Am. J. Hum. Genet. 52:854-865, 1993

Rapid Prenatal Diagnosis of Chromosomal Aneuploidies by Fluorescence In Situ Hybridization: Clinical Experience with 4,500 Specimens

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Summary

Detection of chromosome aneuploidies in uncultured amniocytes is possible using fluorescence in situ hybridization (FISH). We herein describe the results of the first clinical program which utilized FISH for the rapid detection of chromosome aneuploidies in uncultured amniocytes. FISH was performed on physician request, as an adjunct to cytogenetics in 4,500 patients. Region-specific DNA probes to chromosomes 13, 18, 21, X, and Y were used to determine ploidy by analysis of signal number in hybridized nuclei. A sample was considered to be euploid when all autosomal probes generated two hybridization signals and when a normal sex chromosome pattern was observed in greater than or equal to 80% of hybridized nuclei. A sample was considered to be aneuploid when greater than or equal to 70% of hybridized nuclei displayed the same abnormal hybridization pattern for a specific probe. Of the attempted analyses, 90.2% met these criteria and were reported as informative to referring physicians within 2 d of receipt. Based on these reporting parameters, the overall detection rate for aneuploidies was 73.3% (107/146), with an accuracy of informative results for aneuploidies of 93.9% (107/114). Compared to cytogenetics, the accuracy of all informative FISH results, euploid and aneuploid, was 99.8%, and the specificity was 99.9%. In those pregnancies where fetal abnormalities had been observed by ultrasound, referring physicians requested FISH plus cytogenetics at a significantly higher rate than they requested cytogenetics alone. The current prenatal FISH protocol is not designed to detect all chromosome abnormalities and should only be utilized as an adjunctive test to cytogenetics. This experience demonstrates that FISH can provide a rapid and accurate clinical method for prenatal identification of chromosome aneuploidies.

Introduction

Cytogenetic analysis of fetal cells after amniocentesis is routinely offered to women who have an increased risk of carrying chromosomally abnormal fetuses. Indications for such prenatal diagnoses include advanced maternal age, increased risk for fetal trisomy identified by maternal serum screening (Wald et al. 1988; Canick et al. 1990), and fetal abnormalities detected through ultrasound examination. Currently, fetal chromosome abnormalities are diagnosed by examination of banded metaphase chromosomes. This analysis detects chromosome aneuploidies with great accuracy and is also capable of diagnosing chromosome rearrangements. The primary disadvantage of traditional cytogenetics is that amniocytes must be cultured for several days prior to analysis. In certain clinical situations the time required to complete the chromosome analysis may place a significant clinical or emotional burden on the patient and/or the health-care provider. In such cases, a method which provides rapid and accurate identification of the most common fetal aneuploidies would be a useful adjunctive diagnostic test to traditional cytogenetics.

Received September 29, 1992; revision received December 29, 1992.

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0002-9297/93/5205-0002$02.00

Procedures which allow rapid detection of certain chromosomal abnormalities in uncultured fetal cells have been known for some time, as demonstrated by analysis of Barr and Y bodies to detect sex chromosome aneuploidies (Robinson et al. 1979; Penketh et al. 1989; West et al. 1989). Fluorescence in situ hybridization (FISH) to interphase nuclei with chromosome-specific DNA probes can now rapidly and accurately detect the most common autosomal trisomies and aneuploidies of the sex chromosomes (Cremer et al. 1986; Lichter et al. 1988*a*, 1988*b*, 1990; Klinger et al. 1990, 1992; Lichter and Ward 1990; Christensen et al. 1992; Lebo et al. 1992; Zahed et al. 1992; Zheng et al. 1992). The general strategy for utilization of FISH has been extensively reviewed (McNeil et al. 1991; Tkachuk et al. 1991; Trask 1991; Ledbetter 1992; Sawyer et al. 1992). Rapid and accurate detection of chromosomal aneuploidies has been demonstrated in a blinded prospective research study which compared aneuploid detection by FISH for chromosomes 13, 18, 21, X, and Y to results obtained by cytogenetics (Klinger et al. 1992). This study formed the basis for the establishment of a clinical protocol for the application of FISH to prenatal diagnosis.

In the current clinical program, FISH technology was utilized for the rapid detection of aneuploidies of chromosomes 13, 18, 21, X, and Y. This focus was based on the following observations: (*a*) approximately 80% of prenatal genetic amniocenteses are performed because of the presence of an increased risk for the common autosomal trisomies, and (*b*) aneuploidies for these chromosomes are reported to account for greater than 60% of all chromosomal abnormalities detected at amniocentesis and may account for up to 95% of all chromosomal abnormalities which are accompanied by birth defects in newborns (Rhoads et al. 1989; Robinson et al. 1991; Whiteman and Klinger 1991; Lebo et al. 1992; Richkind 1992). The potential for routine application of FISH technology for rapid prenatal diagnosis of chromosomal aneuploidies has been established (Yu et al. 1990; Kuo et al. 1991; Klinger et al. 1992). We herein describe our experience with the first 4,500 cases referred for rapid aneuploidy detection by FISH which was performed as an adjunctive test to cytogenetics.

