the maternal circulation and can be recovered for non-invasive prenatal genetic diagnosis. Although methods for enrichment and isolation of rare intact fetal cells have been challenging, diagnosis of fetal chromosomal aneuploidy including trisomy 21 in first- and second-trimester pregnancies has been achieved with a 50–75% detection rate. Similarly, cell-free fetal DNA can be reliably recovered from maternal plasma and assessed by quantitative PCR to detect fetal trisomy 21 and paternally derived single gene mutations. Real-time PCR assays are robust in detecting low-level fetal DNA concentrations, with sensitivity of approximately 95–100% and specificity near 100%. Comparing intact fetal cell versus cell-free fetal DNA methods for non-invasive prenatal screening for fetal chromosomal aneuploidy reveals that the latter is at least four times more sensitive. These preliminary results do not support a relationship between frequency of intact fetal cells and concentration of cell-free fetal DNA. The above results imply that the concentration of fetal DNA in maternal plasma may not be dependent on circulating intact fetal cells but rather be a product of growth and cellular turnover during embryonic or fetal development.

Keywords: cell-free fetal DNA in maternal plasma/fetal cells in maternal blood/fetal chromosomal aneuploidy/non-invasive prenatal diagnosis/real-time quantitative PCR

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Introduction

Some 80% of Down's syndrome infants are born to women aged < 35 years, and in the United States these women are not routinely offered invasive prenatal testing (American College of Obstetricians and Gynecologists Bulletin, 1996). Even where universal maternal serum analyte screening is offered, current non-invasive screening methods for prenatal diagnosis are

hampered by non-optimal sensitivities and high false-positive rates. At best, maternal serum analyte screening detects 60–70% of trisomy 21 cases with a calculated false-positive rate of 5% (Wald *et al.*, 1988). Consequently, there has not been the desired public health impact of decreasing incidence of liveborn trisomy 21 to the maximal extent (Haddow, 1998). Recovering intact fetal cells from maternal blood thus remains an attractive alternative for non-invasive prenatal genetic testing (de la Cruz *et al.*, 1995; Al-Mufti *et al.*, 1999; Bianchi, 1999).

The sensitivity of aneuploidy detection through analysis of intact fetal cells in maternal blood approaches 75% in some reports, with a false-positive rate estimated between 0.6 and 4.1% (de la Cruz *et al.*, 1998; Bianchi *et al.*, 2002). In support of this approach, it has been shown that pregnant patients so screened would not feel coerced, and doubtless would welcome the near-definitive results of fluorescence in-situ hybridization (FISH) using chromosome-specific probes (Zamerowski *et al.*, 2001). A newer approach involving maternal blood is the assessment of cell-free fetal DNA in plasma, using real-time quantitative PCR.

Sensitivities are reported to be 97–100%, with 100% specificity (Lo *et al.*, 1998a; Honda *et al.*, 2002; Zhong *et al.*, 2000a). Whether as a screening method in routine prenatal care (Hahn and Holzgreve, 2002) or a non-invasive diagnostic test for aneuploidy or single gene mutations, fetal genetic material in the maternal circulation has the potential to revolutionize the detection and management of complicated pregnancies. However, in order to expand the application of intact fetal cells and cell-free fetal DNA as accurate and sensitive non-invasive prenatal diagnostic tools, our understanding of their origins and ultimate fate must be broadened.

Here, the role of intact fetal cells and cell-free fetal DNA in prenatal diagnosis is reviewed, and some of our preliminary data that address the potential relationship between these two phenomena are presented.

Intact fetal cells in maternal blood

The optimal protocol for rare fetal cell selection while limiting the number of maternal cells, has yet to be developed. A relative rarity of fetal cells in the maternal circulation and the lack of a fetal-specific cell marker hamper existing enrichment and isolation protocols. Based on DNA-equivalents using quantitative PCR, the presence of fetal cells in maternal blood in normal pregnancies has been estimated at one to two per ml (Bianchi *et al.*, 1997). Other investigators have reported similar results (Hamada *et al.*, 1993; Reading *et al.*, 1995; Cheung *et al.*, 1996).

