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Simultaneous, Multilocus FISH Analysis for Detection of Microdeletions in the Diagnostic Evaluation of Developmental Delay and Mental Retardation

Azra H. Ligon,[1,*] Arthur L. Beaudet,[1,2,3,4] and Lisa G. Shaffer[1]

Departments of [1]Molecular and Human Genetics, [2]Pediatrics, and [3]Cell Biology and [4]Howard Hughes Medical Institute, Baylor College of Medicine, Houston

Summary

Many microdeletion and contiguous gene–deletion syndromes include mental retardation as a clinical feature. We have developed MultiFISH, a FISH assay using several probes to simultaneously screen for multiple microdeletion syndromes in patients who present with unexplained developmental delay and/or mental retardation. This screening tool can be used to determine whether a particular microdeletion syndrome is involved in the etiology of these clinical phenotypes. In this pilot study we combined probes for the commonly deleted regions of Prader-Willi, Angelman, Williams, Smith-Magenis, and DiGeorge/velocardiofacial syndromes in a single hybridization. The probes were differentially labeled, allowing multicolor detection, and 200 individual samples were screened in a blinded fashion. For all patients found by MultiFISH to have deletions, the deletions were originally identified and/or later confirmed by use of single-probe FISH analysis in our diagnostic cytogenetics laboratory. One patient, who was referred for developmental delay and was shown to have a normal G-banded karyotype, was identified by MultiFISH as having a microdeletion at the DiGeorge/velocardiofacial commonly deleted region. Forty-six of the 200 total samples were tested for microdeletions by use of single FISH probes in the diagnostic laboratory. Ten of these cases were found to have deletions, and all deletions were subsequently detected by use of a MultiFISH screen performed in a blinded fashion. Additionally, for all 200 patients tested by use of MultiFISH, no false-positive deletion results were observed. We demonstrate the ability of this technique to scan for and to identify microdeletions in a proportion of patients whose routine karyotype appears normal yet who are mentally retarded and/or developmentally delayed.

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Address for correspondence and reprints: Dr. Lisa G. Shaffer, Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Room 15E, Houston, TX 77030. E-mail: lshaffer@bcm.tmc.edu

*Present affiliation: Department of Obstetrics, Gynecology, and Reproductive Biology, Brigham and Women's Hospital, Boston.

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Introduction

Developmental delay and/or mental retardation are common clinical indications for chromosomal studies and constitute a large portion of referrals for cytogenetic analysis. Although developmental delay or mental retardation may be seen without associated features, each may also occur as part of recognizable deletion syndromes. Several microdeletion syndromes have been described to date, including Williams syndrome (WS) (deletion of 7q11.23), Prader-Willi syndrome (PWS) and Angelman syndrome (AS) (deletion of 15q12), Smith-Magenis syndrome (SMS) (deletion of 17p11.2), and DiGeorge/velocardiofacial syndromes (DGS/VCFS) (deletion of 22q11.2). The majority of patients with these syndromes have demonstrated deletions at the respective critical regions. Many of these are submicroscopic deletions, at or below the level of cytogenetic resolution, requiring molecular methodologies for their optimal visualization. Some of these microdeletion syndromes are caused by inactivation of a single gene (e.g., Rubinstein-Taybi syndrome); others almost certainly involve multiple genes and can be considered contiguous gene–deletion syndromes (e.g., WS); and others involve an unknown number of genes (e.g., SMS or DGS/VCFS) (Greenberg et al. 1991; Driscoll et al. 1992; Ewart et al. 1993; Nickerson et al. 1995; Frangiskakis et al. 1996; Tassabehji et al. 1996). For some disorders, such as PWS or Rubinstein-Taybi syndrome, only a fraction of the patients have a large deletion detectable by routine cytogenetics or by FISH, and the remainder have other molecular lesions (Breuning et al. 1993; Petrij et al. 1995; American Society of Human Genetics/American College of Medical Genetics Test and Technology Transfer Committee 1996). For other disorders, such as DGS/VCFS, almost all recognized patients have a deletion that is detectable by FISH (Desmaze et al. 1993; Lindsay et al. 1993).