Material and Methods

Probes

Probe sets were developed from unique sequence regions within chromosomes 13, 18, and 21 (Klinger et al. 1992). The chromosome 21 probe set was initiated with D21S71 and, after expansion, consisted of a three-cosmid contig containing 80 kb of nonoverlapping DNA directed at chromosomal region 21q22.3. The chromosome 18 probe set was composed of 109 kb of nonoverlapping DNA contained in a three-cosmid contig within the chromosomal region 18q23. The initial cosmid in the chromosome 18 set contained MBP (gene designation). The chromosome 13 probe contained approximately 97 kb of DNA within the 13q12.3 region. The first cosmid of the set was identified using D13S6 as probe. The noncentromeric targets of these autosomal probes allow detection of free trisomies, trisomies involving Robertsonian translocations, and triploids.

The X chromosome probe set was composed of a single cosmid which hybridizes to the paracentromeric region on the X chromosome. This cosmid contains a 2-kb *Bam*HI fragment which, on the basis of its hybridization location and signal intensity as well as the presence of a *Pst*I site 690 bp from one end of the *Bam*HI fragment, mimics a previously described alpha-satellite repeat on the X chromosome (Waye and Willard 1985). Sequencing of the 2-kb *Bam*HI fragment is in progress. The Y probe was derived from the repetitive clone pDP97 (provided by D. Page, Whitehead Institute Biomedical Research, Cambridge, MA), a subclone of the alpha-satellite repeat present in the cosmid Y97 (Wolfe et al. 1985).

Sample Processing and Hybridization

The methods employed to obtain locus-specific signals within uncultured amniotic fluid cell nuclei have been reported (Klinger et al. 1992). Probe labeling, hybridization under suppression conditions, and detection were as described elsewhere (Landegent et al. 1987; Cremer et al. 1988; Klinger et al. 1990, 1992). To ensure optimal probe performance, labeled probe sets were characterized prior to use, in the following manner: Incorporation of labeled nucleotides was monitored using a radioactive tracer to ensure at least 30% replacement with the derivatized nucleotide. Finished probe size was analyzed using gel electrophoresis, and mean fragment size was required to be between 100 and 250 nucleotides. The specificity and sensitivity of all probe sets were monitored by FISH to metaphase spreads and cultured interphase amniocytes. Finally, each newly labeled probe set was hybridized to uncultured amniotic fluid cells to assess clarity of signal on the basis of background fluorescence versus signal intensity.

Clinical Protocol

All amniotic fluid specimens referred for rapid detection of common aneuploidies by FISH were simultaneously processed for cytogenetic analysis. The cytogenetic analyses were performed by in situ culturing methods, and a minimum of four independent cultures were established for each specimen. Cytogenetic diagnosis was based on examination of GTG-banded chromosomes from at least 15 metaphase cells in 12 or more colonies from a minimum of two independent cultures.

The minimum volume of amniotic fluid required to attempt both FISH and cytogenetic analyses was 18 ml. When less than 18 ml of fluid was received, only cytogenetic analysis was performed.

Protocols employed for the removal of blood cells during the initial stages of our experience provided inconsistent results. Therefore, in order to minimize the risk of maternal cell contamination, after specimen number 1200, analysis was not performed if the amniotic fluid or the spun cell pellet contained visible amounts of blood or other contaminants.

A 5-ml aliquot of amniotic fluid was used for FISH and was processed to permit five independent hybridizations. Each site was hybridized with a single probe set to identify the ploidy status in the amniotic fluid cells for chromosomes 13, 18, 21, X, and Y. After hybridization and detection, slides were counterstained with DAPI and were analyzed by epifluorescence microscopy.

Data Collection

A minimum of 50 hybridized nuclei per probe set were analyzed to determine ploidy levels. All nuclei were initially evaluated under 400× magnification using a 40× oil objective. Clumped nuclei, nuclei with attached cytoplasm or cellular membrane, and nuclei which, on the basis of visual similarity to polymorphonuclear cells, may have been maternal in origin, were not scored. Two independent readers analyzed each case. The initial analysis consisted of examination of at least 30 hybridized nuclei per probe set. This was followed by a second independent analysis in which at least 20 additional hybridized nuclei were examined. This independent evaluation served as the primary source of quality assurance in the analytical process. A nucleus was considered to be monosomic when one hybridization signal was observed. Disomic nuclei had two hybridization signals, and trisomic nuclei produced three signals. Other hybridization patterns—such as the rare four-signal nuclei, representing disomic nuclei in the G-2 phase of the cell cycle—were also recorded. All potentially abnormal specimens were subjected to a third independent evaluation.

After the single occurrence of a false-positive result for a sex chromosome aneuploidy (see Results), all subsequent instances of potential sex chromosome aneuploidies were confirmed by rehybridization with X and Y probes to the appropriate sites prior to notification of the referring physician (Ward et al. 1992). Rehybridization was also utilized in instances of either initial weak signal strength (less than 1% of samples) or results which, on the basis of probe performance, were otherwise inconclusive.

