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The clinical application of interphase FISH in prenatal diagnosis

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Fluorescence *in situ* hybridization (FISH) for five chromosomes (13, 18, 21, X and Y) detected 87 of 107 (81%) of the chromosome aberrations identified by conventional chromosome analysis applied to fetal interphase cells obtained by chorionic villus sampling or amniocentesis. The choice of FISH was solely determined by prospective parents after formal genetic counselling concerning the advantages and disadvantages of FISH analysis. Excluding known familial chromosome aberrations, if FISH analysis revealed normal signals, there was an overall residual risk of 1 in 149 for an undetectable chromosome aberration. This risk varied according to the indication for prenatal diagnosis: 1 in 177 for women of advanced maternal age; 1 in 60 for women at increased risk for Down syndrome based on maternal serum screening; and, 1 in 43 for women whose ultrasound examination revealed fetal anomalies. There were 20 cases of discordance between the FISH results and standard karyotype analysis: four were the outcome of a failure to apply the appropriate FISH probe; 16 were not detectable by the available FISH probes. Of these 16, nine were chromosome abnormalities with clinical significance and seven were familial. If FISH is to become a standard part of prenatal genetic diagnosis, genetic counselling that is sensitive to patient health needs must be based on accurate information about the biological and obstetrical implications of the results of FISH analysis. Copyright © 2000 John Wiley & Sons, Ltd.

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INTRODUCTION

The introduction of fluorescence *in situ* hybridization (FISH) analysis into the clinical practice of prenatal diagnosis has been controversial. The singular benefit of FISH is the rapid detection of aneuploidy by chromosome-specific probes applied to interphase cells. A FISH assay can easily be completed in less than 24 h, compared to the 7–12 days usually required with conventional chromosome analysis. The issue is whether this advantage offsets several of the criticisms about the use of FISH in prenatal diagnosis. At one time the claim was made that the use of FISH in prenatal diagnosis was premature, for it did not provide the accuracy of conventional cytogenetics (Ledbetter, 1992). Aneuploidies of chromosomes 13, 18, 21, X and Y constitute 80–95% of chromosome aberrations associated with birth defects in liveborns (Ratcliffe *et al*., 1970; Jacobs *et al*., 1974). Whereas FISH analysis for these chromosomes had been successfully performed with a high degree of concordance with cytogenetic results (Nederlof *et al*., 1990; Ward *et al*., 1993; Philip *et al*., 1994; Eiben *et al*., 1999), not all chromosome abnormalities, particularly structural rearrangements, can be routinely identified by FISH when compared with conventional chromosome analysis (Evans *et al*., 1999). In response to these criticisms, guidelines have been established for the use of FISH in prenatal diagnosis (Schwartz, 1993; American College of Medical Genetics, 1993). These guidelines recommended that the experimental nature of FISH be described clearly and reiterated at the time of reporting results; that informed consent should be required; and, that patients make no irreversible therapeutic decisions based on FISH analysis alone.

We present a 36-month experience at Prentice Women's Hospital and Maternity Center, Chicago, IL, in which FISH analyses were applied to the routine practice of prenatal diagnosis. A unique feature was that prospective parents solely determined the choice of FISH probes after formal genetic counselling concerning the advantages and disadvantages of FISH analysis. We address the concerns about FISH with regard to its accuracy, the limited number of identifiable chromosomes, the additional cost, and its role in a prenatal diagnosis programme.

MATERIALS AND METHODS

Patient participation

Since February, 1996, all women undergoing either chorionic villus sampling (CVS) between 10 and 12 weeks' gestation or amniocentesis between 15 and 20 weeks' gestation were offered FISH analysis in addition to the obligatory conventional chromosome analysis. Standard genetic counselling about the risks and benefits of CVS or amniocentesis was extended to include information about FISH. This was in three

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forms: (1) a two-page narrative describing the then current status of FISH analysis; (2) genetic counselling by certified genetic counsellors; and (3) a consent form specific for FISH. For the first 18 months, patients were informed that the FISH probes were not approved by the Food and Drug Administration (FSA) of the United States federal government and that costs for FISH analyses would be their responsibility. In June 1998, FISH probes were approved by the FDA, and CPT codes (Current Procedural Terminology) for submitting charges to third party payers (insurance) shortly followed. Throughout the study period, genetic counselling was non-directive with respect to patients' determination of whether they wanted a FISH analysis and which FISH probes they selected.