Although particular unique physical characteristics of some microdeletion syndromes (e.g., the full lips, loquaciousness, and "elfin" features characteristic of WS) may facilitate diagnoses of patients, some syndromes (e.g., DGS/VCFS and SMS) are not as easily identified by

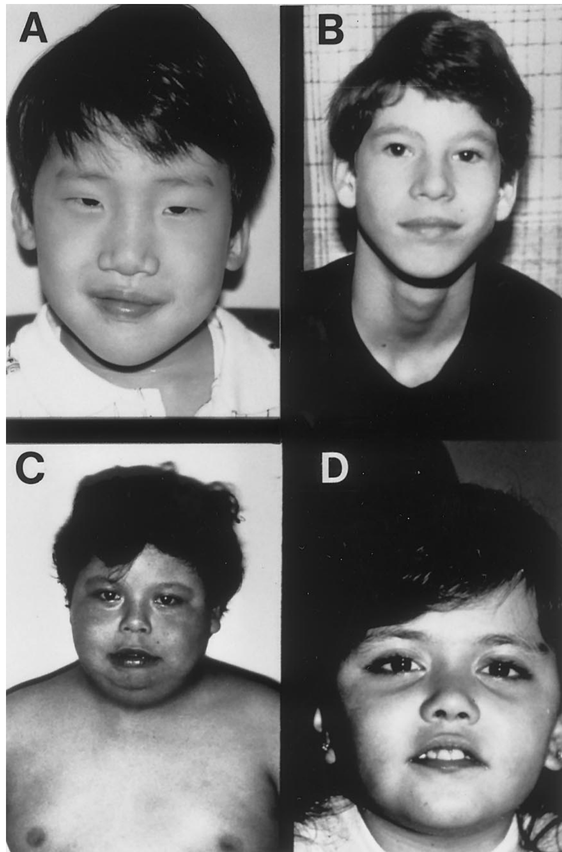

Figure 1 Four patients carrying microdeletions corresponding to syndromes represented in the MultiFISH cocktail. Diagnoses are provided in the appendix.

physical features alone (Lindsay et al. 1995; Greenberg et al. 1996). Furthermore, some features are so subtle as to elude detection even when they are present. This increases the difficulty of clinical identification in patients who present with unexplained developmental delay and/or mental retardation, both of which can be characteristic of multiple syndromes. Shown in figure 1 are four patients, each of whom carries a microdeletion corresponding to one of the syndromes represented in the MultiFISH cocktail. To illustrate the potential clinical difficulty of diagnosing these patients, their respective syndromes are not revealed in the legend accompanying figure 1 but can be found in an appendix at the end of the main text of this paper. If a particular microdeletion is suspected by the clinician, then the presence or absence of that deletion can be tested in a cytogenetics laboratory using FISH (reviewed in Shaffer 1995). However, if several different deletion syndromes appear in the differential diagnosis, the corresponding analyses would require a number of sequential hybridizations using the appropriate individual probes. These sequential analyses are relatively expensive, consume a great deal of technician time and reagents, and require significantly more patient material, compared with single-probe FISH.

We report here the use of MultiFISH—multiple, simultaneous, fluorescently labeled probes for testing microdeletion syndromes—in a study of 200 chromosomal samples referred to our laboratory, for one of several indications, including developmental delay, mental retardation, or fragile X or specifically to exclude a particular microdeletion syndrome. The purpose of this study was to develop a FISH assay that tested multiple loci simultaneously, to determine whether any of the patients referred with nonspecific indications for cytogenetic analysis could be identified as having a known microdeletion syndrome. MultiFISH was performed by use of a cocktail of probes that are routinely used individually to detect deletions in the WS, PWS, AS, SMS, and DGS/VCFS regions. MultiFISH successfully detected patients with previously identified microdeletions and also revealed a deletion in a single patient, referred for developmental delay and mental retardation, for whom no specific genetic disorder was suspected. The work presented here demonstrates that a multiple-probe cocktail, including cosmids corresponding to the critical regions of several deletion syndromes, can be used successfully to identify microdeletions that may otherwise go undetected in a proportion of patients presenting with developmental delay and/or mental retardation.

Material and Methods

Samples

Patient samples were obtained through referrals made to the Kleberg Cytogenetics Laboratory (Baylor College of Medicine), for FISH to exclude a particular microdeletion syndrome ($n = 45$) or for routine banded karyotypes for indications including developmental delay and/or mental retardation ($n = 155$) (see table 1). In addition, metaphases from four normal individuals (controls) were hybridized with each of the four individual probes to be included in the MultiFISH cocktail, in order to establish the single-probe hybridization efficiencies. Metaphase spreads were prepared by use of standard cytogenetic procedures. Cell pellets prepared from lymphocyte cultures were resuspended in an appropriate volume of fixative (75% methanol, 25% acetic acid) prior to preparation of the slides. Slides were flooded with fixative solution, and the cells were then applied with a pasteur pipet while the slides were held at approximately a 30° angle. The slides were dried at room temperature over a period of 5 min and then were checked, by phase microscopy, for a sufficient number of metaphases. Slides were then coded by a cytotechnologist and were entered into the study. If not used immediately, the slides were stored at −20°C.

