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ORIGINAL ARTICLE

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Direct detection of fetal cells in maternal blood: a reappraisal using a combination of two different Y chromosome-specific FISH probes and a single X chromosome-specific probe

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Abstract *Background*: We have recently explored the detection of circulatory male fetal cells directly in maternal whole blood samples by fluorescence in-situ hybridization (FISH). In order to improve the efficacy of fetal cell detection, we have now examined whether this could be enhanced by the use of two different Y chromosome-specific FISH probes (α-satellite and classical satellite III regions) in combination with an X chromosome-specific FISH probe. *Methods*: Nineteen maternal blood samples (median gestational age = 28 weeks, range = 12–37 weeks) were examined in a blinded manner. No enrichment procedure was performed. Following hypotonic treatment and Carnoy's fixation, total nucleated cells were examined by two color FISH with a single X and two Y chromosome-specific probes. Nine cases were examined in parallel by conventional XY-FISH. *Results*: Fetal cell detection was superior when using two Y chromosome-specific probes (specificity = 75%; sensitivity = 91%) when compared to the conventional XY-FISH approach (specificity = 50%; sensitivity = 60%). *Conclusions*: Male fetal cells can be detected in most maternal blood samples examined. Specificity and sensitivity is improved when using a combination of single X and two Y chromosome-specific probes when compared to a conventional XY-FISH protocol.

Keywords Circulatory fetal cells · Maternal blood · FISH

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Introduction

Even though fetal genetic loci such as RhD status or sex can now be reliably determined by the analysis of cell-free fetal DNA in maternal plasma, this approach is currently not suited for the analysis of complex fetal genetic traits, such as those involved in the case of fetal aneuploidies [4, 5]. For this reason the enrichment and analysis of fetal cells from maternal blood samples is currently still the most promising strategy for the non-invasive determination of numerical anomalies of fetal chromosomes, such as those involved in Down's syndrome [5].

Large scale studies, using either MACS (magnetic cell separation) or FACS (fluorescent activated cell sorting), have however indicated that fetal cells, specifically fetal erythroblasts, cannot be reliably retrieved and analyzed from maternal blood samples [2, 3]. These results have raised the question of whether the fetal cells, especially erythroblasts, were indeed present in each pregnancy examined [9]. Furthermore, it appears that the fetal erythroblast may not be the optimal target cell, and that it may be necessary to examine for other more suitable fetal cell types [1, 6].

In this context a recent study has indicated that fetal cells can be detected by the use of fluorescence in-situ hybridization (FISH) in maternal whole blood samples without any form of enrichment [7]. These results are of considerable importance as they yield vital information concerning the number of circulating fetal cells in maternal blood. It is also hoped that by the analysis of these cells, other more optimal target fetal cells types may be identified.

Prompted by these analyses we have recently examined the feasibility of detecting fetal cells directly without any form of enrichment in maternal blood samples [8]. This study indicated that fetal cells were indeed present in the majority of samples examined, and that they were present with astonishingly high frequency (12–20 fetal cells/ml maternal blood) [8]. In our

examination, we also observed that the specificity of detecting male fetal cells could be improved by the use of two different Y chromosome-specific FISH probes (α and III satellite: specificity = 89.5%) when compared to a conventional XY-FISH protocol (specificity = 68.4%) [8]. As the sensitivity of the latter approach was however quite poor (42.9%), we have attempted to further enhance the efficacy of this approach. For this reason, we have examined the use of two similar Y chromosome-specific FISH probes (α and III satellite), but now in combination with a conventional centromeric X chromosome-specific FISH probe (referred to as XYY-FISH). We have also compared this approach to conventional XY-FISH.

Materials and methods

Sample recruitment and processing

Nineteen healthy pregnant women between the 12th and 37th weeks of gestation were recruited for this study, which was approved by the Ethical Review Board of the University of Basel. Written informed consent was obtained in all instances. The maternal whole blood samples, anti-coagulated with EDTA, were processed within 6 h as described previously [7, 8]. In brief, 500 µl of EDTA-blood was washed with 8 ml RPMI 1640 medium (Invitrogen, Basel, Switzerland), incubated with 10 ml prewarmed 0.4% KCl for 20 min at 37°C, fixed several times with freshly prepared pre-chilled Carnoy's fixative (methanol:glacial acetic acid, 3:1), washed with 1% BSA/PBS and transferred by cytocentrifugation onto glass slides (four slides per case). Prior to FISH analysis the slide preparations were digested with 0.005% pepsin for 10 min at RT and then fixed for 10 min in 1% formaldehyde.