Various fetal cell types found in maternal blood have been explored as potential candidates for enrichment and subsequent analysis, including fetal trophoblasts, leukocytes and nucleated erythrocytes. Trophoblast enrichment is impeded not only by limited availability of antibodies specific to placental antigens but also by multinucleated morphology (Covone *et al.*, 1984; Bertero *et al.*, 1988; Hawes *et al.*, 1994; Schueler *et al.*, 2001). Leukocytes may persist from previous pregnancies, and isolation strategies are also limited by the lack of availability of unique cell markers or HLA antigens to differentiate maternal from fetal leukocytes (Shroder and de la Chapelle, 1972; Ciaranfi *et al.*, 1977). Nucleated red blood cells (NRBC) have the advantage of a relatively short half-life of 25–35 days; thus, cells of fetal origin are unlikely to persist from prior pregnancies (Pearson, 1967). NRBC are also relatively abundant in first-trimester blood, with unique cell morphology and complete chromosomal complement. Consequently, the vast majority of research for fetal cell recovery for non-invasive prenatal diagnosis has focused on the fetal NRBC (Bianchi *et al.*, 1990; Simpson and Elias, 1993; Zheng *et al.*, 1993; Reading *et al.*, 1995; Simpson *et al.*, 1995; Sohda *et al.*, 1997). However, given that the majority of NRBC are often identified as maternal (XX) using FISH, a growing concern has been that only a small proportion, if any, of the recovered NRBC were actually of fetal origin. More recent studies using single-cell PCR have shown that although fetal NRBC exist, as many as 50% of NRBC are maternal in origin (Troeger *et al.*, 1999a). In addition, many of these cells are undergoing normal physiological apoptosis (Hristoskova *et al.*, 2001) and, therefore, not surprisingly give rise to unstable or fragmented DNA that is not suitable for molecular cytogenetic analysis (Hahn *et al.*, 1998; Sekizawa *et al.*, 2000). Thus, an alternative fetal cell type, e.g. progenitor cells, may be more suitable and representative of all pregnancies.

The frequency of nucleated fetal cells in maternal blood increases from 0.0035% to 0.008% in the second and third trimesters respectively (Hamada *et al.*, 1993). Using FISH on whole-blood smears, the frequencies of fetal cells (all types) in maternal blood in the first, second and third trimesters were found to be 0.27, 3.52 and 8.56×10^{-6} respectively. With increasing gestational age, small but significant increases in both NBRC yield (from 100 to 1000 cells in 40 ml blood that morphologically resulted in NRBC) and purity (0.1% at 6 weeks to 1% at term) were also demonstrated (Ganshirt-Ahlert *et al.*, 1998). Similarly, a relative increase in fetal NRBC in maternal blood in the second trimester (15–16 weeks gestation) compared to the first trimester (11–12 weeks gestation) was demonstrated, further suggesting that non-invasive prenatal diagnosis using intact fetal cells may be technically more feasible in the second trimester (Rodriguez de Alba *et al.*, 2001). Nonetheless, very few fetal cells are present in the maternal circulation. Thus, enrichment is necessary to increase the concentration of fetal cells, therefore reducing the number of adventitious maternal cells that complicate fetal FISH analysis. Purity need not reach 100% for accurate and practical diagnosis.