Sample preparation

Following CVS, 3–5 mg of villus tissue was placed in 5 ml of trypsin–pepsin for 2 h at 37°C; after vigorous pipetting, the dispersed single cells were suspended in 0.5 ml of basic salt solution (BSS) and 8 ml of warmed hypotonic solution (37°C) was added for 20 min; following centrifugation and suspension in 0.5 ml of hypotonic solution, the cells were fixed (3 parts methanol: 1 part acetic acid) for 30 min at room temperature. For amniocentesis, usually 15–20 cc of amniotic fluid was obtained, from which 2–4 cc were set aside for FISH analysis. Amniotic fluid cells were then exposed to hypotonic solution, followed by fixation as described for chorionic villus tissue. After replacing the original fixative with fresh fixative, villus and amniotic fluid cells were dropped onto oblique slides, which were then dried at 60°C for 5–10 min and dehydrated in 100% ethanol. Usually, two slides were prepared for each patient sample.

FISH probes were commercial products (AneuVysion Assay Kit) available from Vysis, Inc. (Downer's Grove, IL, USA) and applied according to the manufacturer's instructions. The probes were comprised of two sets, one set containing alpha satellite probes for the X, Y and 18 chromosomes and the second set containing probes spanning the retinoblastoma gene (RBI) at 13q14 and the region 21q22.13–q22.2.

Each set was applied to one slide side-by-side. Positive and negative control slides were applied in accordance with FDA requirements. Generally, hybridization was performed overnight but with the use of the Hybrite techology (Vysis, Inc.), hybridization was performed in 5 min for the first set (chromosomes X, Y and 18) and in 1 h for the second set (chromosomes 13 and 21). There were no differences in results for the different hybridization times.

FISH analysis

For the first 12 months of the study period, two technicians evaluated 30 different hybridized nuclei for each of the five chromosomes. Nuclei were evaluated under 625X magnification using a 65X oil objective. The number of nuclei displaying 1, 2, 3 or 4 hybridization signals was recorded for each of the five probes. For the remaining 18 months, one technician was responsible for FISH analysis of 30 cells for each of the five probes; similar to the experience of Eiben *et al.* (1999), we have found no difference between analysing 30 and 50 cells. The laboratory standard for equivocal results for identifying aneuploidy was set at $\geqslant 5\%$ of the cells.

Conventional chromosome analysis

Four primary cultures from each chorionic villus or amniotic fluid cell sample were established. Cytogenetic analyses of G-banded chromosomes were performed by the *in situ* technique. In all cases, 15 cells from 15 different colonies in at least two different primary cultures were analysed for chromosome number; five cells were karyotyped through the microscope; and, two cells were captured by computer imaging (PSI system) and analysed from prints for chromosome structural integrity.

RESULTS

During the 36-month study period between February 1996 and February 1999, 3969 women with singleton pregnancies underwent prenatal diagnostic testing. In offering FISH, this laboratory was responding to a growing number of physician and patient requests as well as the implementation of FISH by other local laboratories. A total of 2336 women (59%) elected to have FISH analysis of either their villus or amniotic fluid cells. Fifty-four per cent of the patients elected to have FISH analysis performed using five probes (X, Y, 13, 18 and 21); the rest selected three or fewer probes, with 26% selecting to be given the results of only one of the FISH probes. The consistent reason for their choice of having a FISH analysis was the rapid turnaround-time of 24 h for preliminary results regardless of the reason for undergoing prenatal testing.

Indications for prenatal diagnosis were organized into five categories by descending order of frequency (Table 1). The incidence of chromosome aberrations detected by FISH was compared to conventional chromosome analysis for each of the FISH probes selected by the prospective parents. Overall, FISH detected 87 of 107 (81%) of the chromosome aberrations identified by conventional chromosome analysis. There were four instances in which prospective parents elected not to include the FISH probe that would have resulted in detection of the chromosome aberration (Table 1). In all instances, prospective parents were informed of any abnormal FISH results even though they may not have selected or paid for the specific probes identifying a chromosome aberration.

If the five available FISH probes had been applied routinely rather than by parental selection, 84% of abnormal fetal chromosome complements would have been identified. In all cases where a numerical

Table 1—Comparison of chromosome abnormalities detected by FISH with conventional karyotyping methods for prenatal diagnosis

Indication	Total no. of cases	Karyotype		FISH		Detection rate[a]
		Normal	Abnormal	Detected	Undetected	
Advanced maternal age	1642	1585	56	46	10(2) [b]	82%
Maternal serum screening for Down syndrome	494	474	20	14	6(2)	70%
Maternal serum screening for trisomy 18	16	15	1	1	0	100%
Previous chromosome anomaly	32	29	3	3	0	100%
Ultrasound anomalies	152	125	27	24	3	89%

[a]Detection rate = (no. FISH detected)/(no. abnormal karyotypes).
[b]Figures in parentheses represent cases in which the patient elected not to include the FISH probe that would have resulted in detection of the chromosome abnormality.

chromosome aberration was indicated by FISH analysis, the abnormal number of signals consistently exceeded 96% of the total cell population under study.

