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Recommended Citation

Moatter, T., Khilji, Z., Murad, F., Munim, S. (2007). Analysis of amniotic fluid specimens for common chromosome disorders using interphase fluorescence in situ hybridization. *Journal of Pakistan Medical Association*, 57(4), 189-192.

Available at: https://ecommons.aku.edu/pakistan_fhs_mc_pathol_microbiol/757

Analysis of amniotic fluid specimens for common chromosome disorders using interphase fluorescence in situ hybridization

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Abstract

Objective: The aim of the study was to examine the usage of multi colour FISH technology as an adjunct to conventional cytogenetics for the prenatal diagnosis of aneuploidy in interphase nuclei from high risk pregnancies.

Methods: Amniotic fluid samples were collected for interphase FISH analysis using DNA probes for chromosomes 13, 18, 21, X and Y. All the probes were directly labeled with fluorescent molecules. Fluorescent signals were observed under a microscope. A minimum of 100 nuclei with defined hybridization signals were counted for each probe.

Results: Seventy-eight amniotic fluid samples were received for FISH analysis. The average age of mothers and their gestational ages were 33 years and 17.5 weeks respectively. Triple test screening was positive in 39.5% of the women followed by advanced maternal age and ultrasonographic abnormalities. Interphase FISH was performed on 76 specimens whereas 2 samples were rejected because of blood contamination. Aneuploidy was identified in 6 out of 76 specimens. Two cases of trisomy 21, two cases of trisomy 18 and one case of monosomy X were detected. In addition, one case showed 10% mosaicism for trisomy 21. Initially 4 (5.3%) samples were uninformative due to technical reasons but gave acceptable scoring signals when reanalyzed.

Conclusion: This study has demonstrated that interphase FISH is a rapid and a reliable technique for the enumeration of chromosome number in uncultured amniocytes. Clinicians can use it for making early decisions necessary for the management of high risk pregnancies ultimately saving patients from anxiety and psychological stress (JPMA 57:189;2007).

Introduction

Prenatal diagnosis for chromosomal abnormalities is primarily accomplished by conventional cytogenetic banding of metaphase chromosomes of foetal cells obtained from amniotic fluid. Chromosomal aneuploidies including trisomy 13, 18 and 21, monosomy X and 47, XXY account for more than 80% of live born cytogenetic abnormalities.^{1,2} Cytogenetic analysis is associated with technical difficulties. A major disadvantage of the conventional cytogenetics is the time taken for culture of foetal cells and it range between 10 days to 3 weeks and in some cases culture failure can result which necessitate another invasive procedure.³ Foetal cells for chromosome diagnosis are obtained either by amniocentesis which is done around 16 weeks of gestation or chorionic villus sampling at about 9-11 weeks of gestation. Both these procedures carry an associated risk of 0.1-0.5% for abortion.⁴

The time required for chromosomal analysis might place significant clinical or psychological burden on the patient. Interphase fluorescence in situ hybridization technology in such cases can provide rapid and accurate diagnosis of aneuploidies.⁵⁻⁷ FISH involves hybridization of selected chromosome specific DNA sequences that have been labeled with fluorescent dyes to chromosome preparations. In 1991, the first developed probes were derived from DNA of flow-sorted whole chromosomes and used for prenatal diagnosis of trisomy 13, 18 or 21 on uncultured amniotic fluid cell nuclei.⁸ Several studies have shown that FISH

was highly effective for rapidly determining the number of specific chromosomes in interphase cells, within 48 hours compared to conventional cytogenetics which usually takes 8-10 day.^{9,10} In 2004, the United Kingdom National Screening Committee recommended that the new screening program for trisomies 13, 18 and 21 need not include karyotyping and can offer prenatal diagnosis for Down's syndrome with FISH or PCR as rapid diagnostic tests.¹¹

Chromosomal abnormalities lead to a significant genetic disease burden on our society, especially due to lack of awareness among the general population and also because of lack of diagnostic and management facilities. To our knowledge this is the first report from Pakistan which presents data on the use of multicolor commercially available FISH probes. The aim of the present study was to examine in high risk pregnancies usage of multicolor FISH technology as an adjunct to conventional cytogenetics for the prenatal diagnosis of aneuploidy in interphase nuclei.

Material and Methods

From January 2004 to December 2005, amniotic fluid samples were collected from women who had reported to the clinics at the Aga Khan University Hospital. FISH was not performed on samples if visible blood contamination was present. The gestational age at amniocentesis and the indications for testing were recorded. Indications for testing included advanced maternal age, abnormal values of

biochemical markers, foetal anomalies observed during ultrasound scan, previous child with Down's syndrome or children with chromosomal abnormalities in the family and parents with chromosomal rearrangements. FISH for aneuploidy of chromosomes 13, 18, 21, X and Y was performed using Aneuvysion assay kit according to the manufacturer's instructions (Vysis, Abbott, USA).