Most enrichment strategies involve sequential steps. A preliminary separation such as Ficol-Paque or Percoll-gradient density centrifugation is typically first employed to remove mature red blood cells and granulocytes, leaving peripheral mononuclear cells. Different gradient densities are likely to influence the type, number and quality of cells recovered (Sekizawa *et al.*, 1999; Troeger *et al.*, 1999b; Samura *et al.*, 2000; Prieto *et al.*, 2001). Further enrichment can usually be achieved using one or a combination of different methods, including magnetic activated cell sorting (MACS) (Ganshirt-Ahlert *et al.*, 1992; Busch *et al.*, 1994), ferrofluid suspension (Steele *et al.*, 1996), charge flow separation (Wachtel *et al.*, 1996) or fluorescence-activated cell sorting (FACS) (Price *et al.*, 1991; Simpson and Elias, 1993, 1995; Simpson *et al.*, 1995; Zheng *et al.*, 1995; Lewis *et al.*, 1996; Sohda *et al.*, 1997; Wang *et al.*, 2000a). The general principle is to employ negative and/or positive selection criteria to remove unwanted maternal cells, retrieving the desired fetal cells using monoclonal antibodies (mAb) that recognize cell-surface antigens. Selection for intracellular proteins is another option, but fewer data have been reported in this respect. Although all of the methods listed above have been used successfully to isolate fetal cells, none has resulted in 100% enrichment.

The application of FISH to detect fetal chromosomal abnormalities (mainly aneuploidy) through maternal blood analysis offers an alternative, non-invasive means of testing all pregnancies with the potential for a much lower false-positive detection than the 5% currently tolerated for maternal serum analyte screening (de la Cruz *et al.*, 1998). Unlike other screening methods, the analysis of fetal cells in maternal blood can provide direct evaluation of fetal cells from an ongoing pregnancy, potentially rendering genetic counselling less ambiguous for most affected couples. The detection of the major fetal aneuploidies (trisomy 13, 18 and 21) has been accomplished using FISH (Bianchi *et al.*, 1992; Elias *et al.*, 1992; Ganshirt-Ahlert *et al.*, 1993; Simpson and Elias, 1993; Simpson *et al.*, 1995; Bischoff *et al.*, 1998). Although some early studies employed sequential or multi-colour FISH, enabling detection of up to three chromo-

somes simultaneously (X, Y and usually either 21 or 18), effective prenatal testing in the general population will likely require concurrent assessment of all the common aneuploidies. Thus, a five-colour FISH protocol has been developed that enables simultaneous analysis of all five-chromosomes following a single hybridization reaction (Bischoff *et al.*, 1998).

In 1994, a prospective multi-centre National Institute of Child Health and Human Development fetal cell study was initiated to assess the utility of fetal cells in maternal blood for diagnosis or screening of fetal chromosomal abnormalities. After the first 5 years, interim data analysis was performed on 2744 maternal samples (Bianchi *et al.*, 2002). Overall, fetal male cells were correctly identified in 41.4% of cases ($n=1292$) when the fetus was euploid. However, among confirmed aneuploid cases the detection rate was 74.4%, suggesting that fetal cells are relatively more abundant and, hence, more readily recoverable following enrichment. Indeed, independent studies using PCR methods to quantify the number of intact fetal cells present have confirmed that among aneuploid pregnancies, particularly trisomy 21, fetal DNA was elevated several fold compared with euploid cases (Bianchi *et al.*, 1997). Mean DNA equivalents were six-fold higher in numbers of fetal cells observed in maternal whole blood in cases of trisomy 21, compared with the one to two fetal cells per millilitre of maternal blood expected for euploid pregnancies.

To date, the analysis of intact fetal cells in maternal blood has not been applied clinically, because recovery is inconsistent and sensitivity requires further improvement. There is, however, confidence that when cells of chromosomal status differing from that of the mother are found, these cells are truly fetal. In one report, FISH on recovered fetal cells was used to exclude fetal aneuploidy in a case in which the mother had a balanced translocation (46,XX,t(1;6)(p31;q14) (Wang *et al.*, 2000b). Gamma-globin- or zeta-globin-positive cells were selected by FACS and recovered nuclei analysed for gender and the number of chromosomes 1 and 6. Based on the finding of two signals for each X, 1 and 6, fetal aneuploidy was excluded and confirmed at birth. In addition, fetal cell analysis can complement extant invasive prenatal diagnostic techniques. Low-grade fetal cell mosaicism has been observed that was not evident on chorionic villus sampling. In one case, two XY and seven XXY cells were detected among 2000 flow-sorted XX-maternal cells (Bischoff *et al.*, 1995). Routine cytogenetic analysis of cultured chorionic villus cells had detected only normal 46,XY fetal cells. Only after additional FISH analysis of 250 cultured cells was the low-grade mosaicism of XXY cells (four cells) detected. Therefore, the presence of abnormal fetal cells in some maternal cases may reflect biological mechanisms involved in selecting against abnormal cell lines—presumably the same phenomenon as exists in confined placental mosaicism (Rodriguez de Alba *et al.*, 2000).

