Original Article

Accuracy Assessment of Interphase Fluorescence In-Situ Hybridization on Uncultured Amniotic Fluid Cells

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Abstract

Background: Parental anxiety while waiting for the results of amniocentesis has been investigated by many authors. It seems that the implementation of faster techniques such as fluorescence in-situ hybridization (FISH) will have some benefits in reducing this anxiety. Besides the patients' attitudes to choosing this method, gynecologists who are the persons responsible for treatment, must feel comfortable about prescribing FISH techniques.

Materials and Methods: This study, using a simple methodology, was undertaken to evaluate the results of FISH tests on the amniotic fluid from 40 pregnant women undergoing cesarean surgery. Two sets of probes including X/Y cocktail and 13, 21 and 18 were applied on different slides.

Results: The results of FISH tests were compared with the reports of the pediatrician about the health condition of the newborn. Complete conformity between the two sets of findings, have convinced our gynecologists of the benefit of prescribing this method to reduce the anxiety of patients at risk of having abnormal offspring due to chromosomal anuploidies.

Conclusion: As has been documented by many authors, conventional chromosome analysis has great advantages over fluorescence in situ hybridization of interphase amniocytes, but reducing the anxiety of parents is a good reason for employing the FISH technique.

Keywords: Amniotic Fluid, FISH, Prenatal Test

Introduction

Amniocentesis is one of the two principle techniques for sampling fetal material for prenatal diagnosis. The amniotic fluid contains cells of fetal origin that can be cultured for diagnostic tests. Amniocentesis is performed typically at the 15th to 16th week after the first day of the last menstrual period, but it has been performed as early as 10 to 14 weeks in some centers. On average, tissue culture requires 10-14 days before the chromosome analysis can be started. The inevitable delay between amniocentesis and fetal chromosome diagnosis is the cause of much parental anxiety. Sometimes, in case of an aberrant finding, insufficient time remains post-diagnosis for optional termination of the pregnancy. On the other hand, early amniocentesis versus mid-trimester amniocentesis is associated with an increased risk of spontaneous abortion of 2.6 percent in the early group versus 0.8 percent in the mid-trimester group (1). If couples are to have an opportunity to consider termination of pregnancy when an abnormality is found in the fetus, they need to be provided with the information at the earliest possible opportunity. Because prenatal diagnosis is always a race against time, the rate of culture failure, which is about 1%, can be a concern (2). Also, there is the possibility of misleading results due to overgrowth of maternal cells, estimated to occur in around 0.2% of cultures (3). In certain clinical situations, the time required to complete the chromosomal analysis might be too long and place additional clinical and emotional burden on the patient and health care providers. Due to these drawbacks a method which provides rapid and accurate identification of aneuploidies would be a useful adjuvant to the conventional cytogenetic diagnostic tests.

The introduction of fluorescence in-situ hybridization (FISH) and fluorescence-based PCR reactions, enabled identification of aneuploidies in interphase cell nuclei of uncultured amniocytes. The advantage of using FISH with direct fluorescence labeled DNA probes include: its

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high specificity and sensitivity; its more rapid performance; its ability to simultaneously detect several probes using different fluorochromes; and its applicability in both interphase and metaphase nuclei. The simultaneous detection of X and Y chromosome with specific probes is likely to be the most reliable method for identifying the sex of embryo and aberrations of the sex chromosomes. With several modifications of the FISH technique and the use of direct label fluorescence DNA probes, the whole FISH procedure has been reduced to a few hours (4).

Despite these advantages and a lot of progress in FISH techniques and the production of highly specific and reliable probes in the last decade, including FDA approval of the AneuVysion assay kit (Vysis, Inc. USA) to enumerate chromosomes 13, 18, 21, X, Y for prenatal diagnosis, there are a lot of concerns about sensitivity, specifity and the predictive values of FISH tests in prenatal diagnosis, and little attention has been paid to using this technique in routine prenatal diagnosis.

