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13. ABSTRACT *(Maximum 200 Words)*

The three major goals of this study were to: 1) validate volumetric MRI in the measurement of plexiform neurofibromas; 2) obtain a body of normative data on the growth rate of plexiform neurofibromas; 3) develop an infrastructure to support future clinical trials. Although the first aim will not be fully achieved until all of the data are available and analyzed, we have made significant progress by demonstrating tumor volumes can be reliably measured by multiple observers. The reproducibility study has been completed and submitted for publication. Regarding the second aim, this year saw a continued increase in patient enrollment mainly due to the addition of three new clinical sites. Most plexiform neurofibromas have been imaged only once or twice, but we have included data representing longitudinal follow-up of one tumor as an example of the type of growth data we are hoping to collect. Finally, with respect to building infrastructure, we have a stable group of participating centers and have streamlined the process of signing on new centers. We have also worked collaboratively with the group at the National Cancer Institute who is running a clinical trial of a farnesyl protein transferase to coordinate the natural history study with this clinical trial. The study got off to a slow start due to administrative problems related to the process of obtaining IRB approval for centers but data is now steadily accumulating, making it clear that the objectives of the study will be achieved.

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Introduction

This report marks completion of the third year of this project. The three major goals of this study were to: 1) validate volumetric MRI in the measurement of plexiform neurofibromas; 2) obtain a body of normative data on the growth rate of plexiform neurofibromas; 3) develop an infrastructure to support future clinical trials. Although the first aim will not be fully achieved until all of the data are available and analyzed, we have made significant progress by demonstrating tumor volumes can be reliably measured by multiple observers. The reproducibility study has been completed and submitted for publication. Regarding the second aim, this year saw a continued increase in patient enrollment mainly due to the addition of three new clinical sites. Most plexiform neurofibromas have been imaged only once or twice, but we have included data representing longitudinal follow-up of one tumor as an example of the type of growth data we are hoping to collect. Finally, with respect to building infrastructure, we have a stable group of participating centers and have streamlined the process of signing on new centers. We have also worked collaboratively with the group at the National Cancer Institute who is running a clinical trial of a farnesyl protein transferase to coordinate the natural history study with this clinical trial. The study got off to a slow start due to administrative problems related to the process of obtaining IRB approval for centers but data is now steadily accumulating, making it clear that the objectives of the study will be achieved.

Progress Report for Statement of Work by Task

Task 1. **Complete development of study infrastructure – Months 1-6**

a. **IRB approval at all clinical sites**

Table 1 lists the participating clinical centers, the principal investigator at each site, and the IRB approval status. The IRB column refers to approval by the local IRB; the "Army" column refers to approval by the army IRB. Several centers have been dropped from the study. These are: Hadassah University Hospital, Mayo Clinic, Mt. Sinai School of Medicine, Toronto Hospital for Sick Children, University of Manchester, University of Padova, University of Pittsburgh, and University of Texas. The major reason for drop out was lack of follow-through by the local investigator to complete the necessary paperwork for IRB approval. Two new centers have been added, both to replace dropout and to accommodate investigators who have expressed a strong desire to participate. These are: National Cancer Institute and Massachusetts General Hospital.

During this past year we have streamlined the process of enrolling new centers, based on our experience in guiding the centers through the process of preparing the IRB submission and designing the consent form so that it will be likely to be approved both by the local center and the Army IRB.

Center	PI	# Pts	IRB	Army
Children's Hospital Boston - 107	Bruce Korf	22	Yes	Yes
Children's Hospital Medical Ctr - 173	Robert Hopkin	7	Yes	Yes
Children's Hospital of Oklahoma - 178	John Mulvihill	12	Yes	Yes
Children's Memorial Hospital - 177	Joel Charrow	12	Yes	Yes
Children's National Medical Ctr- 170	Roger Packer	22	Yes	Yes
Guy's Hospital - 187	Rosalie Ferner	17	Yes	Yes
Klinikum Nord Ochsenzoll - 160	Victor-Felix Mautner	30	Yes	Yes
Mass General - 106	Mia MacCollin	0	Yes	Yes
Mass General - 189	Bruce Korf	4	Yes	Yes
National Cancer Institute - 181	Brigitte Widemann	12	Yes	Yes
New Children's Hospital - 112	Kathryn North	16	Yes	Yes
Texas Children's Hospital - 172	Sharon Plon	5	Yes	Yes
University of British Columbia - 100	Jan Friedman	7	Yes	Yes
University of Utah - 117	David Viskochil	14	Yes	Yes
Washington University - 169	David Gutmann	23	Yes	Yes

Table 1. Status of IRB approval of participating clinical centers.

b. **Complete clinical data entry forms and test electronic transfer of clinical data**

Data entry forms were completed by the end of the first year, and have not changed.

c. **Organize package of materials for pathology review and tissue repository**

This task was completed by the end of the second year and has not changed. A detailed protocol for submission of tissue specimens has been produced and is available for download on our website.

d. **Set up listserv and website**

The study website has been operational for over a year at www.nfstudies.org. The only significant change in the past year relates to the location of the MRI Database tool. This year the database tool was transferred to our website in order to improve the site's utility. Also, a new feature was added for centers to search for patient visits as well as scans (Figure 1). This form searches all records in our patient database and returns real-time information pertaining to the patient in question. This tool is password protected to ensure only appropriate parties can access this information.