Cell-free fetal DNA in maternal plasma

Intact fetal cell analysis necessitates extensive isolation and enrichment protocols, whereas analysing for fetal DNA does not, and is more abundant in maternal plasma (Lo *et al.*, 1997). The analysis of maternal plasma also is more amenable to general screening programmes and could be used in conjunction with other maternal serum analytes [hCG, unconjugated estriol (uE3), alpha-fetoprotein (AFP), pregnancy-associated plasma protein-A

(PAPP-A)] (Ariga *et al.*, 2001; Costa *et al.*, 2001). Sensitivity could also be greater. In one study (Lo *et al.*, 1998a), the mean concentration of fetal DNA in maternal plasma detected was 21.2 times higher than that predicted on the basis of expected number of intact fetal cells (1–2 cells/ml; Bianchi *et al.*, 1997). Increasing gestational age also correlates with increased cell-free fetal DNA; concentrations are generally low in the first trimester, and rise in the second and third trimesters (Lo *et al.*, 1998a). Cell-free fetal DNA concentrations correspond to 3.4% of the total maternal plasma (range 0.39 to 11.9%) during the gestational ages from 11 to 17 weeks, but to 6.2% (range 2.33 to 11.4%) in the late third trimester. This can be compared with the much lower frequencies (0.0035 and 0.008%) of nucleated fetal cells in maternal blood in the second and third trimesters respectively (for a review, see Lo, 2000). The sharp increase in fetal DNA in maternal plasma and serum over the last 8 weeks of pregnancy that has been demonstrated (Lo *et al.*, 1998a) could indicate a gradual breakdown of the maternal fetal interface/placental barrier (Bianchi, 2000a,b). Finally, certain intact fetal cells (e.g. CD34⁺, CD38⁺) that are potentially isolated from maternal blood may have persisted from previous pregnancies and transfusions and compromise diagnosis (Bianchi *et al.*, 1996); however, the short half-life of plasma fetal DNA should remove even the theoretical fear of error based on carry-over from previous pregnancies.

Several studies to assess the sensitivity and specificity of fetal DNA in first- and second-trimester maternal plasma have now been reported (Costa *et al.*, 2001; Sekizawa *et al.*, 2001; Zhong *et al.*, 2001; Honda *et al.*, 2002), with relatively large sample sizes ($n=121$, 302 and 237 and 81 respectively). Real-time PCR was employed to quantify plasma fetal DNA based on Y-specific sequences. Overall, 95–100% sensitivity with 100% specificity was observed. Sensitivity in first-trimester (7–12 weeks) euploid samples was less efficient, with detection of 70–95%. The reduced detection rate in the first trimester could most likely be explained by the presence of relatively lower amounts of fetal DNA secondary to turnover of relatively fewer fetal cells. Nonetheless, cell-free fetal DNA is clearly abundant and readily detectable compared with rare intact fetal cells. As will be demonstrated below, cell-free fetal DNA can be found in a significant number of cases in which intact fetal cells are not detected.