Slide preparation

Approximately 5 ml of the amniotic fluid obtained by amniocentesis was spun and the cell pellet was resuspended in 5 ml of trypsin/EDTA and incubated at 37°C water bath. Following centrifugation and 0.56% KCl pretreatment, fixative was added drop by drop to the cells. The suspension was then centrifuged and resuspended in 100 µl fresh fixative, 10 µl of which was used for slide preparation. Slides were then pretreated in 2X SSC for 20 min at 60°C and later digested with pepsin prepared in 0.01N HCl for 20 min at 37°C. Subsequent to rinsing in phosphate buffered saline (PBS) pH 7.5, slides were fixed in 0.95% formaldehyde and then finally washed in PBS at room temperature for 5 min. The specimens were dehydrated through a graded series of ethanol (70%, 85% and 100%) and following air drying step, DNA was denatured in 50% formamide at 73±1 °C for 7 min. Next the slides were placed in 70% ethanol jar for 1 min and this step was repeated with 85% and 100% ethanol then excess ethanol was drained from the slides.

DNA probes

Slides were placed on a 45°C slide warmer and then 10 µl of each well mixed centromeric enumeration probe (CEP) 18/X/Y and locus specific identifier (LSI) 13/21 probes were applied on two separate well defined areas. Cover slips were placed over the probe solution and sealed with rubber cement. The slides were hybridized at 37°C in a hybridization chamber (Hybrite, Vysis, Abbott, USA) for at least 16 hours. Next day after removing the rubber cement the slides were washed in 0.4X SSC/0.3% NP-40 at 73±1°C for 2 min. The slides were air dried in the dark, then to each target area 10 µl of DAPI II (125 ng/ml in antifade) counterstain, was applied before covering it with a glass coverslip. The signals were enumerated using a Nikon E800 microscope with single band pass filters for Spectrum orange, Spectrum green, Spectrum aqua and DAPI that allow visualization of orange, green, aqua signals against a blue background according to the manufacturer's instructions. A minimum of 100 nuclei with defined hybridization signals were enumerated. Cases with aberrant FISH signals were considered as inconclusive and were not counted.

Results

A total of 78 amniotic fluid samples were received in our molecular cytogenetics laboratory from Jan 2004 - Dec 2005 for prenatal analysis of aneuploidies in high risk pregnancies. In two cases samples were rejected because of contamination with maternal red blood cells. The age of the

mothers varied from 22 to 45 years (mean 33 years). At the time of amniocentesis gestational age ranged between 16-21 weeks (mean 17.5 weeks). Several indications for FISH assays were recorded (Table). The main referral reason was positive triple test screening results which accounted for approximately 39.5% whereas high maternal age and ultrasonographic abnormalities were indicated in 25% and 11.8% cases respectively. Among the ultrasonography abnormalities the most common were pleural effusion, nuchal thickening (range 5.2-8.4 mm) heart defect, skeletal dysplasia and omphalocele. Surprisingly, 23.7% women opted for screening because of the presence of a child with Down's syndrome in the family.

When compared to conventional karyotyping which takes 10 to 20 days (mean time 14 days) FISH assay was completed within 24-48 hours and were mostly reported within 36 hours after the receipt of the sample. Signals from both CEP (X, Y, 18) and LSI (13, 21) probes were clearly defined and easily recognized under the microscope. Moreover, signal from chromosome 18 probe was more diffuse and stringy compared to compact and sharp signals from LSI probes (Figure). In FISH preparations, nuclei of amniocytes showed well defined margins and were evenly stained with DAPI. In all sample preparations 100-200 nuclei were screened for all type of probes. The results of the FISH assay were considered uninformative if there was undetermined hybridization pattern or technical difficulties were encountered in doing the analysis. Numerical aberrations were identified in 6 out of 76 specimens. There were two cases of trisomy 21, two cases of trisomy 18, one case

Table. Indications for prenatal diagnosis.

Indication	Number	Percentage
Triple test screening positive	30	39.5
Advanced maternal age	19	25
Previous Down syndrome child	18	23.7
Abnormal ultrasound findings	9	11.8
Total	76	100

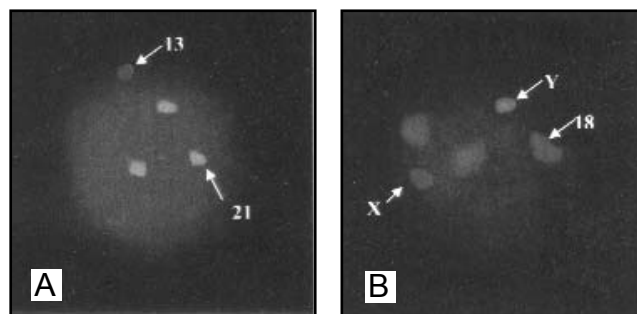


Figure. (a) A representative photograph of an amniocyte nucleus hybridized with chromosome 13 (green color) and 21 (red color) specific LSI probes. Three red signals point toward trisomy 21. (b) An interphase nucleus shows three signals of chromosome 18 specific CEP probe (aqua blue) indicative of Edward's syndrome.