FISH analysis

XYY-FISH

All 19 samples were examined by FISH analysis using centromeric and III satellite Y chromosome-specific probes (Yα satellite rhodamine and Yqh classical satellite III rhodamine) in combination with a conventional centromeric probe for the X chromosome (Xα satellite fluorescein) (Qbiogene, Basel, Switzerland).

XY-FISH

Conventional dual-color FISH for the X and Y chromosomes was accomplished using a commercial chromosome enumeration cocktail (CEPX spectrum green (α-satellite)/CEPY spectrum orange (α-satellite), (Vysis/ABBOTT Diagnostics, Baar, Switzerland).

All FISH analyses were performed according to the manufacturer's instructions, using FISH probes diluted 1:100 in cDenHyb-1 (Insitus Biotechnologies, Alberquerque, NM, USA) [8]. For XYY-FISH, codenaturation was performed at 80°C for 2 min, while that for XY-FISH was carried out at 72°C for 8 min. All hybridizations were carried out at 37°C for 6 h. Post-hybridization washes were performed in 50% formamide/1.5x SSC at 45°C (5 min) and in 1.5x SSC at 45°C (2 min). After a final wash with distilled water, the slides were counterstained with 0.01% DAPI/Glycerol (Sigma, Fluka Chemie GmbH, Buchs, Switzerland).

For each hybridization analysis, male cord blood control slides were examined in parallel to monitor the quality of the FISH performance, yielding average efficiencies of greater than 98% for each FISH protocol.

All samples were analyzed in a blinded manner using a triple pass filter for the first scan and the subsequent confirmation of individual X and Y chromosome-specific FISH signals using the appropriate single band pass filters at 630× magnification (Axiovision 3.1, Carl Zeiss, Zürich, Switzerland).

In order for a cell to be classified as fetal, the clear presence of specific signals for both X and Y FISH chromosomes had to be present. Results from the FISH analysis were only compared to the fetal gender as determined from birth outcome once the evaluation was complete.

Results

Our analysis of the 19 maternal blood samples by the combination of two Y chromosome-specific FISH probes in combination with a FISH probe for the X chromosome (XYY-FISH) indicated that male fetal cells could be detected with 75% specificity and 91% sensitivity (Table 1), indicating that male fetal cells could be detected in 10 out of the 11 pregnancies with male fetuses. Our parallel analysis of nine of these samples by conventional XY-FISH indicated that male fetal cells could be detected with 50% specificity and 60% sensitivity (Table 1), which is in good agreement with our previous results [8]. On the other hand, when the same samples were analyzed by our new XYY-FISH approach, male fetal cells could be detected with 100% specificity and 100% sensitivity. Therefore, the use of two different Y chromosome-specific FISH probes in combination with an X chromosome-specific FISH does appear to yield a better result than that obtained by conventional XY-FISH. Furthermore, it appeared that more male fetal cells were detected by the XYY-FISH approach, where 1 to 4 fetal cells were recorded per slide, (yielding an average concentration of between 8 to 32 fetal cells per ml of maternal blood), compared to the 1 to 2 fetal cells that were recorded per slide using conventional XY-FISH. We also observed that the signals for the Y chromosome were not as bright and readily identifiable using the conventional XY-FISH approach (Fig. 1a) when compared to those detected by the novel XYY-FISH procedure (Fig. 1b).

Table 1 Detection of male fetal cells in maternal blood by two different FISH strategies

Case	Number of male fetal cells detected by XYY-FISH/ml maternal blood	Number of male fetal cells detected by XY-FISH/ml maternal blood	Gender of fetus
1	24	12	Male
2	16	12	Male
3	0	8	Female
4	0	0	Female
5	0	16	Female
6	0	0	Female
7	32	0	Male
8	32	0	Male
9	32	20	Male
10	8	n.t.	Female
11	0	n.t.	Male
12	0	n.t.	Female
13	8	n.t.	Male
14	16	n.t.	Male
15	32	n.t.	Male
16	8	n.t.	Male
17	0	n.t.	Female
18	24	n.t.	Male
19	8	n.t.	Female
Sensitivity	91%	60%	
Specificity	75%	50%	