Analytical Criteria

Analysis of specimens referred for aneuploidy detection by FISH on uncultured amniocytes resulted in three different clinical outcomes: (1) an "informative" result which was reported to the physician, (2) an "uninformative" report which was transmitted to the physician, or (3) no molecular cytogenetic analysis attempted, because of inadequate volume or visible contamination.

Informative disomic samples were defined as those in which a minimum of 50 hybridized nuclei per probe set were analyzed (250 nuclei per specimen), and greater than or equal to 80% of all nuclei from each hybridization demonstrated a disomic autosomal signal pattern and a normal sex chromosome pattern. If hybridization with any probe resulted in greater than 20% nonmodal signals, the entire sample was reported as uninformative.

Informative abnormal specimens were defined as those in which greater than or equal to 70% of nuclei which hybridized with the relevant probe demonstrated a nondisomic pattern. Such analyses led to the generation of reports which stated that the hybridization pattern was consistent with a specific autosomal trisomy, a sex chromosome aneuploidy, or, when all probes demonstrated a trisomic pattern, a triploid chromosome constitution. Other probes within abnormal samples were required to meet the greater-than-or-equal-to-80% normal signal distribution. Potential trisomic specimens which did not meet these criteria were reported as uninformative.

These reporting criteria were based on (1) a cutoff which was 3 SDs from the nonoverlapping discriminator between disomic and trisomic samples as defined in the previous study and (2) the observation that the joint probability of detecting three chromosomal domains in a given trisomic nucleus is less than that of detecting two chromosomal domains in a disomic nucleus

(Klinger et al. 1992). These stringent criteria were designed to minimize the possibility of false-positive and false-negative results. These criteria increase the number of samples, particularly trisomics, which fall into the "uninformative" category. However, during the initial clinical application of FISH for aneuploidy detection, it was felt that an increased rate of "uninformative" potential trisomic results was preferred to the potential risk of false-positive results.

Uninformative reports were generated when the outcome from any one of the five independent hybridizations failed to meet either the 80% reporting criterion, for a disomic pattern, or the 70% reporting criterion, for an abnormal pattern. In addition, if potential maternal cell contamination was observed, e.g., the presence of a mixture of XY and XX nuclei or the presence of polymorphonuclear cells, an uninformative report was generated.

Data Reporting

After analysis of the specimen, the FISH results were correlated with patient and clinical information, and written results were then reported to the referring physician. The report described the ploidy status of chromosomes 13, 18, 21, and the sex chromosome complement. Total processing and analysis required approximately 24 h, and greater than 98% of results were reported to the referring physician within 2 working days from receipt of specimen. The cytogenetic result was reported to the physician on completion of karyotypic analysis, approximately 1 wk later. All clinical FISH reports included our stated policy that irreversible therapeutic action should not be based solely on the results of FISH and that FISH analysis was performed only as an adjunctive test to traditional cytogenetics.

Results

Assay Characteristics

This study reports on a total of 4,500 amniotic fluid samples which were submitted to our molecular cytogenetics laboratory for the rapid detection of common aneuploidies by using FISH followed by routine cytogenetic analysis. In these samples chromosome analysis was successful in 99.9% of cases. The time from amniocentesis to completion of the cytogenetic analysis was the same in both the cytogenetics-only group and the FISH-plus-cytogenetics group, averaging less than 8.5 d. Therefore, the removal of 5 ml of amniotic fluid for in situ hybridization, from a total volume of at least 18 ml, did not affect cytogenetic analysis.

FISH analysis was not attempted on 2.3% of the samples because of the presence of contaminating blood or on 1.5% of samples because of insufficient volume. Of the 4,329 remaining samples tested, analysis was successful and resulted in the generation of an informative report in 3,901, or 90.2%, of attempted tests. These results are described in tables 1 and 2.

Accuracy.—As shown in table 1, there were 3,782 samples reported as disomic for chromosomes 13, 18, and 21 with a normal sex chromosome constitution which were confirmed by cytogenetics. A result of autosomal trisomy or triploidy was obtained by FISH and was cytogenetically confirmed in 87 patients. There were no instances of false-positive autosomal results. There were seven instances of false-negative results as defined by undetected presence of autosomal trisomies. These data result in an aneuploidy detection rate of 92.5% for informative specimens. The undetected autosomal trisomies included an undetected trisomy 13 male fetus, a trisomy 18 male fetus, and five undetected trisomy 21 female fetuses. Three of these false negatives (the trisomy 18 and two trisomy 21's) were due to a combination of increased nuclear fluorescent background coupled with intense autofluorescence of microscope objectives. The failure to detect the trisomy 13 fetus remains unexplained, since the initial clinical impression at the time of ultrasound was consistent with the presence of a trisomy 13 fetus and since serial hybridizations failed to demonstrate a trisomic hybridization pattern. The remaining three undetected trisomies were from female fetuses and may have been due to either undetected maternal cell contamination or inefficient detection of the third signal.