Advanced maternal age

In the case of the most frequent indication for prenatal diagnosis, advanced maternal age (35 years or older), FISH analysis detected 82% of the abnormal karyotypes. In two cases, the numerical chromosome aberration involved the X chromosome and since the parents did not want to know the sex of the fetus, probes for the X and Y chromosomes were excluded by their choice (Table 2, cases 2 and 4). If the entire set of five probes were to have been routinely applied rather than by parental choice, the detection rate for advanced maternal age would have increased by less than 2%, to 85.7%.

Table 2—Discrepancies between FISH and conventional karyotype analyses

Karyotype	FISH Probe	Indication	Reason for discrepancy		Comment
			Chromosome probe not requested by patient	Anomaly not detectable with w/probes	
47,XY,+21	X	AMA	X		
45,X/46,XY	13,21	MSS for DS[a]	X		
47,XX,+21	X,Y,18	Pelviectesis	X		Confined placental mosaicism
47,XXX	13,21	AMA	X		
46,XX+21p-	13,21	MSS for DSS		+	Familial
47,XX,+22	13,21	AMA		+	
47,XX+mar	X,Y.13,18,21	AMA		+	
46,del(X)(p11.3p22.3)/ 46,XX	X,Y,13,18,21	AMA		+	
47,XY,+10/ 46,XY	X,Y,13,18,21	AMA		+	Confined placental mosaicism
46,XX,t(5;21)	X,Y,13,18,21	AMA		+	Parents normal
46,XX,+13q mar	X,Y,13,18,21	MSS for DS		+	Familial
46,XY,t(3;21)	X,Y,13,18,21	MSS for DS		+	Parents normal; balanced translocation but multiple anomalies at birth
46,XY,inv (2)(p11q13)	X,Y,13,18,21	Cystic hygroma		+	Familial
46,XY,t(X;8)	X,Y,13,18,21	AMA		+	Familial
46,XY,t(8;11)	X,Y,13,18,21	AMA		+	Familial
47,XX+inv(18)dic(18;18)(q23;q23)	X,Y,13,18,21	AMA		+	Parents normal
45,XY,t(13;14)	X,Y,13,18,21	MSS for DS		+	Familial
46,XY,t(2;4)	X,Y,13,18,21	MSS for DS		+	Familial
47,XX,+der(10) t(3;10)	X,Y.13,18,21	Cystic hygroma		+	Familial
46 XY,inv(6)	X,Y,13,18,21	AMA		+	Parents Normal

[a]MSS, maternal serum screen; DS, Down syndrome.

Table 3—Detection of chromosome abnormalities by FISH when prenatal diagnosis is indicated by various ultrasound anomalies

Ultrasound anomaly	Total no. of cases	Karyotype		FISH	
		Normal	Abnormal	Detected	Undetected
Choroid plexus cyst	46	44	2	2	0
Cystic hygroma	7	3	4	2	2
Congenital heart defect	5	4	1	1	0
Echogenic bowel	8	8	0	0	0
Hydrops	6	5	1	1	0
IUGR	12	10	2	2	0
Oligohydramnios	7	6	1	1	0
Omphalocoele	2	2	0	0	0
Pelviectesis	19	18	1	1	0
Possible bladder anomalies	1	0	1	1	0
Multiple congenital anomalies[a]	39	25	14	13	1

[a]Multiple congenital anomalies: two or more affected systems.

Increased risk for Down syndrome

The second most frequent indication for prenatal diagnosis, a risk for Down syndrome of 1 in 250 or greater based on multiple marker screening (alpha-fetoprotein, unconjugated oestriol and human chorionic gonadotrophin), yielded the lowest FISH detection rate for chromosome anomalies, 60% (Table 1). The actual detection rate would have been 70% if appropriate FISH probes had been applied, but in two of the eight cases the parents failed to select the correct and available FISH probe (Table 2, cases 1 and 2).

Ultrasound anomalies

FISH probes selected by prospective parents detected 89% of the chromosomally abnormal fetuses present in 27 of the 152 pregnancies undergoing prenatal diagnostic testing because of structural anomalies or oligohydramnios (Tables 1 and 3). The three karyotypically abnormal fetuses that FISH analysis did not detect are detailed. Two fetuses undergoing prenatal diagnosis because of cystic hygroma carried chromosome rearrangements not identifiable by the five FISH probes (Table 2, cases 13 and 19); one case, however, was known to be familial. The third case (Table 2, case 3) involved confined placental mosaicism for trisomy 21, which was not detected by conventional chromosome analysis of villi nor present in amniotic fluid cells.