The aim of the present study was to evaluate the reliability of FISH as method for prenatal diagnosis and, more specifically, to attract the attention of gynecologists and pediatricians to the advantages of FISH techniques in comparison to conventional techniques. There are relatively few published articles that compare the results of conventional cytogenetic methods and FISH in prenatal diagnosis, but usually the numbers of abnormal cases in the studies were small and the studies encountered difficulties in the follow-up of patients. In this study we took the amniotic fluid samples at the time of delivery to determine the constitution of the sex chromosomes and enumerate chromosomes 13, 21, 18. The results were compared with reports from the gynecologists and pediatricians.

Materials and Methods

Five milliliters of fresh amniotic fluid were obtained from forty patients undergoing cesarean surgery by the gynecologist. All the pregnancies were due to infertility treatment by intercytoplasmic sperm injection (ICSI).The samples were coded before delivery to the genetics lab. To ensure that the analyses were performed blind, the results of clinical examinations by a pediatrician, the sex of the newborns together with the name of patient and the selected codes were collated by a third researcher. The ethical committee of the Institute approved the study. Informed consent was obtained from all patients after they had been given detailed information prior to the procedure.

Sample preparation

For each sample, 2-5 ml of amniotic fluid was centrifuged for 10 min at 1000 rpm. The pellet was resuspended in 5 ml of prewarmed trypsin-EDTA (Sigma, USA), gently vortexed, and incubated at 37 C for at least 15 min. Following centrifugation for 10 min at 1000 rpm, the pellet was treated with hypotonic solution (0.56% KCl) at 37 C for 20 min in a water bath. Following centrifugation at 1000 rpm for 10 min, the supernatant was removed and the pellet was resuspended by slowly adding 3 ml of methanol/glacial acetic acid (3:1). The sample was vortexed and the suspension was centrifuged at 1000 rpm for 10 min. The supernatant was decanted and replaced by 1 ml of fresh fixative solution. At this stage, the specimen could be either stored for a long time at -20 C or for 1 h at 4 C before being spread. One or two drops of the cell suspension were dropped on a marked area of a clean slide which was placed on a slide warmer at 45 C.

Hybridization and detection

Commercially available FISH probes specifically designed to enumerate the X, Y, 13, 21 and 18 chromosomes were used for this research (Vysis, USA). The CEP probes (alpha satellite) were used for chromosomes X, Y and 18, and locus specific (LSI) probes were used for chromosomes 13 and 21 due to probability of cross hybridization between them.

Prior to hybridization slides were incubated at 37 C in 2x SSC (standard saline citrate) for 10 min and then in pepsin solution (0.005% pepsin-0.01N HCl) for 15 min. After washing the slides in 1x PBS (phosphate buffer saline) at room temperature for 5 min, they were transferred to 1% formaldehyde for 5 min at 4 C and then washed in PBS and dehydrated by immersion for 2 min in 70%, 85% and 100% ethanol.

Following slide pretreatment, 10ul of each set of probe mixtures (X-Y and 18-13-21) were placed on two marked areas of each slide, covered by coverslips and sealed with rubber cement. Probes and slides were co-denatured at 75 C for 5 min, and hybridization was performed at 37 C overnight. After incubation, the coverslips were removed, and slides were washed in 0.4xSSC/0.3% NP-40 at 73 C for 2 min and then in 2xSSC/0.1% Np-40 at room temperature for 1 min. The slides were air dried in the dark, counterstained with DAPI II, and covered with coverslips.

FISH analysis

Microscopic evaluation was performed using an epifluorescence microscope equipped with single band pass filters for DAPI, green, red and aqua spectrums (Nikon Eclipse E-800). For each of the cases 30-50 cells were evaluated for presence and enumeration of X, Y, 18, 13, 21 chromosome signals.

Table 1: Comparisons of results from FISH on uncultured amniocytes and reported sex and health conditions of newborns