An abnormal sex chromosome constitution was detected by FISH and was cytogenetically confirmed in 20 patients, for a detection rate of 100% for informative specimens. There was a single false-positive sex chromosome result. In this case the initial hybridization resulted in a single X signal and no Y signal. This result was interpreted to be consistent with monosomy X. Subsequent ultrasound examination demonstrated the presence of a male fetus, and concurrent cytogenetic analysis revealed a 46,XY karyotype. Rehybridization of the sample with X and Y probes demonstrated a normal male pattern, indicating that the initial FISH result was incorrect.

Maternal cell contamination.—In four cases, the FISH analysis indicated a disomic female fetus, and subsequent cytogenetic analysis revealed a 46,XY

Table 1

Results of Prenatal Aneuploidy Detection by FISH in 3,901 Subjects

	No. of Informative Results				
	True Negative[a]	True Positive[b]	False Positive[c]	False Negative[d]	Maternal Contamination[e]
Autosomes	3,807	87	0	7	0
Sex chromosomes	3,876	20	1	0	4
Overall	3,782	107	1	7	4

NOTE.—Informative results were obtained on 90.2% of attempted analyses.

[a] Absence of aneuploidy for the tested chromosomes 13, 18, 21, X, and Y, as reported by FISH and confirmed by cytogenetics.

[b] Presence of aneuploidy for the tested chromosomes, as reported by FISH and confirmed by cytogenetics.

[c] Abnormal report, by FISH, which was shown to be normal on cytogenetics.

[d] Normal results by FISH which were diagnosed as aneuploid for the tested chromosomes by cytogenetics.

[e] FISH reported a disomic female, and cytogenetics revealed a 46,XY karyotype.

karyotype. These discrepancies were the result of undetected maternal cell contamination. Three of these instances occurred within the first 1,200 samples. During this early phase, specimens which contained visible contaminating blood were treated with ammonium chloride prior to processing. When it became apparent that this treatment did not efficiently remove contaminating maternal nucelated blood cells, a policy was implemented not to perform FISH on visibly bloody specimens. Since the institution of this policy, only a single instance of undetected maternal cell contamination has been encountered in the subsequent 3,300 cases.

Uninformative results.—A total of 9.8% of the assays were considered uninformative on the basis of our reporting criteria. A total of 2.3% of samples were uninformative because of insufficient numbers of nuclei present to complete the full analysis, while 3.8% of samples displayed maternal cell contamination. In 2.8% of the samples, uninformative results originated from technical problems such as contaminating cytoplasm surrounding the nuclei, weak hybridization signals, increased background fluorescence caused by excessive amniotic fluid debris, or absence, due to unknown causes, of hybridization signals for a single probe. In the

Table 2

Performance Characteristics for Informative FISH Results

	Autosomes	Sex Chromosomes
Positive predictive value[a]	100% (87/87)	95.2% (20/21)
Negative predictive value	99.8% (3,807/3,814)	100% (3,876/3,876)
Sensitivity	92.6% (87/94)	100% (20/20)
Specificity	100% (3,807/3,807)	99.9% (3,876/3,881)
False-negative rate	7.4% (7/94)	0% (0/20)
False-positive rate	0% (0/3,807)	.003% (1/3,876)
Maternal cell contamination		.1% (4/3,880)
Overall accuracy	99.8% (3,807+87/3,901)	99.9% (3,876+20/3,901)

NOTE.—Results are based on ploidy analysis by FISH for chromosomes 13, 18, 21, X, and Y, as compared with confirmatory traditional cytogenetic analysis. Triploid chromosome constitution scored as a single autosomal abnormality even though all autosomes and the sex chromosomes demonstrated an abnormal hybridization pattern.

[a] Described by Goldman (1987).

Table 3

Chromosome Abnormalities Present in 4,500 Patients Referred for FISH plus Cytogenetics: All Aneuploidies

	Trisomy 13	Trisomy 18	Trisomy 21	Triploidy	Sex Chromosomes	Overall
Informative results:						
True positives	12	30[a]	38[b]	7	20[c]	107
False negatives	1	1	5	0	0	7
False positives	0	0	0	0	1	1
Accuracy	92.3%	96.8%	88.4%	100%	95.2%	92.5%
Uninformative results	2	10[d]	17	2	1	32
Overall detection rate	80.0%	73.2%	63.3%	77.8%	95.2%	73.3%

[a] Includes one case of isochromosome 18q.
[b] Includes one case of tissue-limited mosaicism for i(21q).
[c] Four cases of XXY, 4 cases of XXX, and 12 cases of monosomy X.
[d] Includes a partial trisomy 18 resulting from a der(21)t(18;21)(p11;q11) which demonstrated 62% trisomic nuclei with the chromosome 18 probe.

remaining uninformative 15 samples (0.9%), the hybridization results for any one of the probes failed to meet the reporting criteria of modal signal distribution as described in Material and Methods. The failure of any single probe to produce a consistent hybridization pattern therefore occurred in less than 0.2% of all hybridizations within this study (0.9% divided by five probes).