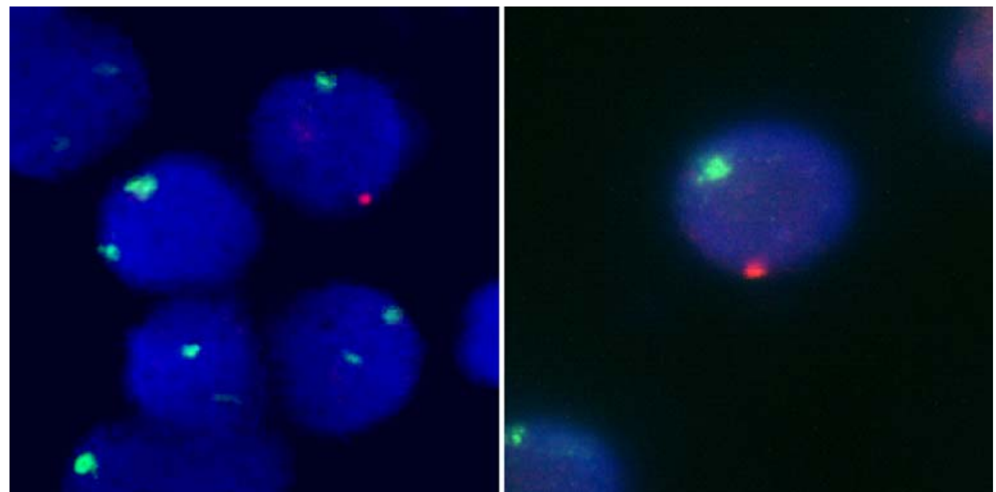

Fig. 1 **a** Detection of a male fetal cell in maternal blood by conventional XY-FISH. **b** Detection of a male fetal cell in maternal blood by the use of two Y chromosome-specific (α- and III-satellite) FISH probes in combination with a FISH probe specific for the X chromosome (XYY-FISH)

Discussion

Previous studies have indicated that male fetal cells can be detected directly in maternal whole blood samples by conventional XY-FISH or PRINS (primed in-situ labelling) [7]. Although these studies yielded important information concerning the number of circulating fetal cells in maternal blood, the results are possibly skewed by the fact that they were not conducted in a blinded manner.

In a recent study, we have used a similar approach to that described by Krabchi and colleagues, in that we also used conventional XY-FISH to detect the presence of such circulatory male fetal cells, but performed our analysis in a blinded manner concerning the sex of the fetus [8]. This analysis indicated that male fetal cells could be detected with a specificity of 68.4% and sensitivity of 52.4%. By switching to a protocol in which male fetal cells were detected solely on the basis of two independent Y chromosome-specific FISH signals (α- and III-satellite), we achieved a specificity of 89.5% [8]. However, the sensitivity was slightly decreased to 42.9% [8]. Although this study did indicate that male fetal cells were present in a large proportion of pregnancies with male fetuses, we were anxious to improve upon the accuracy of our method. For this reason we have again used two similar Y chromosome-specific FISH probes (α- and III-satellite), but now in combination with an X chromosome-specific FISH probe.

Our current investigation indicates that circulatory male fetal cells can now be detected with 75% specificity

and 91% sensitivity, which is a considerable improvement over previous results. The efficacy of this approach also becomes readily apparent in our parallel assessment of the same samples by conventional XY-FISH, where male fetal cells could only be detected with 50% specificity and 60% sensitivity.

Our data are also quite encouraging with regard to the number of male fetal cells detected per ml of maternal blood, in that these may also be slightly higher than our previous estimates (8–32 vs. 12–20). Akin to our previous study, male fetal cells could be detected in the majority of maternal blood samples examined. Although encouraging, it is clear that these results will have to be confirmed in a larger study. It will be particularly important to determine at what stage of pregnancy circulatory cells can be detected reliably, since in our current study we examined a broad spectrum of gestational ages. It will also be important to determine their frequency early in pregnancy at those stages where they may be useful as the basis for a non-invasive prenatal diagnosis, particularly in the first trimester (5). Furthermore, the fetal source (placental or hemopoietic) of these cells is also of considerable interest, as this could lead to the development of new tools permitting their efficacious isolation from maternal blood samples.

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