Sample code	Appearance	Gender Report	FISH result for gender	Conformity	FISH results for Autosomes	Pediatrician reports
1	Bloody	Male	XY	Ok	Normal	Normal
2	Bloody	Female	XX	Ok	Normal	Normal
3	Clear	Female	XX	Ok	Normal	Normal
4	Clear	Female	XX	Ok	Normal	Normal
5	Clear	Female	XX	Ok	Normal	Normal
6	Clear	Male	XY	Ok	Normal	Normal
7	Clear	Male	XY	Ok	Normal	Normal
8	Clear	Female	XX	Ok	Normal	Normal
9	Clear	Male	XY	Ok	Normal	Normal
10	Clear	Male	XY	Ok	Normal	Normal
11	Clear	Male	XY	Ok	Normal	Normal
12	Clear	Female	46XX/45X	Ok!	Normal	Normal
13	Clear	Male	XY	Ok	Normal	Normal
14	Clear	Male	XXY	Ok!	Normal	Normal
15	Clear	Female	XX	Ok	Normal	Normal
16	Clear	Female	XX	Ok	Normal	Normal
17	Clear	Male	XY	Ok	Normal	Normal
18	Clear	Female	XX	Ok	Normal	Normal
19	Clear	Female	XX	Ok	Normal	Normal
20	Clear	Female	XX	Ok	Normal	Normal
21	Bloody	Female	XX	Ok	Normal	Normal
22	Clear	Female	XX	Ok	Normal	Normal
23	Clear	Female	XX	Ok	Normal	Normal
24	Clear	Female	XX	Ok	Normal	Normal
25	Clear	Male	XY	Ok	Normal	Normal
26	Clear	Male	XY	Ok	Normal	Normal
27	Clear	Male	XY	Ok	Normal	Normal
28	Clear	Female	XX	Ok	Normal	Normal
29	Clear	Female	XX	Ok	Normal	Normal
30	Clear	Female	XX	Ok	Normal	Normal
31	Bloody	Male	XY	Ok	Normal	Normal
32	Clear	Female	XX	Ok	Normal	Normal
33	Clear	Male	XY	Ok	Normal	Normal
34	Clear	Male	XXY	Ok!	Normal	Normal
35	Clear	Female	XX	Ok	Normal	Normal
36	Clear	Female	XX	Ok	Normal	Normal
37	Clear	Female	XX	Ok	Normal	Normal
38	Clear	Male	XY	Ok	Normal	Normal
39	Clear	Male	XY	Ok	Normal	Normal
40	Clear	Male	XY	Ok	Normal	Normal

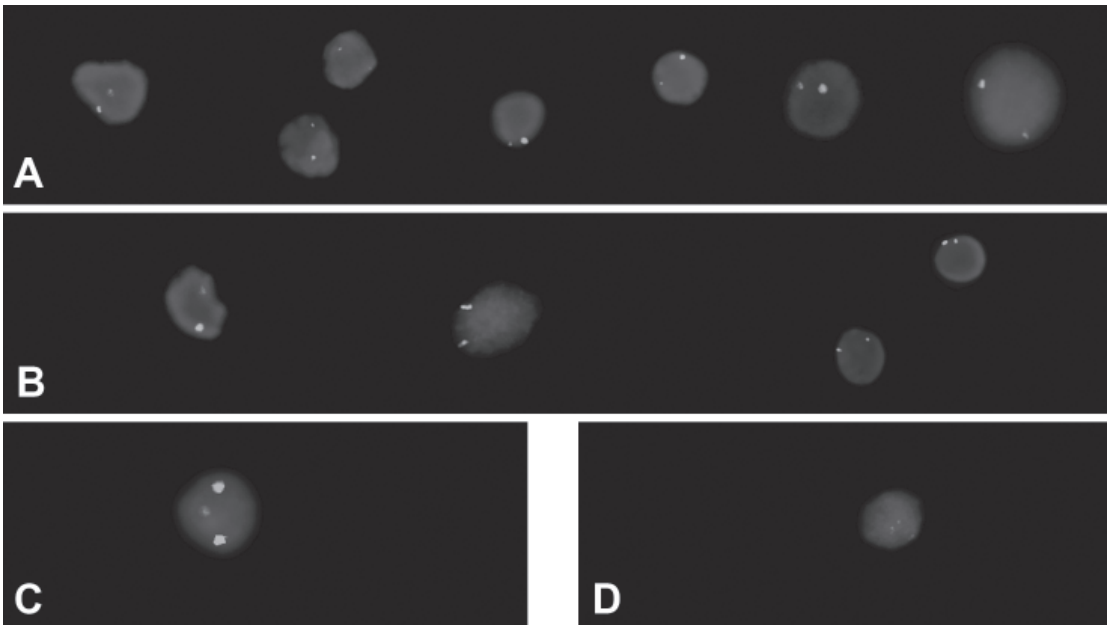

Figure 1: FISH images of X (green) and Y (orange) probes on amniocytes. A) XY, B) XX, C)XXY Klinefelter, D) YYY (from a case with mosaicism of sex chromosome). (This figure has also been printed in full-color at the end of the issue)

Using the code assigned to the FISH samples the results of the FISH tests were married to the data complied by the third researcher.