of monosomy X and one sample mosaic for trisomy 21. The mosaic sample was repeated to confirm the result. A FISH image from a case of trisomy 21 is shown in figure 1a,

it demonstrates three signals (red) for chromosome 21 and two signals (green) for chromosome 13. Figure 1b, shows a FISH image of Edward's syndrome with three chromosome 18 signals (aqua blue). Overall 70 FISH specimen were normal, initially 4 (5.3%) were uninformative due to technical reasons, when these samples were reanalyzed satisfactory results were obtained.

Discussion

Conventional cytogenetics was first used to show chromosomal aneuploidy in 1959 by studies of Lejeune and colleagues, who described an extra chromosome in cultured fibroblasts from patients with Down's syndrome.¹² Additional chromosomal syndromes which involved sex chromosomes including Turner syndrome were also described in 1959.¹³ Amniocentesis was first performed in 1952 and in 1972 it became the standard for obtaining foetal cells and today the most common reason for prenatal diagnosis is increased risk of having a child with trisomy 21.¹⁴

Standard cytogenetics is associated with a number of technical difficulties. The major disadvantage of karyotyping is the long turnaround time to obtain the test results due to the cell culture requirement. This delay puts women under increased anxiety and depression. In addition, fear of a false positive result also leads to psychological pressure. According to Rausch and colleagues, this anxiety was the cause of decreased participation in maternal serum screening in subsequent pregnancies.¹⁵ Studies have shown that awaiting cytogenetic results has been determined to be a most anxiety provoking aspect of prenatal diagnostic procedures and this concern is often reported by physicians as a reason for not offering prenatal screening to women.^{16,17}

In 1992, FISH assay on uncultured amniocytes was used for detection of numerical aberrations in high risk pregnancies as a choice for patients with increased emotional and psychological pressure.^{18,19} Klinger et al in 1992 were the first to study applications of FISH on interphase nuclei of amniocytes using probes for 13, 18, 21 X and Y followed by a larger study carried out by Ward et al in 1993.^{18,20} Since then many studies have been published in the literature. Hulten et al predicted a risk for false positive result is less than 1 in 30 000 and that of a false negative result to be 1 in 4000.²¹ FISH was shown to diagnose aneuploidies within 24-48 hours following amniocentesis which decreases the time interval between sampling and diagnosis. This is of tremendous value in prenatal diagnosis of urgent high-risk pregnancies. After extensive clinical validation, the Food and Drug Administration has approved

Aneuvysion FISH assay for the detection of aneuploidies of chromosome 13, 18, 21, X and Y²² in 1999. Presently, FISH is the most common types of molecular method for prenatal diagnosis because of its short turn-around time which provides a major benefit for pregnancy management especially in emotionally distressed women who have a previous Down's child. As observed in the present study, a large number (23%) of women who have opted for FISH based prenatal screening had a previous child with Down's syndrome. In 2004, the UK National Screening Committee recommended FISH for screening for Down's syndrome as an independent test.¹¹ Therefore, it is important that all high risk pregnancies based on indications including abnormal ultrasonography, advanced maternal age, biochemical screening and a history of Down's syndrome in the family must undergo prenatal screening. This would protect the parents from emotional trauma and financial liability for special care and treatment for the child in future. These factors stress the need for rapid results to the clinician and patient leading to better pregnancy and anxiety management. In this study one mosaic sample showed 10% of nuclei carrying 3 signals of LSI 21, whereas no direct relationship was found between the degree of mosaicism judged by FISH and conventional cytogenetics. In some studies it has been proposed that it might be due to variations in signals which can be attributed to parameters such as sample quality, slide preparation and hybridization or detection efficiency rather than to low level mosaicism.²³

In spite of its rapid technological advantages FISH has certain limitations compared to standard karyotyping which can detect structural chromosomal abnormalities and gives a complete chromosome picture undetectable by present FISH approaches. Maternal cell contamination may cause problems in FISH assay. It has been estimated that 1-2% of amniotic fluids contain visible blood stained which can lead to false negative diagnosis of foetal aneuploidy.²⁴ In a study carried out by Jobanputa et al presence of maternal cell contamination was approximately 2% (range 0% - 10%) estimated from the number of nuclei with XX signals in karyotypically male samples.²⁵ Therefore, as a precautionary measure laboratories should discard any heavily blood contaminated samples.

In conclusion, FISH usage on interphase nuclei of amniocytes is beneficial for early diagnosis of genetic disorders. Moreover, FISH assay is rapid and technically easy to perform, it should be considered for the genetic diagnosis all management of high risk pregnancies in Pakistan.

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