Table 1

Summary of MultiFISH Results

Indication[a]	No. of Patients Deleted by MultiFISH/ Total No. of Patients Tested
DDMR/FRAX[b]	1/155
Exclude WS[c]	4/9
Exclude AS[d]	0/8
Exclude PWS[e]	1/7
Exclude SMS	2/7
Exclude DGS/VCFS	3/13
Exclude MDS[f]	0/1

[a] DDMR = developmental delay and/or mental retardation; FRAX = fragile X syndrome; and MDS = Miller-Dieker syndrome.

[b] Includes cases referred for multiple congenital anomalies as well as for dysmorphism.

[c] Includes 1 patient also referred to exclude VCFS.

[d] Includes joint referrals to exclude VCFS (1 patient), PWS (1 patient), FRAX (1 patient), SMS (1 patient) and Rett syndrome (1 patient).

[e] Includes referrals to exclude AS (1 patient).

[f] A specific probe for MDS was not included in the MultiFISH cocktail.

Probe Descriptions

Cosmid cELN272 mapping to chromosome 7q11.23 (Ewart et al. 1993) corresponds to the 5′ portion of the elastin gene and detects deletions in >90% of WS patients (Nickerson et al. 1995). Cosmids c102 and c106, which identify the SNRPN locus (Nakao et al. 1994), were combined to identify deletions in 15q12, in the PWS/AS region. Cosmid F5 (Lindsay et al. 1995) identifies the commonly deleted DGS/VCFS region at 22q11.2. Cosmids cSMS92C10 and cSMS62F2, mapping to the FLI locus (Chen et al. 1995), were combined to identify deletions of 17p11.2, a region commonly deleted in SMS. All cosmids were grown in the appropriate media supplemented with either ampicillin (50 μg/ml final, for cosmids c102 and c106) or kanamycin (25 μg/ml final, for the remaining cosmids) and were purified by use of the Qiagen system of cosmid isolation.

Probe Labeling

The cosmid for DGS/VCFS was labeled by use of biotin and were detected by use of avidin-FITC, producing a signal that fluoresced green. Probes for SMS and WS were labeled with digoxigenin and were detected by use of anti-digoxigenin conjugated to rhodamine, which fluoresced red. The probes for PWS/AS were combined in a 1:1 ratio of biotin- and digoxigenin-labeled cosmids. After detection, this produced fluorescent yellow signals on chromosome 15. For the nick-translation reactions, 1 μg of each cosmid DNA was labeled in a 40-μl reaction containing 10 μl of dNTP mix (2.4 μl each of 10 mM dATP, dCTP, and dGTP, 1.6 μl of 10 mM dTTP; 8.0 μl of either digoxigenin 11-dUTP or biotin 16-dUTP [final volume 120 μl] [Boehringer Mannheim]), 4 μl of 10 × nick-translation buffer (500 mM Tris pH 7.5, 10 mM DTT, 100 mM $MgCl_2$), 2 μl of DNA polymerase I (5 U/μl stock), and 3.2 μl of a 1:250 dilution of DNase I (1 mg/ml stock). Reactions were vortexed briefly to combine them and then were spun to collect them. Nick-translation reactions were performed at 15°C for 1 h and then were stopped by addition of 2 μl of 0.5 M EDTA and heat inactivation at 65°C for 10 min. Completed reactions were then vortexed to combine the EDTA and were spun down and stored at 4°C. Precipitations included the following cosmids (final concentrations in the MultiFISH cocktail are given in parentheses): cELN272-DIG label (35 ng/μl), C102-DIG label (15 ng/μl), C102-BIOTIN label (15 ng/μl), C106-DIG label (15 ng/μl), C106-BIOTIN label (15 ng/μl), F5-BIOTIN label (20 ng/μl), SMS92C10-DIG label (15 ng/μl), and SMS62F2-DIG label (15 ng/μl), plus 20 μl of sonicated salmon sperm DNA (10 mg/ml stock), 15 μl of Cot-1 DNA (1 mg/ml stock), and 400 μl of 100% ethanol. After precipitation, the pellets were washed with 70% ethanol and then were dried. Pellets were resuspended in either 10 μl (half slide; 1 × probe) or 20 μl (full slide; 1 × probe) of 50% hybridization buffer (50% formamide, 10% 20 × SSC, 10% dextran sulfate).