Performance characteristics.—Table 2 outlines the performance characteristics for informative FISH results with the described probes. The FISH analysis demonstrated a positive predictive value of 100% for the presence of autosomal trisomies and of greater than 95% for sex chromosome aneuploidies. The total false-positive rate for autosomes was 0%, and that for the sex chromosomes was 0.103% (one false-positive plus four undetected maternal contaminations). The overall accuracy was therefore 99.8% for informative autosomal results and 99.9% for informative sex chromosome results.

Synopsis of abnormal samples.—Results for all specimens with the relevant chromosome abnormalities are presented in table 3. A total of 73.2% (107/146) of all aneuploidies were identified by FISH, reported as abnormal, and confirmed on completion of the cytogenetic analysis.

Thirty-two (21.8%) of all aneuploidies were reported as uninformative. Twenty of these were due to a hybridization pattern which did not meet reporting criteria: 6 demonstrated trisomic hybridization signals in 35%–50% of hybridized nuclei, and 14 specimens demonstrated 50%–68% trisomic nuclei. The hybridization pattern for these 14 specimens, while highly suggestive of a trisomic condition, did not meet reporting criteria for the presence of an abnormality and were reported as uninformative.

Five autosomal trisomies and one sex-chromosome aneuploidy were uninformative because of insufficient numbers of nuclei for complete analysis. Two additional autosomal trisomies and two triploid specimens were uninformative because of maternal cell contamination. Two trisomic samples were reported as uninformative because of poor hybridizations with probes not involved in the trisomic condition.

Those chromosome abnormalities which were diagnosed by cytogenetics but which this FISH protocol was not designed to detect are outlined in table 4. Four mosaics involving the tested chromosomes were reported as uninformative because of a hybridization pattern where the percentage of observed three-signal nuclei fell below the 70% reporting criterion. Two low-level sex chromosome mosaics were not detected; nor were two autosomal mosaics for chromosomes not included in the probe set. Sixteen inherited chromosomal abnormalities, including 13 which were balanced, were diagnosed cytogenetically. Eight de novo chromosome abnormalities were detected, three of which appeared to be balanced chromosomal rearrangements.

Assay Efficiency

The clinical data generated using FISH with the sex chromosome probes demonstrated a high degree of specificity. When the hybridization pattern was consistent with a normal male sex chromosome constitution, greater than 86% of samples demonstrated a single X signal and a single Y in greater than 94% of hybridized

Table 4

Additional Chromosomal Abnormalities Diagnosed by Cytogenetics

Category	No.
Mosaics reported as uninformative:	
Trisomy 13 mosaic	1
Sex-chromosome mosaics: X/XX	3
Total	4
Mosaics reported as disomic by FISH:	
XX/XXX	1
XY/XXY	1
Trisomy 20 mosaic	1
Trisomy 16 mosaic	1
Total	4
Inherited chromosome abnormalities:	
Translocations:	
Balanced	10[a]
Unbalanced	3
Inversions	3
Total	16
De novo chromosome abnormalities:	
Apparently balanced	3
Unbalanced	5
Total	8

NOTE.—The probe set and protocol utilized in this study were not designed to detect these chromosome abnormalities.

[a] Includes four cases where one parent was a known Robertsonian translocation carrier.

nuclei. More than 87% of female samples had two X signals in greater than 94% of hybridized nuclei. A "Y" signal was rarely seen in female specimens; only 2% of such specimens demonstrated such a signal, and it was never present in greater than 4% of the nuclei.

Informative specimens with abnormal sex chromosome constitutions demonstrated similar patterns. In fetuses with monosomy X, greater than 96% of hybridized nuclei had a single X signal and no Y signal. In all patients with XXX, greater than 90% of nuclei demonstrated three X signals. The seven triploid patients and the four XXY patients showed an abnormal pattern of hybridization in greater than 76% of hybridized nuclei.

The efficiency of hybridization/detection for the three autosomal probes in 11,765 hybridizations is presented in figure 1. The efficiency was assessed from the observed percentage of nuclei with three signals in those hybridizations subsequently demonstrated to be trisomic by cytogenetics (fig. 1, *top*) versus the percentage of nuclei with three signals from hybridizations subsequently identified as disomic by cytogenetics (fig. 1, *bottom*). In a significant majority of autosomal trisomies (fig. 1, *top*), three signals were present in greater than 50% of hybridized nuclei. As shown in the bottom panel of figure 1, less than 0.3% of normal disomic hybridizations had greater than 20% "three-signal" nuclei. This bimodal distribution may form the basis for future modifications of the reporting criteria.

Referral Pattern

FISH studies were requested on 23% of all amniotic fluid samples submitted for cytogenetic analysis. The physician referral patterns for FISH plus cytogenetic analysis versus cytogenetics alone are shown in table 5. The most frequent referral for rapid aneuploidy detection by FISH was advanced maternal age. However, physicians were more apt to request rapid aneuploidy detection when ultrasound abnormalities were detected or when the amniocentesis was performed as a result of an increased risk for trisomy based on maternal serum screening.