Previous pregnancy with a chromosome aberration

The three cases of a recurrence of a conception with a chromosome aberration were identified by FISH analysis (Table 1).

Increased risk for trisomy 18

A case of trisomy 18 was identified following FISH analysis, based on maternal serum values of 0.75 MoM or less for alpha-fetoprotein, 0.6 MoM or less for unconjugated estriol, and 0.5 MoM or less for human chorionic gonadotrophin (Table 1).

DISCUSSION

The present study documents that FISH analysis of villus and amniotic fluid cells is extremely accurate in detecting numerical aberrations for chromosomes 13, 18 21, X and Y. In the 2335 patients involving more than 6000 individual FISH analyses, there were no false positives (excluding one case of confined placental mosaicism) and no false negatives, consistent with the findings of Ward *et al.* (1993) and Eiben *et al.* (1998). Discordance between the FISH analysis and conventional karyotyping involved either confined placental mosaicism (one case), structural chromosome aberrations (13 cases), numerical chromosome aberrations for which the FISH probes were not designed to identify (two cases) as well as the four cases for which the available FISH probe was not selected by the parents (Table 2).

Of the 16 cases of discrepancy remaining after excluding the instances in which there was a failure to apply the appropriate probe, seven were not clinically significant and were primarily familial in origin. Of the 107 chromosome aberrations comprising the present study, nine (8.4%) clinically significant karyotypic abnormalities could not be detected by FISH. The overall detection efficiency of all chromosome abnormalities by FISH was 85%, higher than the 'in principle' figure of 69.4% efficiency reported by Evans *et al.* (1999) in their retrospective assessment of 146 128 cases from eight centres and four countries. The 16 cases that would have been undetected included four cases of previously unknown familial

Table 4—Change in risk pre- and post-FISH analysis, based on selection of probes by parents versus the application of probes for chromosomes 13, 18, 21, X and Y

Indication	Incidence: chromosome aberration	Residual risk of chromosome aberration if FISH probes normal based on:	
		Parent selection	All 5 probes
Advanced maternal age	1 in 29	1 in 177	1 in 199
Increased risk for Down syndrome	1 in 25	1 in 60	1 in 80
Ultrasound anomaly	1 in 6	1 in 43	1 in 43

translocations, of which one was chromosomally unbalanced.

Excluding known familial cases, if FISH analysis revealed normal signals, there remained 1 in 149 chance of an undetectable chromosome aberration capable of producing an adverse pregnancy outcome (15 in 2238, including three cases where parents failed to select the FISH probe capable of identifying numerical chromosome aberration). This risk varied according to the indication for prenatal genetic diagnosis.

For women of advanced maternal age, the frequency of fetal chromosome aberrations was 3.4%, or 1 in 29 (Table 4). Applying FISH on the basis of patient selection alone, the risk of a chromosome aberration being present following a normal FISH analysis was 1 in 177. If FISH analysis had been routinely applied for the entire set of five probes, there was a slight decrease in the risk to a 1 in 199 chance of a chromosome aberration being present that could not be identified by FISH analysis. And, if the two known familial translocations were excluded, the risk would actually be 1 in 265 (Table 4).

For women at increased risk of Down syndrome based on maternal serum multiple marker screening, the odds of the fetus being chromosomally abnormal was 1 in 25 (Table 4). Following a normal FISH analysis based on parental selection, the residual risk of an undetected chromosome abnormality was reduced to 1 in 60. If the five probes were routinely applied, the chance of a chromosome aberration being present after a normal FISH analysis would be 1 in 80 (6 in 480 cases).

For women undergoing prenatal genetic diagnosis for fetal anomalies detected by ultrasound, the risk of a chromosome aberration being present was 17.8% or approximately 1 in 6. With a normal FISH analysis, this risk was reduced to 1 in 43 and this would not have changed substantially if FISH had been applied routinely. It can be concluded that parental selection of FISH probes was successful in detecting chromosome aberrations, if compared to the theoretical application of all available FISH probes. Nevertheless, while FISH analysis in prenatal genetic diagnosis can be reassuring, we concur with Evans *et al.* (1999) that its application should only be provided in the context of complete and accurate genetic counselling and that there is a continued need for chromosome analysis based on established procedures.