Slide Denaturation and Hybridization

Prior to hybridization, the slides were dehydrated in a series of ethanol washes (70%, 90%, and 95%, for 2 min each) and then were air dried. Slides were denatured in 70% formamide, 2 × SSC at 70°C for 2 min (plus 1°C for every additional slide) in coplin jars. Denatured slides were immediately immersed in ice-cold 70% ethanol for 2 min, followed by successive 2-min washes in ice-cold 80%, 90%, and 100% ethanol and air drying. Probes were denatured at 70°C for 10 min prior to placement on the slides. After the addition of glass coverslips sealed with rubber cement, the slides were hybridized overnight at 37°C in a humidified chamber.

Washing and Detection

Hybridized slides were washed in a 50% formamide, 2 × SSC solution for 15 min at 43°C, followed by two 5-min washes in 2 × SSC at 37°C and a 3-min rinse (all performed in coplin jars) in 4 × SSC, 0.1% Tween-20 (final concentrations) at room temperature. Slides were detected by the following steps: 60 μl (per slide) FITC-avidin (Oncor), 20 min at 43°C; 60 μl (per slide) mouse anti-digoxigenin antibody diluted 1:250 in anti-avidin; 60 μl (per slide) anti-mouse IgG-digoxigenin diluted 1:200 in TNB buffer (100 mM Tris pH 8.0, 150 mM NaCl, 0.05% Tween 20, 0.5% blocking reagent [Boehringer Mannheim]); and 60 μl (per slide) anti-digoxigenin rhodamine (Boehringer Mannheim) diluted 1:100

in FITC-avidin. Washes of 3 min as described above were performed between each detection/amplification step. Metaphases were visualized after being counterstained with DAPI.

Scoring

Twenty metaphases were scored for each slide. For each metaphase the presence or absence of four sets of homologous signals was scored: two sets of yellow signals on chromosome 15 (for PWS/AS); two sets of red signals on chromosome 7 (for WS); two sets of red signals on chromosome 17 (for SMS), distinguishable from chromosome 7 on the basis of size and morphology; and two sets of green signals on chromosome 22 (for DGS/VCFS). In a small fraction of samples, one homologue of a chromosome pair would not fluoresce in a given metaphase. However, scoring of additional metaphases, for a total of 20, would then confirm that no deletion was present. Patients who were deleted for a particular locus were deleted for a single homologue in each of the 20 cells (100% of metaphases) scored.

Digital-Imaging Microscopy

Metaphase chromosomes were visualized, and the MultiFISH results were scored on a Zeiss Axiophot fluorescence microscope using a triple-bandpass filter. This allowed the simultaneous visualization of the different-colored signals included in the MultiFISH cocktail. Digital images were obtained with the use of a Perceptive Scientific Instruments Powergene 810 probe system. Captured images were then printed on a Tektronix Color/Monochrome Phasar II SDX printer.

Results

Two hundred cultured lymphocyte samples were screened by use of the MultiFISH cocktail of probes, which included single cosmids as well as cosmid contigs. The indications for chromosomal studies in each of these cases were either to exclude specific microdeletion syndromes by FISH (e.g., PWS or SMS) or to assess the possibility of a chromosomal basis for features including developmental delay, mental retardation, dysmorphic facies and/or multiple congenital anomalies (table 1). All patients received a routine chromosome analysis ($n = 200$) and, if requested in the referral, a specific FISH assay for a suspected microdeletion, by use of the relevant individual FISH probes ($n = 45$). At the time of the routine cytogenetic study, additional slides were made and submitted for MultiFISH analysis. In each case, the MultiFISH samples were scored in a blinded fashion, and 20 metaphases per patient were scored. Since this assay was not designed to address the issue of mosaicism, patients were scored as either deleted or not deleted for a particular probe. All patients deleted for a given probe (and therefore "positive" for the corresponding gene-deletion syndrome) were found to be deleted for that probe in each of the 20 metaphases scored. By MultiFISH, 11 of the 200 samples analyzed were found to have a deletion. When samples were decoded, it was found that 10 of the 11 samples deleted for a probe had been submitted to the cytogenetics laboratory for analysis by FISH and had been identified in parallel as being deleted, by use of single-probe FISH. As discussed below, MultiFISH also identified one patient with a deletion of the DGS/VCFS region. Single-probe FISH was subsequently performed and confirmed the deletion.

After the MultiFISH scoring was complete, indications for cytogenetic referral were matched to the individual patients via the laboratory identification numbers. The majority (189/200) of patients screened were not found to be deleted for any of the tested loci. This category included the 154 patients (77.0%) referred for reasons other than to rule out a specific deletion (table 1). A representative normal MultiFISH result, in which all signals are present, is shown in figure 2. For patients not deleted for any of the tested loci, a total of eight signals could be visualized, whereas only seven signals were observed in patients deleted for one of the tested loci.