Referrals for rapid aneuploidy detection were more than fourfold higher (12% vs. 2.7%) when the primary indication for amniocentesis was the presence of fetal abnormalities as detected on ultrasound examination. Fetal ultrasound abnormalities were present as a primary or secondary indication in a total of 15% of all specimens referred for FISH analysis but were present in only 3.3% of pregnancies requesting traditional cytogenetics alone.

Rapid aneuploidy detection was requested more frequently (29% vs. 20%) when the initial referral was due to an increased risk of a trisomic fetus based on a maternal serum screening profile or based on a first-degree relative with a trisomy. In contrast, a fourfold decrease in FISH referrals was noted in pregnancies where the primary risk factor was an elevation in maternal serum alpha-fetoprotein. This was expected, as this FISH protocol is of little clinical utility when the primary indication for prenatal diagnosis is an increased risk for open neural tube defects.

Discussion

The present study describes the first large-scale clinical application of FISH for the rapid identification of aneuploidies for chromosomes 13, 18, and 21 and the sex chromosomes. FISH analysis was performed on uncultured amniotic fluid cells as an adjunctive test to traditional cytogenetics, on request of referring physicians. The results of the FISH analyses were routinely reported to the referring physician within 2 d of receipt

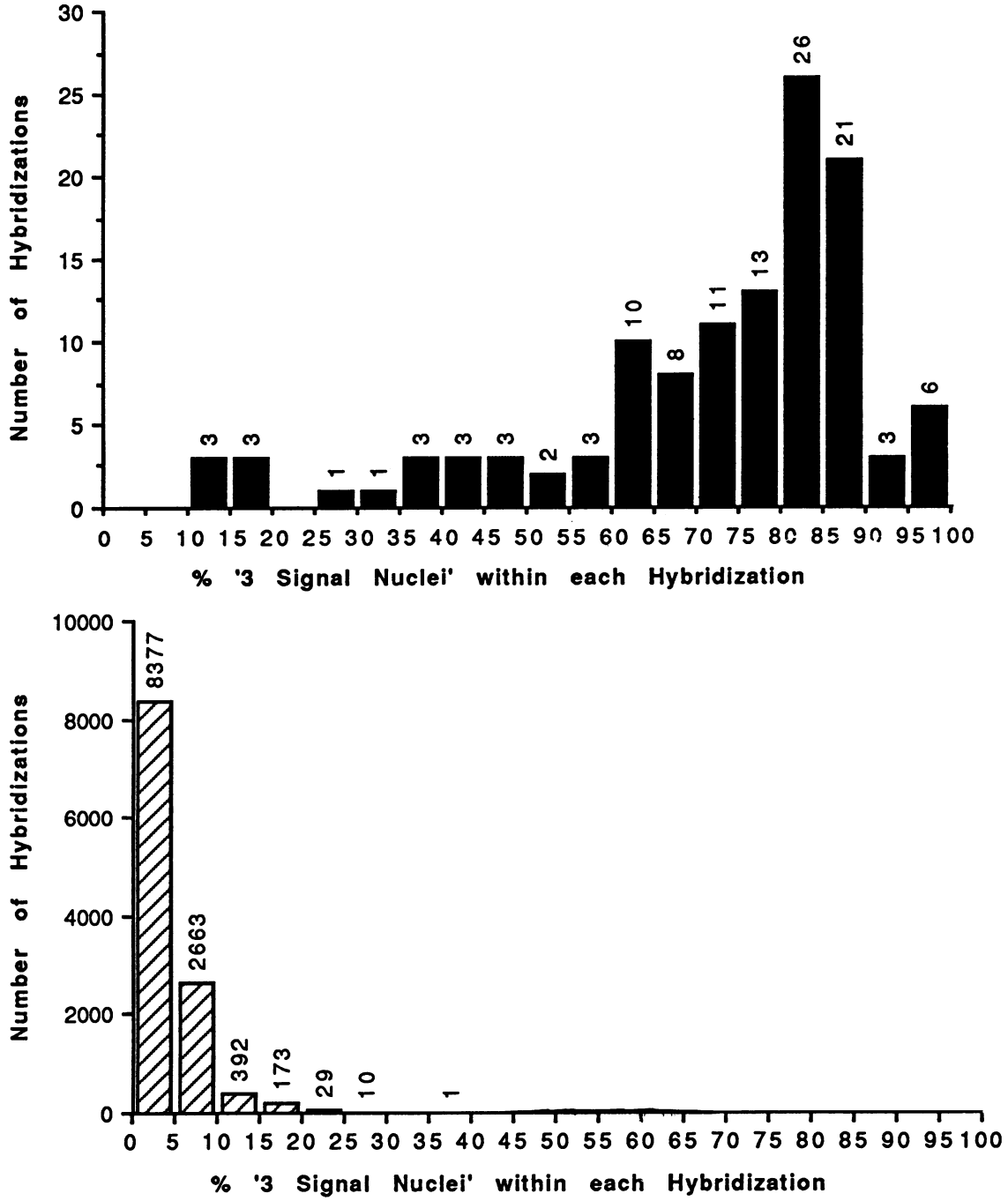

Figure 1 Distribution of "three-signal nuclei" from uncultured amniotic fluid cells after hybridization with autosomal probes directed at chromosomes 13, 18, and 21. Shown is the number of hybridizations in which a specific percentage of three-signal nuclei was observed. Distributions are presented for autosomal hybridizations in samples identified as trisomic (*top panel*) or disomic (*bottom panel*) by cytogenetics. Samples in which intermediate hybridization patterns produced uninformative results are included. Samples which were uninformative because of either maternal cell contamination or very low numbers of nuclei were omitted (less than 6.1% of total processed samples).