The quantity of fetal DNA is elevated when the fetus is aneuploid. Using real-time PCR, one group (Lo *et al.*, 1999) demonstrated a 2-fold increase in fetal DNA levels for trisomy 21, compared with euploid cases. Subsequent studies have supported these observations on confirmed trisomy 21, but not on trisomy 18 (Zhong *et al.*, 2000b), suggesting that different fetal growth and placental pathologies result in different levels of fetal DNA. Analysis of archival maternal serum samples has also shown a 1.8-fold increase in the amount of fetal DNA levels in trisomy-positive pregnancies as compared with gestational age-matched controls. In aggregate, these findings point to the possible use of fetal DNA as an additional aneuploidy screening analyte. To this end, another group (Farina *et al.*, 2002) demonstrated a significant correlation between early gestational age (10–12 weeks) and fetal DNA concentrations among 63 euploid pregnancies that were normally distributed. However, variation in DNA extraction levels was demonstrated by our group and by others (Hahn *et al.*, 2001), indicating a need for further evaluation.

Cell-free fetal DNA can be readily amplified and analysed using PCR-based methods, thereby providing an attractive alternative to fetal cell isolation and assessment. In order to measure small amounts of target DNA relative to background (often excess) genomic DNA, sensitivity can be enhanced with fluorescent-based real-time PCR for quantitative analysis. Although the accuracy of real-time PCR improves with an increased starting template amount (Stenman and Orpana, 2001), the technique is still not suitable for application to maternal whole-blood samples, in which perhaps only one fetal cell exists per 10^5 to 10^6 maternal cells. When the target DNA is present in very low copy numbers (e.g., 1–3 copies/ml), real-time PCR similarly may be at the limit of assay sensitivity; thus, vicissitudes of sampling are likely to affect detection rate. Detection of the target DNA may therefore be influenced not only by the amount of material used per test but also by the number of aliquots tested. The concentration of background DNA can further influence the detection rate of low copy number sequences (Stenman and Orpana, 2001). Consequently, assays must be conducted at least in triplicate in order to ensure reliable results. It is also critical to confirm the sensitivity of any PCR quantification experiment given that trace amounts of contaminating DNA may be amplified just as a dilute sample of target DNA is amplified.

Until recently, the inability of PCR to distinguish readily between maternally inherited fetal DNA and native maternal DNA has been a diagnostic impediment. That is, there has been a lack of any comparable equivalent to Y-specific DNA that could serve as a facile internal control to ensure the presence of fetal DNA in the sample being assessed for aneuploidy. Clinically, this limits current cell-free fetal DNA prenatal assessment to unique fetal gene sequences not present in the maternal genome, such as rhesus D antigens (in Rh-negative mothers) (Faas *et al.*, 1998; Lo *et al.*, 1998b; Bischoff *et al.*, 1999; Zhong *et al.*, 2000a). In general, PCR is not helpful when the disease is either X-linked or autosomal recessive and both parents carry the same mutation. Further evaluation of 'blinded' specimens will be required to determine the sensitivity of detecting quantitative differences between loci in a given specimen, as has been recently demonstrated (Zimmermann *et al.*, 2002). Results from recent studies (Poon *et al.*, 2002) also warrant further evaluation to determine the efficiency of using differential DNA methylation to distinguish between fetal DNA in maternal plasma.

Source and significance of intact fetal cells and cell-free fetal DNA

The mechanism(s) underlying variations in the amounts of intact fetal cells and cell-free fetal DNA in the maternal circulation in both normal and abnormal pregnancies remain to be elucidated (Bianchi and Lo, 2001). As alluded to above, elevated numbers of fetal cells as well as elevated levels of cell-free fetal DNA in the maternal circulation have been detected in patients with certain aneuploidies (Bianchi *et al.*, 1997; Lo *et al.*, 1999; Zhong *et al.*, 2000b). Pathological changes in the Down's syndrome placenta, such as villus oedema, might explain these increased values. Elevated cell-free fetal DNA levels have also been found in a variety of pregnancy complications, such as pre-eclampsia, polymorphic eruptions of pregnancy and other medical complications of pregnancy (Holzgreve and Hahn, 2002).