Forty-five of the samples (22.5%) included in the survey were referred for analysis of a specific microdeletion. Thirty-five of these cases were found to be nondeleted for the tested loci both by MultiFISH and by single-probe FISH. All cases found to be deleted for the tested loci by independent single FISH studies were also detected by MultiFISH analysis. In all 10 of these cases, the clinical cytogenetics laboratory had identified a deletion by using the appropriate single-probe FISH. Each case was also simultaneously submitted for blinded analysis by MultiFISH. In no instance was any patient incorrectly classified as deleted for the tested loci (i.e., there were no false positives) by MultiFISH. This result demonstrates that individual deletions identified by use of MultiFISH are consistent with the results obtained by use of traditional, single-probe FISH.

In order to establish a new diagnostic procedure for use in a cytogenetics laboratory, analytical sensitivity and specificity must first be determined. The analytical sensitivity for this assay is the probability that the test will detect a deletion when a deletion is truly present (Leaverton 1991, pp. 7–27). For MultiFISH, this can be calculated by use of the known deletion-patient samples that were entered into this study. The appropriate deletion was detected in 100% of 11 specimens with known deletions, when the MultiFISH assay was used; therefore, the sensitivity of this test, as measured in this population of samples, is 100%.

The analytical specificity for this assay is the probability of scoring a sample as a nondeletion when a deletion is truly not present (i.e., when there are two normal

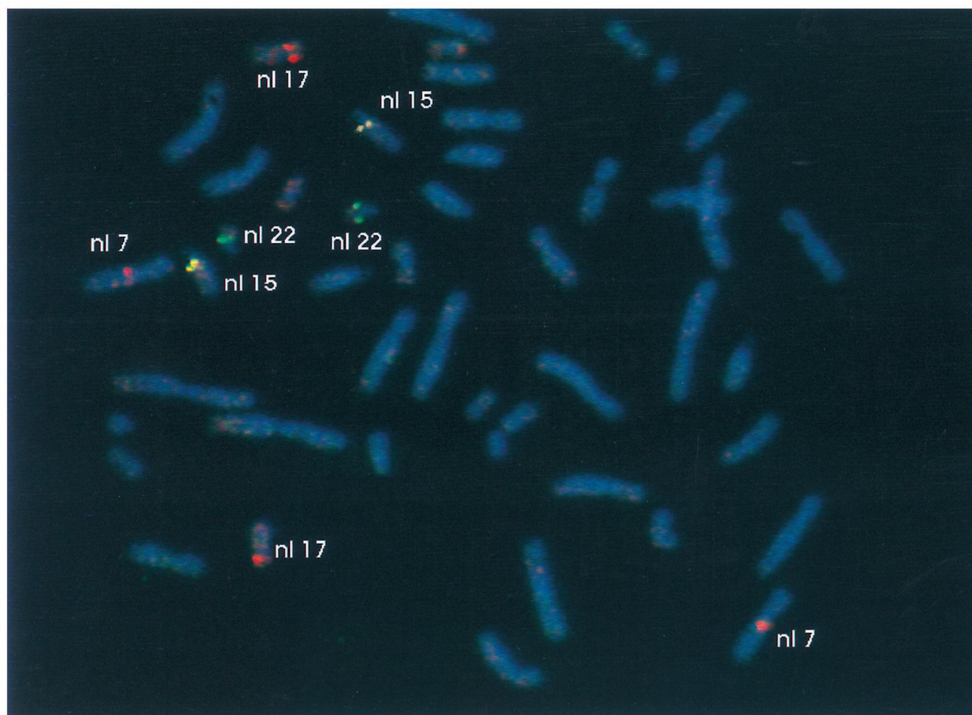

Figure 2 Representative normal MultiFISH result. Signals correspond to critical regions for the following syndromes: WS, chromosome 7 (nl 7 [*red*]), DGS/VCFS, chromosome 22 (nl 22 [*green*]), PWS/AS, chromosome 15 (nl 15 [*yellow*]), and SMS, chromosome 17 (nl 17 [*red*]).