In the past, FISH analysis for prenatal diagnosis has been criticized for several reasons. One concern related to maternal cell contamination following CVS or amniocentesis, with reports in the case of uncultured amniotic fluid cells of 20% or greater (Winsor *et al.*, 1996; NuB *et al.*, 1994) and as high as 80% in blood-stained specimens (Christensen *et al.*, 1993). Undetected maternal decidua in the case of CVS specimens and maternal leukocytes in amniotic fluid that fail to grow in tissue were presumed sources of maternal cell contamination. Significant maternal cell contamination could lead to serious diagnostic errors for genetic disorders due to either chromosomal or single gene mutations. The level of maternal cell contamination is in fact directly related to the obstetrical procedure, not to the FISH analysis, and were consistently less than 2% when CVS or amniocentesis was performed by an experienced operator or with supervison (Hockstein *et al.*, 1998). The concern about maternal cell contamination highlights several advantages of FISH over conventional chromosome analysis. FISH analysis provides a more accurate estimate of chromosome mosaicism than conventional chromosome analysis. FISH can rapidly analyse more interphase cells in comparison to conventional cytogenetics, which requires metaphase cells following long-term tissue culture. In the case of chromosome mosaicism, FISH is also not subject to possible differential selection as a consequence of tissue culture.

Another criticism of the application of FISH in prenatal diagnosis relates to cost versus benefits. The use of FISH in prenatal diagnosis programmes has been questioned because it fails to identify all chromosome aberrations, requires additional labour and expense beyond conventional karyotyping, and, according to established guidelines, should not be the basis of any final decisions concerning pregnancy status, particularly elective termination. FISH was offered in the present study with the belief that it provides considerable relief of anxiety for those receiving a normal result and an indication for the need to prepare for those receiving an abnormal result. This approach was originally supported by assertions that FISH would be clinically justifiable in situations of great parental stress, e.g. in patients with increased risk of Down syndrome based on maternal serum multiple marker screening (Ward *et al.*, 1993) or just to lower anxiety levels in general (Schwartz, 1993).

The reasons FISH was selected included speed of

receipt of results (56%), advance preparation (16%) and emotional needs (13%). The reasons FISH was not selected included cost (46%), preliminary nature of results (31%), and lack of a benefit that could not be awaited (31%) (Tucker, 1997. The efficiency of FISH analysis for common aneuploidies in relieving the anxiety of patients awaiting prenatal diagnosis results, Master Thesis, Northwestern University Graduate School; Tucker *et al.*, manuscript in preparation).

All women choosing FISH reported anxiety relief following receipt of results (100% versus 54% for controls at the same time frame, 3–4 days after prenatal diagnosis procedures). FISH was highly valued by those who selected it (3.88 on a 4-point scale) and even valued by some that did not. The efficacy of FISH analysis in relieving anxiety of patients and their partners awaiting results of prenatal diagnosis was investigated in conjunction with the present study (Tucker, 1997, Master Thesis; Tucker, 1998) and revealed the following: Patients choosing FISH had a significantly higher initial level of anxiety than those declining FISH, based on the State Trait Anxiety Inventory developed by Marteau and Bekker (1992). Providing FISH results significantly reduced anxiety in the 'high anxiety' group (women at increased risk for Down syndrome, abnormal ultrasound or maternal anxiety) but not for women of advanced maternal age. Nevertheless, the anxiety levels of the 'high anxiety' group still remained higher than women declining FISH. The overwhelming positive response to FISH and the unanimous report of decreased anxiety should contribute to any discussion on the value and clinical indications of FISH analysis in prenatal diagnosis programmes (Tucker, 1997, Master Thesis). The cost prohibitive aspect of FISH analysis also must be examined concomitantly, as it played a significant role in both control and study patients' levels of satisfaction (Tucker, 1997, Master Thesis).

Finally, based on the results of the present study, the question arises as to whether certain current guidelines should be re-evaluated. Consider the following scenario: at 20 weeks' gestation, maternal serum multiple marker screening indicates an increased risk for Down syndrome; an ultrasound evaluation reveals physical changes compatible with trisomy 21; and, FISH analysis shows three signals indicating three copies of chromosome 21 in uncultured amniotic fluid cells. Given that serum and ultrasound findings are insufficient to provide a secure diagnosis, the issue is whether therapeutic decisions require waiting for completion by a conventional chromosome analysis. Cost-benefit analyses in such cases require further study by genetic professionals. If FISH is to become a standard part of prenatal genetic diagnosis, genetic counselling must continue to be sensitive to patient health needs and must be based on accurate information about the biological and obstetrical implications of the FISH results. If any therapeutic decision were to be made on the basis of FISH results alone, the decision must be reached by a clear understanding of the nature of FISH analysis and the chromosome abnormalities detected.

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