Cell-free DNA could arise from either placental cells or from intact fetal cells that secondarily undergo apoptosis. In the latter situation, T-cell-mediated maternal cellular immune system attack would occur. Irrespective of this, apoptosis is presumably the common pathway underlying the presence of cell-free fetal DNA in the maternal circulation. The detection of apoptotic fetal cells in the plasma of pregnant women has been demonstrated (van Wijk *et al.*, 2000), and this also suggests that circulating apoptotic fetal cells are the source of cell-free fetal DNA. In further support of this theory, it has been shown that 42.7% of fetal NRBC (fNRBC) detected in maternal blood were undergoing apoptosis (Sekizawa *et al.*, 2000). This high percentage might help to explain the rarity of intact fetal cell detection, compared with the high levels of cell-free fetal DNA in maternal plasma (Lo *et al.*, 1998a; Hahn *et al.*, 2000). These conclusions, however, have yet to be confirmed by comparative or quantitative studies that assess the frequency of all intact fetal cells and cell-free fetal DNA concentrations within maternal blood specimens. If apoptotic intact fetal cells were the source of cell-free fetal DNA, a correlation between intact fetal cell number and the amount of cell-free fetal DNA would be expected.

Relationship between intact fetal cells, cell-free fetal DNA and detection of aneuploidy: the Baylor experience

If both intact fetal cells and cell-free fetal DNA are to be combined for improved diagnostic accuracy, it would be pivotal to know whether the two phenomena truly share a linear relationship. To help clarify this issue, a pilot study was performed to determine whether the frequency of detection of intact fetal male cells correlates with the amount of cell-free fetal DNA (Y sequence-specific) in maternal blood.

After obtaining approval from the institutional review board and the patients having provided their informed consent, maternal blood specimens were collected ($n=70$) prior to an invasive procedure. The mean gestational age was 15.0 ± 3.9 (range 10.0–23.4) weeks. The samples included 47 male singletons, three male multiple gestations (two twins and one triplet), and 23 female singletons. In four additional male singleton cases, chromosomal anomalies were detected following amniocentesis (47,XY,+18; 47,XY,+7; 46,XY/46,XYdel1q; 46,XY/45,X).

Maternal blood samples were processed for enrichment of intact cells using MACS for CD71-positive selection (Takabayashi *et al.*, 1997), followed by FISH analysis using X- and Y-chromosome-specific probes to identify male (XY) fetal cells (Bischoff *et al.*, 1998). In addition, for analysis of presence and quantitation of cell-free fetal DNA, plasma DNA was also recovered using the QIAamp Blood Kit (Qiagen, Basel, Switzerland). Real-time PCR using the TaqMan Assay (Applied Biosystems) was performed using the GeneAmp 7700 Sequence Detection System. Quantitation of both the Y-chromosome-specific DYS1 (Y49A clone) sequences (used to measure the amount of male fetal DNA) and the ubiquitous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequences was performed (Bianchi *et al.*, 1997; Lo *et al.*, 1998a).

The FISH and real-time PCR results are summarized in Table I. Intact fetal male (XY) cells were detected in 13 of the 47 (28%) male cases. The number of fetal male cells detected in the 13 cases ranged from one to seven cells, representing a frequency of

Table I. Detection efficiency of fetal intact male (XY) cells by FISH and Y-sequences by real-time PCR

Fetal gender	Method of detection ^a	Fetal male detection rate (%)	Mean Y-sequence ^b (Geq/ml)
Male cases (n=47)	FISH only	2/47 (4.3) ^c	NA
	FISH and PCR	11/47 (23.4)	37.6 ± 46.8
	PCR only	27/47 (57.4)	409 ± 1385
	Neither FISH nor PCR	7/47 (14.9)	NA
Female cases (n=23)	FISH only	5/23 (21.7) ^d	NA
	FISH and PCR	0/23 (0.0)	NA
	PCR only	4/23 (17.4) ^e	2.4 ± 0.483
	Neither FISH nor PCR	14/23 (60.9)	NA
Multiple male gestation cases			
	Twins (n=2)		
	Triplet (n=1)		
Chromosomally abnormal cases (n=4)			

^aIdentification of method(s) which resulted in positive detection of fetal genetic material. Intact fetal cells were identified by FISH; cell-free fetal Y-sequence DNA (DSY1 locus) was identified by real-time PCR using the TaqMan assay.