chromosomes) (Leaverton 1991, pp. 7–27). All cases scored as deleted for the tested loci had their respective deletions confirmed by use of single-probe FISH; thus, on a patient-result basis, the specificity was 100%. For FISH, specificity on a per-chromosome basis provides a measure of hybridization efficiency. The hybridization efficiency was calculated in two ways, by use of the data for individual probes and by use of the data for pooled probes, for the nondeletion group of patients and the deletion group of patients. The results are shown in table 2. First, the hybridization efficiency of each individual probe was calculated for the nondeletion individuals ($n = 189$). The calculated hybridization efficiencies were 94.7% (WS probe), 96.7% (DGS/VCFS probe), 96.9% (SMS probe), and 98.0% (PWS/AS probe). Furthermore, the combined overall hybridization efficiency of the MultiFISH assay was determined to be 96.6%. This number indicates that >96% of metaphases scored had all eight signals and that <4% of cells analyzed had fewer than eight (i.e., either six or seven) signals. For purposes of comparison, each individual cosmid (or cosmid contig) was also hybridized to normal cells from four control individuals, and a total of 100 metaphases/probe were scored, in order to calculate probe hybridization efficiencies. The following results were observed: 94% (WS probe), 97% (DGS/VCFS probe), 98% (SMS probe), and 98% (PWS/AS probe). Clearly, by comparison with single-probe FISH, hybridization efficiency was not compromised in MultiFISH. Furthermore, for the individuals deleted for the tested loci ($n = 11$), we used their "nondeleted" chromosomes to calculate the hybridization efficiency (expected number of hybridization signals is seven). The hybridization efficiency of each probe ranged from 98.6% (WS probe) to 99.3% (PWS/AS probe), with 98.9% being the overall collective hybridization efficiency of the MultiFISH assay. There was

Table 2

Probe Specificity

	No. of Signals Observed/No. of Signals Expected (%)	
	Patients Not Deleted for Probe[a]	Patients Deleted for Probe[b]
WS	7,157/7,560 (94.7)	355/360 (98.6)
PWS/AS	7,410/7,560 (98.0)	417/420 (99.3)
DGS/VCFS	7,309/7,560 (96.7)	395/400 (99.2)
SMS	7,329/7,560 (96.9)	357/360 (98.8)
All loci	29,205/30,240 (96.6)	1,524/1,540 (98.9)

[a] Maximum signals possible per patient is 8.
[b] Does not include deleted chromosome (i.e., maximum number of signals per patient is 7).

a significant difference, in probe hybridization efficiency, between the individual probes within the group not deleted for the tested loci ($\chi^2_3 = 39.87$, $P < .0001$) but not within the group deleted for the tested loci ($\chi^2_3 = 0.17$, $P \approx .99$). This discrepancy may be due to sample size. Regardless, these differences did not affect the overall ability of this assay to discriminate between samples deleted for the tested loci and samples not deleted for the tested loci.

Finally, the MultiFISH assay identified a deletion in the DGS/VCFS region in a single patient whose karyotype was apparently normal. This particular patient was referred for chromosomal analysis because of developmental delay and mental retardation. Since no specific disorder had been suspected by the clinician, a FISH analysis to rule out any microdeletions had not been requested. Analysis of this sample by MultiFISH showed hybridization of the F5 probe to only a single chromosome 22 homologue in 100% of the metaphases examined. By use of the single F5 cosmid probe corresponding to the DGS/VCFS commonly deleted region, the results of MultiFISH for this patient were subsequently confirmed.

Discussion

Geneticists have been well served by diagnostic tests that detect a large number of disorders, as exemplified by banded karyotype analysis, amino acid analysis, and organic acid analysis. It would be desirable to increase the general sensitivity of chromosomal analysis, in order to detect the large number of known microdeletion syndromes and to detect as yet undiscovered microdeletions. Three diagnostic difficulties exist: first, some of the syndromes do not have distinct and consistent features that can guide the clinician toward requesting a specific diagnostic test; second, variability in the clinical presentation, such as subtle or atypical features as exemplified in figure 1, may lead to underrecognition of certain disorders; and, third, many clinical evaluations are performed by neurologists, cardiologists, geneticists, and other clinicians, who may not be as experienced as a skilled dysmorphologist in recognizing subtle or nonspecific features. This problem is especially relevant in the case of very young infants, for whom the characteristic features of a disease may not yet have manifested and in whom developmental delay and/or mental retardation cannot be adequately assessed. Although various strategies are being explored to increase the detection of microdeletions, pooled probes for MultiFISH analysis could represent one attractive approach to this complex diagnostic problem.