Results

Table 1 shows the results from the FISH procedures on the uncultured amniocytes together with data on the sex and health condition of the newborns in our research project. Of the 40 samples analyzed by FISH, 22 samples (55%) were XX, 16 samples (40%) were XY, two sample were XXY(5%), and one sample (2.5%) had a 7% sex chromosome mosaicism (46XX/45X). Findings from the FISH tests for sex chromosomes completely confirmed by the reports and there were no instances of false results. The XXY cases have been confirmed by the conventional cytogenetic test but no karyotype has been performed to confirm the sex chromosome mosaicism due to lack of cooperation from the parent. There were no special clinical reports about possible abnormal conditions due to trisomy 21 (Downs syndrome), trisomy 13 and trisomy 18 which are disorders compatible with postnatal survival.

Discussion

Amniotic fluid samples obtained for amniocentesis do not contain any fetal cells in division and have to be grown in vitro to obtain cells at the metaphase stage. Over the last three decades, improved technology for prenatal diagnosis by karyotyping has mainly involved devising methods for reducing culture time. Now in some laboratories average reporting time for the culture of amniotic fluid samples has been reduced to 13-14 days. It is recognized that long waiting times for the results may cause much psychological suffering and this has been one of the main reasons for the introduction of molecular methods for prenatal diagnosis of common chromosome disorders. This type of approach does not require cell culture and reports can routinely be issued within 1-2 days. The most time consuming part of the interphase FISH procedure concerns fluorescence microscopy, and involves spot counting of 25-50 nuclei.

The present study was designed to show the power of FISH as a molecular cytogenetic technique for prenatal diagnosis. The short period of time between sampling and reporting the results is of interest to gynecologists involved in the treatment of patient. Several studies have reported the successful application of this technique in prenatal diagnosis, but its implementation as a standard procedure requires documentation of its reliability and accuracy which can only be addressed by collecting data from a variety of large studies. In this study we chose a simple method of evaluating the technique and showing its reliability to specialists other than geneticists.

Complete agreement between the results of the FISH tests and reports of the sex and results from the clinical examinations of newborns in this study are likely to persuade our perinatologists and gynecologists to prescribe this method as the method of choice for reducing the anxiety of patients at risk of having abnormal offspring due to chromosomal anuploidies.

It seems that improvement in the manufacture of the probes has reduced the false negative and false positive observed previously in studies using FISH techniques for prenatal diagnosis. Authors such as Ward (5) and Bryndorf (6) reported 0.1-0.2 false negative and false positive in their analysis, but other authors such as Thilaganathan (7) and Feldman (8) reported no false negatives or positives when using newer versions of probes such as Aneuvysion. Of course false negatives have been obtained even when using these kinds of probes (9, 10, 11). Different reasons have been reported for these mistakes such as cross hybridization, pericentromeric and centromeric deletions, and reduced copy number of alpha satellite sequences, which can be avoided mostly by using locus specific probes. Maternal cell contamination, poor technical procedures and constitutionally abnormal chromosomes are some of the other reasons behind false negative results, some of which are hard to avoid.

There are efforts underway for ultrarapid (12, 13) and automated (14, 15, 16) FISH examination of amniocytes so that this test can be offered to a broader patient population while providing fast and reliable results.

The findings of different scientists confirm the need for conventional chromosome analysis to complement FISH results. However, the work reported in this paper indicates that FISH analysis on uncultured amniocytes can play an important role in counseling and decision-making, especially in cases at risk for aneuploidies.

Conclusion

Overall, the findings of different scientists confirm the need for conventional chromosome analysis to complement FISH results but these literatures indicate that FISH analysis on uncultured amniocytes could play an important role in counseling and decision-making, especially in the cases at risk for aneuploidies.