^bMean amount of Y-specific DNA sequences detected using real-time PCR and the TaqMan assay.

^cIn two cases, only intact fetal male (XY) cells were identified by FISH using centromere-specific probes to chromosomes X and Y.^dIn five female cases, only intact male (XY) cells were identified by FISH. One XY-cell was identified in each case.

^eIn four female cases, Y-chromosome-specific sequences were detected in only one of three wells amplified. FISH= fluorescence in-situ hybridization; PCR=polymerase chain reaction; NA=not applicable.

0.01% to 0.16% fetal cells in maternal blood. In 11 of these 13 cases, Y-specific fetal DNA sequences in maternal plasma were also detected. However, correct detection of cell free-fetal DNA alone based on positive Y-sequences was 38 of 47 cases (81%). Failure to detect fetal DNA in all cases was likely result of either low levels of fetal DNA in first-trimester cases, high concentrations of contaminating maternal DNA concentrations, or inefficiency of the DNA extraction protocols. No correlation was found between the amount of total DNA (GAPDH) and increasing gestational age. When comparing samples in which intact fetal cells were (n=13) or were not detected (n=34), it was surprising that no difference was found with respect to the quantity of cell-free fetal Y-specific DNA (Figure 1). This suggested there may not be any relationship between the two types of fetal (intact cells and DNA) material present in maternal blood.

Among female cases, FISH and real-time PCR results were consistent in 14 of 23 cases (61%), i.e., with no detection of either intact XY cells or Y-specific DNA sequences (Table I). Of the nine cases with inconsistent results, four were positive for Y-sequences by PCR. In the remaining five cases, one intact XY-cell was observed in each sample. Based on our selection criteria for CD71⁺ cells, these XY-intact cells were likely to have resulted from FISH hybridization background and not from a previous pregnancy. Overall, choosing cut-off limits for positive detection at a mean of >3 Geq/ml Y-sequence DNA and >1 XY-cell, the results of all 23 female cases would be consistent for the two tests.

There were three cases with multiple male gestations (two twins, one triplet) and four cases with abnormal fetal chromosome

complement. No correlation was observed between the frequency of intact fetal cells and quantity of cell-free fetal DNA (Table I). Analogous to previous findings (Zhong *et al.*, 2000b), the pregnancy with trisomy 18 failed to show a relative increase in Y-sequence quantity (5.3 Geq/ml) compared with gestational age-matched euploid cases. The above results imply that the concentration of fetal DNA in maternal plasma is not dependent on circulating intact fetal cells but rather is a product of growth and cellular turnover during embryonic or fetal development.

The sensitivity of the real-time PCR/TaqMan approach is limited in detecting very low copy number target sequences. The nine male cases having no Y-sequences in any of the three wells nonetheless showed some of the highest GAPDH copy numbers (ranging from 52 000 to 221 500 Geq/ml). Thus, the quantity of GAPDH (background DNA) copies per ml of plasma in each case is likely to influence the ability to detect low concentrations of Y-sequences. This may be analogous to difficulties encountered in scoring rare intact fetal cells by FISH. If the frequency of intact fetal cells is low (<1% of enriched cell population), the manual scoring of 10³-10⁴ of cells becomes laborious and, hence, inefficient. Improved sensitivity in detecting cell-free fetal DNA could similarly be addressed by concentrating more plasma DNA or by increasing the number of reactions (wells) for analysis. It has been shown (Chiu *et al.*, 2001; Hahn *et al.*, 2001) that methods of purifying plasma DNA from maternal blood can influence the detection of low copy number sequences. Other variables include the initial volume of plasma, centrifugation speed and primer/probe concentrations. Irrespective of these variables, cell-free fetal DNA in maternal plasma is clearly more