Table 5

Patient Referral Pattern: Combined FISH plus Cytogenetics, versus Cytogenetics Alone

Test Requested	Frequency of Primary Indication						
	Advanced Maternal Age	Advanced Maternal Age plus Abnormal Ultrasound	Abnormal Ultrasound	Increased Trisomy Risk[a]	Elevated Maternal Serum Alpha-Fetoprotein	Maternal Anxiety	Other
Cytogenetics	59%	.6%	2.7%	20%	9.0%	2.7%	6.0%
FISH plus cytogenetics	49%	3.0%	12%	29%	2.0%	2.7%	2.3%

NOTE.—Data constitute the primary referral pattern in 4,500 consecutive samples.

[a] Includes pregnancies at increased risk of trisomy based on abnormal maternal screening profile or family history.

of specimen. The clinical results on these first 4,500 specimens demonstrate that FISH analysis of uncultured amniocytes can enhance standard cytogenetics by allowing accurate identification of autosomal trisomies, sex chromosome aneuploidies, and the presence of triploidy in significantly less time than is required by current cytogenetic technologies.

Previously, the obstacles to the application of FISH for rapid detection of aneuploidies in uncultured amniotic fluid cells have been the low sensitivity and specificity of existing DNA probes and inefficient sample-handling protocols (Ferguson-Smith 1991). For the current study, the prenatal diagnostic application of the FISH assay was made possible by the development of DNA probes with high signal-to-noise ratio, good spatial resolution of the fluorescent signals, and high hybridization/detection efficiencies in association with the development of novel cell-handling techniques (Klinger et al. 1992).

As with metaphase cytogenetics, interphase FISH analysis requires examination of multiple cells. However, unlike metaphase analysis, the current determination of chromosomal ploidy levels by FISH is based on a statistical analysis of signal pattern after hybridization. It was therefore necessary to define specific criteria for assignment of ploidy in uncultured amniotic fluid cells. Previous statistical analysis indicated that specimens in which greater than 77% of examined nuclei demonstrate two signals were disomic, whereas trisomies were unequivocally identified as those hybridizations in which greater than 42% of nuclei for any given probe displayed three hybridization signals (Klinger et al. 1992). We believed that more conservative criteria were appropriate for the initial clinical application of FISH for aneuploidy detection, and we chose to use the stringent reporting criteria described in Material and Methods. Analyses which did not meet these criteria were reported as uninformative.

In the present study, an informative result was generated in greater than 90% of analyzed specimens. When informative results were obtained, the concordance rate between the FISH and the cytogenetic analyses was greater than 99.8%. If the FISH analysis resulted in a disomic normal pattern, the results were reported with an accuracy of 99.8% for absence of autosomal trisomies and with an accuracy of 100% for the absence of sex chromosome aneuploidies. The stringent criteria for the generation of an informative report resulted in no false-positive autosomal results, a positive predictive value of 100%. One false-positive sex chromosome aneuploidy was encountered. The positive predictive value for sex chromosome aneuploidies was therefore greater than 95%. These performance characteristics demonstrate the strength of FISH for the detection of aneuploidy in uncultured amniotic fluid cells.

The false-negative rate for sex chromosome aneuploidies was 0%, and undetected maternal cell contamination occurred in less than 0.1% of samples. For informative specimens the sensitivity for identification of autosomal trisomy was 92.6%. This was less than anticipated on the basis of initial investigations (Klinger et al. 1992; Lebo et al. 1992). Undetected maternal cell contamination, increased background fluorescence due to excessive cellular debris, and autofluorescence of the microscope lenses (see below) contributed to the observed false negatives, thereby decreasing the sensitivity rate. Additionally, at least one false negative (and possibly three) was the result of decreased hybridization efficiencies. It has been demonstrated that any decrease in hybridization efficiency has a dramatic effect on the

proportion of three-signal nuclei versus two-signal nuclei observed in trisomic samples (Klinger et al. 1992). Continued enhancement of existing hybridization protocols and continued expansion of our clinical experience should significantly reduce the false-negative rate in the future.

The strict reporting criteria used to define and report samples as abnormal in this study led to a detection rate of 73.2% for all aneuploidies. With the expanding clinical data set, it may be appropriate to begin to reevaluate these criteria. If the criteria for reporting a sample as abnormal were changed to include all samples where greater than 50% of hybridized nuclei were trisomic, the detection rate would improve to 82.9% (107+14/146). This change in the reporting criteria would, therefore, enhance the detection rate without altering the false-positive or false-negative rates. Such modifications are currently under investigation.