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References

1. The Canadian early and midtrimester amniocentesis trial(CE-MAT) group Randomised trial to assess safety and fetal outcome of early and midtrimester amnicentesis. Lancet. 1998; 351: 242-247.
2. Thein AT, Abdel-Fattah SA, Kyle PM, Soothhill PW. An assessment of the use of interphase FISH with chromosome specific probes as an alternative to cytogenetics in prenatal diagnosis Prenat Diagn. 2000; 20: 275-280.
3. Winsor ETJ, Silver MP, Theve R, Wright M, Ward BE. Maternal cell contamination in uncultured amniotic fluid. Prenat Diagn. 1996; 16: 49-54.
4. Jiu J, Zheng XZ, Baramki TA, Yazigi RA, Compton G, Katz E. Ultrarapid detection of sex chromosomes with the use of fluorescence in situ hybridization with direct label DNA probes in single human blastomeres, spermatozoa, amniocytes, and lymphocytes. Fertil steril. 1998; 70(5): 927-932
5. Ward BE, Gersen SL, Carelli MP, McGuire NM, Dackowski WR, Weinstein M, et al. Rapid prenatal diagnosis of chromosomal aneuploidies by fluorescence in situ hybridization: clinical experience with 4500 specimens. Am J Hum Genet. 1993; 52: 854-865.
6. Bryndorf T, Christensen B, Vad M, Parner J, Carelli MP, Ward BE, et al. Prenatal detection of chromosome aneuploidies in uncultured chorionic villus samples by FISH. Am J Hum Gen. 1996; 59: 918-926.
7. Thilaganathan B, Sairam S, Ballard T, Peterson C and Meredith R. Effectiveness of prenatal chromosomal analysis using multicolor fluorescent in situ hybridisation. BJOG. 2000; 107: 262-266.
8. Feldman B, Ebrahim SA, Hazan SL, Gyi K, Johnson MP, Johnson A, et al. Routine prenatal diagnosis of aneuploidy by FISH studies in high-risk pregnancies. Am J Med Genet. 2000; 90: 233-238.
9. Cheong LeungW, Chitayat D, Seaward G,Windrim R, Ryan G, Barrett J, et al. Role of amniotic fluid interphase fluorescence in situ hybridization (FISH) analysis in patient management. Prenat Diagn. 2001; 21: 327-332.
10. Tepperberg J, Pettenati MJ, Rao PN, Lese CM, Rita D, Wyandt H, et al. Prenatal diagnosis using interphase fluorescence in situ hybridization (FISH): 2-year multicenter retrospective study and review of the literature. Prenat Diag. 2001; 21: 293-301.
11. Witters I, Devriendt K, Legius E, Matthijs G, Van Schoubroeck D, Van Assche FA, et al. Rapid prenatal diagnosis of trisomy 21 in 5049 consecutive uncultured amniotic fluid samples by fluorescence in situ hybridisation (FISH). Prenat Diagn. 2002; 22: 29-33.
12. Liehr T, Ziegler M. Rapid prenatal diagnostics in the interphase nucleus: procedure and cut-off rates. J Histochem Cytochem. 2005; 53(3): 289-291.
13. Choolani M, Ho SS, Razvi K, Ponnusamy S, Baig S, Fisk NM, et al. Fast FISH: technique for ultrarapid fluorescence in situ hybridization on uncultured amniocytes yielding results within 2 h of amniocentesis. Mol Hum Reprod. 2007; 13(6): 355-359.
14. Lev D, Daniely M, Zudik A, Preisler E, Hoffmann N, Kaplan T, et al. Automatic scanning of interphase FISH for prenatal diagnosis in uncultured amniocytes. Genet Test. 2005; 9(1): 41-47.
15. Evans MI, Sharp M, Tepperberg J, Kilpatrick MW,

Tsipouras P, Tafas T. Automated microscopy of amniotic fluid cells: detection of FISH signals using the FastFISH imaging system. Fetal Diagn Ther. 2006; 21(6): 523-527.

16. Wauters J, Assche EV, Antsaklis A, Tepperberg J, Sharp SM, Kilpatrick MW, et al. Fully automated FISH examination of amniotic fluid cells. Prenat Diagn. 2007; 27(10): 951-955.