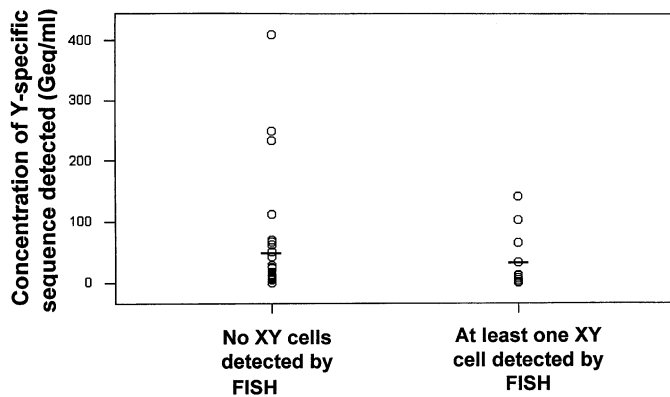


Figure 1. Comparison between the concentration of Y-specific fetal DNA (Geq/ml) detected in cases with either intact XY cells identified ($n=13$) or not ($n=34$). No significant difference was found between the mean concentration of Y-sequences measured by the real-time PCR/TaqMan assay in cases with (31.9 ± 45.0 Geq/ml) or without (46.7 ± 89.4 Geq/ml) intact XY cells ($P=0.466$, not significant).

abundant and at least four times more likely to be detected as compared with intact fetal cells.

In intact cell analysis, both false-negative (e.g. failure to detect XY-cells among confirmed male cases) as well as false-positive (e.g. detection of Y-sequences in known female cases) results were observed. False-negative (intact cell) FISH results could reflect either the rarity of intact fetal cells in maternal circulation or a low yield of the enrichment protocol. False-positive FISH findings may result from vanishing twins, sample contamination, or possibly from autosomal cross-hybridization with the Y-specific probes (Lo *et al.*, 1993). Similarly, for cell-free DNA real-time PCR specimens with very low copy number of target sequences approach the limits of sensitivity of the assay (see above). Although the operator can adjust threshold values and consequently remove false-positive detection, the detection of low Y-sequence quantities in potentially positive male cases would also be reduced by this adjustment. Thus, faced with an unknown patient specimen, the sensitivity and specificity of the assays must rely on the concentrations for each of the standard controls and set cut-off values.

A caveat is that the present study specifically addressed enrichment of intact fetal NRBC. That lack of correlation between total numbers of intact fetal cells and levels of cell-free fetal DNA might reflect the fact that although fetal leukocytes, trophoblasts and other intact fetal cells are not accounted for by the present method, they still contribute substantially to the cell-free fetal DNA pool. If steady half-lives are assumed for cell fetal DNA and intact fetal cells in the maternal circulation, and that the cell-free DNA originates from the intact cells, then a correlation between the two is logical and may still exist.

Conclusions

Although efficient recovery of intact fetal cells is not optimal, intact fetal cell quantities are increased and more readily detected by FISH in chromosomally abnormal pregnancies, specifically for trisomy 21. Similarly, cell-free fetal DNA is present in relatively abundant amounts in all pregnancies, and mutant fetal gene

sequences are detectable following relatively simple DNA extraction procedures and PCR-based methods. Quantitative analysis of total DNA (fetal and maternal) in maternal plasma throughout gestation may also enable prenatal screening of fetal aneuploidy, in which cell-free DNA appears to be increased. Cell-free fetal DNA may be a more robust approach than intact cell analysis. In our hands, the two approaches were shown not to correlate. An analysis of intact cells for cell-free DNA by PCR would also allow the evaluation of single gene mutations. Once optimized, the use of either of these approaches should be a highly attractive addition to non-invasive aneuploidy screening and/or testing.

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