We have demonstrated that multiple probes hybridized concomitantly in a FISH analysis can be useful in the simultaneous examination of several frequently deleted regions. Typically, FISH analysis using a single probe is performed when a clinician suspects a particular microdeletion or contiguous gene–deletion syndrome in a patient. Ideally, the results of FISH analysis provide a molecular cytogenetic confirmation of a clinical diagnosis that is already suspected by the physician. However, making such a diagnosis is often complicated when a patient presents with nonspecific or nondiagnostic findings, such as mental retardation or global developmental delay. This problem is evidenced in table 1, which includes seven cases, each referred to exclude multiple genetic syndromes that can include developmental delay as a component. Furthermore, as a specific example, patient C (fig. 1) was referred to the clinical cytogenetics laboratory to be evaluated for both PWS and WS. Subtle facial features were suggestive of both PWS (almond-shaped eyes) and WS (full lips). Although this patient's short stature was suggestive of either of these syndromes, his obesity was certainly more typical of PWS (for the correct diagnosis, see the appendix).

Here we have described the identification of a DGS/VCFS deletion through MultiFISH. Although a physical exam of this 2-year-old child did identify some dysmorphism (flat nasal bridge, broad forehead, and hypertelorism), no features that were particularly suggestive of VCFS were present. Routine cytogenetic analysis of this child resulted in an apparently normal G-banded karyotype, and, since the clinician did not suspect any deletion syndrome, an appropriate FISH analysis was not performed. However, analysis of this child by use of MultiFISH identified a deletion at the DGS/VCFS critical region. Discovery of the deletion provided this family with a diagnosis and potentially avoided a great deal of time and expense in additional testing to determine a cause for this child's developmental delay. As a result, appropriate medical evaluation can now be obtained, potential future complications of this disease can now be predicted, and recurrence-risk estimates can be made for the parents. For VCFS in particular, MultiFISH can potentially be very useful in the identification of affected individuals, since the physical features are not always conspicuous enough to allow a diagnosis; this is especially true in young children (Lindsay et al. 1995) and in children of varied ethnicities. It is precisely this type of patient for whom the MultiFISH analysis would be most beneficial.

There are many microdeletion syndromes for which critical or commonly deleted regions have been identified and for which good-quality FISH probes have been developed. These syndromes include the disorders being assayed with this first version of a MultiFISH deletion detection panel: PWS, AS, WS, DGS/VCFS, SMS, and Miller-Dieker syndrome, which was not represented in MultiFISH (Kuwano et al. 1991, 1992; Desmaze et al. 1993; Ewart et al. 1993; Lindsay et al. 1993; Chen et

al. 1995; reviewed in Shaffer 1995). When any of these conditions is suspected in a patient, FISH analysis using the appropriate probe is frequently used to confirm the diagnosis. However, if more than one such syndrome is to be tested for deletions, then multiple sequential FISH analyses must be performed. In contrast, cohybridization of several critical-region probes saves time, requires less sample material and fewer slides, and allows for simultaneous analysis of some of the more common gene-deletion syndromes. Hybridization of probes labeled in different color combinations, as described here, further facilitates analysis by fluorescent microscopy, when coupled with a triple-bandpass filter.

Establishing a new FISH test in a diagnostic cytogenetic laboratory requires that the assay demonstrate both analytical sensitivity and specificity. By examining the efficacy of individual FISH probes versus the MultiFISH assay as performed here, we have shown that neither sensitivity nor specificity has been compromised; both were 100%. The hybridization efficiency (i.e., the percent of normal metaphases in which all four sets of signals could be visualized) of MultiFISH was 96%–99%. When all eight signals could not be seen, it was typically a single signal that was absent (this occurred in the remaining <4% of metaphases examined). More remarkably, however, each of the cases deleted for the tested loci demonstrated hybridization of only a single homologue in 100% of the metaphases examined. Certainly, as in FISH analyses using single probes, good-quality slides with many metaphases allow for clearer interpretation of the results.

The MultiFISH assay should be viewed as a screening tool that increases one's ability to detect chromosomal abnormalities. As such, a normal MultiFISH analysis does not exclude the possibility of other chromosome abnormalities and should therefore be performed in conjunction with routine cytogenetics. This is evidenced by the finding of chromosome anomalies—such as trisomy 21, fragile X, del(13)(q21q32), and inv(20)(q12q13.3)—through routine cytogenetic analyses in certain cases of the 200 included in this study. Clearly, chromosome analysis has a great advantage of uncovering gross abnormalities, since the whole genome is assessed. In the approach that we present here, our detection is obviously limited to the syndromes for which corresponding probes are included. The complementation of routine cytogenetics and FISH is currently practiced with FISH aneuploidy screening, the results of which are routinely followed by karyotype analysis (Klinger et al. 1992). Likewise, any deletion detected by MultiFISH should also be confirmed by use of the appropriate single-probe FISH as part of a laboratory's quality-assurance program.