One unexpected occurrence was the development of autofluorescence within the microscope lenses during the initial phases of this study. This phenomenon was documented by the manufacturer, who is working to correct the problem. Currently the 40× oil objectives are replaced four times a year.

The applications of molecular genetic FISH technology for prenatal diagnosis are beginning to be addressed. When compared with traditional cytogenetics following amniocentesis, advantages afforded by FISH include (1) rapid results which are generated within 2 d, compared with 7–14 d for traditional cytogenetics, (2) the observation that FISH is less labor intensive than cytogenetics, and (3) the applicability of FISH to uncultured specimens or to specimens with a low mitotic index (Pinkel et al. 1988; Kuo et al. 1991; Klinger et al. 1992; Ledbetter 1992; Richkind 1992; Sawyer et al. 1992). In our experience, the results of the FISH studies have also assisted the clinical cytogenetics laboratory in the assignment of priority status for analysis of high-risk specimens.

The potential advantages of rapid aneuploidy detection for the health-care provider and patient are as follows: (1) FISH may be the optimal strategy for rapid confirmation of potential numerical chromosome aneuploidies when ultrasound reveals fetal abnormalities; (2) FISH coupled with traditional cytogenetics may, in certain circumstances, aid genetic counseling (Bajalica et al. 1992; Klever et al. 1992); and (3) early receipt of normal results has a positive effect on maternal/fetal attachment and decreases anxiety levels (Caccia et al. 1991). The reduction of maternal anxiety through the rapid delivery of normal disomic results may be particularly advantageous for those women who exhibit high levels of anxiety during the time required for prenatal diagnosis. High anxiety has been recorded, for example, in women who initially believed that their pregnancy was not at increased risk and who then received abnormal maternal serum screening results which increase both their risk for trisomy and their anxiety level (Evers-Kiebooms et al. 1988; Abuelo et al. 1991). Rapid diagnosis is also useful when there are significant constraints between the time of amniocentesis and the time in which therapeutic decisions and actions may be taken. In the absence of consistent prenatal health care, amniocentesis is often, out of necessity, performed after 20 wk of gestation. Termination of pregnancy is generally not an option after 24 wk of gestation, which leaves a short time for identification of chromosome abnormalities and an even shorter time for the patient and health-care provider to make clinical decisions and to initiate action if desired. Under such circumstances, FISH provides both the patient and the health-care provider with valuable and timely information. Examination of the referral pattern for FISH presented in this study indicates that this technology is being appropriately utilized for the identification of potential aneuploid fetuses in situations of great stress, i.e., following an abnormal ultrasound or determination of increased trisomy risk by serum screening.

The application of FISH to prenatal diagnosis is not a panacea. As used in this study, this technology detects aneuploidies caused by monosomies, free trisomies, trisomies associated with Robertsonian translocations, triploidy, and other numerical chromosome abnormalities involving chromosomes 13, 18, 21, X, and Y. The technology described here was not designed to detect other cytogenetic abnormalities such as mosaics, translocations, or rare aneuploidies. Potential disadvantages of this technology include increased maternal anxiety after an uninformative result and the negative effect of receiving a disomic FISH result followed by the identification of a chromosomal lesion not identified by the current protocol.

A total of 178 chromosomal abnormalities were diagnosed by cytogenetics within the current study. These abnormalities included aneuploidies, mosaics, inherited balanced and unbalanced translocations, and de novo structural abnormalities. Aneuploidies for the tested chromosomes accounted for 146, or 82%, of these abnormalities. There were 13 cytogenetic abnormalities which are not typically associated with fetal phenotypic abnormalities, including 3 inherited inversions and 10 inherited balanced translocations. Thus,

of the 165 chromosome abnormalities associated with a significant risk for phenotypic abnormalities at birth, the probe set used could have potentially identified 88% (146/165). Actual detection rates were influenced by choice of stringent reporting criteria and by technical issues as presented earlier in Material and Methods and Results.

The results of the present study demonstrate the clinical diagnosis of the common fetal aneuploidies in significantly less time than is possible by standard cytogenetics. We believe that, at this time, FISH should not be used as an independent, stand-alone technology for prenatal diagnosis. The existence of chromosome abnormalities which are not detected by current FISH protocols and the lack of widespread experience with this new technology require that the FISH protocols be utilized as an adjunctive test to traditional cytogenetic analysis. The utility of FISH for rapid detection of aneuploidies continues to be explored. As new probes and sample-handling protocols are developed, prenatal diagnostic applications of FISH undoubtedly will expand.

Acknowledgments

The authors would like to acknowledge the technical assistance of Ann Boone, Roseanne DiMaggio, Matthew DeRiso, Paula Finn, Patricia Manos, Karen Pavelka, Mary Richard, Donna Shook, Robert Theve, and Pao-Ling Wu.

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