There are certainly limitations to MultiFISH, most of which apply to the interpretation of any FISH results. It should be noted, for example, that not all patients with mutations at critical genes involved in contiguous gene–deletion syndromes will have deletions. For example, only 70% of PWS patients are deleted for a paternal copy of chromosome 15q11-q13 (American Society of Human Genetics/American College of Medical Genetics Test and Technology Transfer Committee 1996). Of the remaining cases, ~28% show maternal uniparental disomy, and 2% will have a mutation affecting the imprinting center (American Society of Human Genetics/American College of Medical Genetics Test and Technology Transfer Committee 1996). Although both of the latter mechanisms can preclude expression from the paternal PWS allele, neither type of mutation will be ascertained by use of this type of FISH analysis. These cases will ultimately require molecular studies (i.e., parent-of-origin studies and methylation studies), in order to allow the mutation to be uncovered. Additionally, rare patients with small, atypical deletions may not be ascertained by MultiFISH, since detection requires that probes be located entirely within the deleted regions.

Clearly, MultiFISH can identify only deletions for which the specific probes have been included. The constitution of the probe cocktail may of course be adjusted to reflect other deletion syndromes of interest, as long as specific, good-quality probes are available. Therefore, MultiFISH can be considered only as reliable as its constituent probes. It might be argued that clinicians can suspect the diagnoses in question and order the appropriate, specific tests, thereby making a screening diagnostic approach inappropriate. We believe that this perspective is flawed, for multiple reasons: (1) it overestimates the ability of even the best clinicians to diagnose the recognizable syndromes, as evidenced by figure 1; (2) current practices may diagnose typical clinical cases and fail to diagnose atypical cases, so that the full clinical spectrum for some disorders may be presently unknown; (3) many physicians evaluating developmentally delayed infants and children are not experienced dysmorphologists; (4) there are syndromes that simply cannot be recognized clinically in a consistent manner yet are easily diagnosed by laboratory testing (e.g., SMS) (Greenberg et al. 1996); (5) cytogenetic laboratories vary in the ability to recognize subtle deletions (e.g., recognition of del(15)(q11.2q13) can be inherently difficult) (Delach et al. 1994); and (6) if multiple probes can be tested for a small or modest incremental cost relative to that of testing with a single probe, a higher quality of diagnostic evaluation can be performed. MultiFISH has the greatest value for disorders that are difficult to recognize clinically and/or for which deletions are difficult to detect cytogenetically. Examples of disorders that can be especially difficult to diagnose include α-thalassemia/mental retardation contiguous-gene deletion syndrome, which maps to chromosome

16p (Wilkie et al. 1990), and the chromosome 1p36.3–deletion syndrome (Keppler-Noreuil et al. 1995; Shapira et al. 1997).

In order to increase the utility of this technique, it would be desirable to develop various MultiFISH panels corresponding to specific subsets of disorders. For example, a cardiac MultiFISH panel might include probes corresponding to syndromes that feature cardiac abnormalities (e.g., WS, DGS/VCFS, and SMS). We believe that there is a definite need for screening procedures for identification of a broad range of subtle cytogenetic abnormalities not detected by banded karyotype analysis and that the MultiFISH approach provides a useful strategy to do so. However, it is certain that none of these methods represents a substitute for proper referrals for specialized clinical evaluation and counseling.

Finally, the number of different probes assembled in the cocktail may be limited by the different color combinations available through direct/indirect labeling and detection methods. However, ultimately we would like to increase the number of different colors to further facilitate the scoring process and to allow us to incorporate more unique probes in a given cocktail. To date, multicolor FISH analysis has been described for applications ranging from detection of common aneuploidies in uncultured amniotic-fluid cells to differential painting of all the 24 human chromosomes (Klinger et al. 1992; Ledbetter 1992; Ried et al. 1992; LeBeau 1996; Speicher et al. 1996). In these cases, simultaneous hybridization of multiple probes offers advantages similar to those of MultiFISH. All protocols afford the opportunity to detect anomalies of several chromosomes within a single hybridization and, as such, should enhance the utility of FISH analysis and should increase the ability to detect cytogenetic abnormalities in a diagnostic setting.

Appendix

Specific diagnoses of patients are as follows: patient A (VCFS), del(22)(q11.2q11.2); patient B (WS), del(7)(q11.23q11.23); patient C (WS), del(7)(q11.23q11.23); and patient D (SMS), del(17)(p11.2p11.2).

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