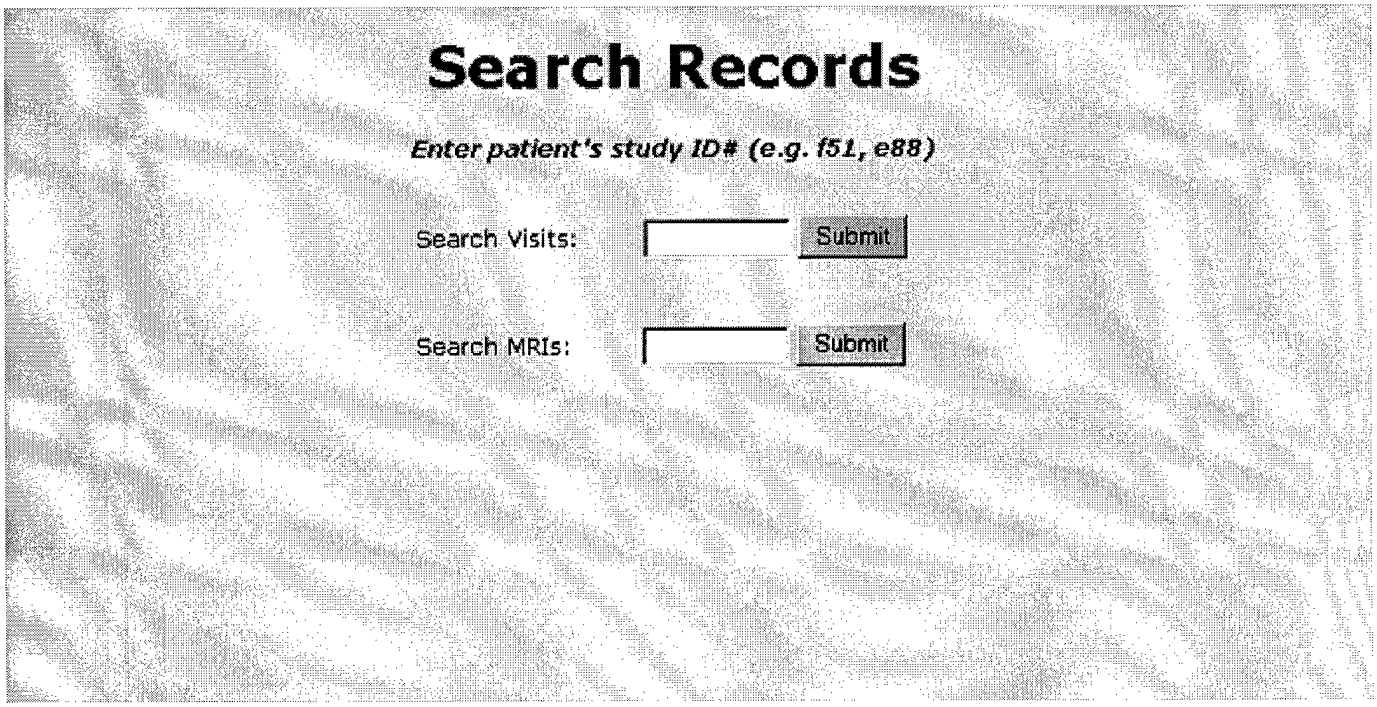

Figure 1. Screenshot of search page for patient database.

e. **Test MRI data transfer**

Each center has submitted test data for the NF1 Study either by optical disk or through File Transfer Protocol (FTP).

f. **Purchase workstation and prepare data entry forms at WorldCare.**

The workstation was purchased in November of 1998. Documentation was provided in last year's progress report.

WorldCare has maintained the NF1 Natural History Study infrastructure by ensuring that on site project systems are constantly prepared for data collection and analysis. To this end efforts have been made to bring up to date existing hardware and software responsible for all aspects of project functionality. Included are equipment for sending and receiving images such as optical drives, translators and servers related to file transfer protocol. Additionally, the image analysis suite has been updated and is running on a recently purchased, cutting edge computer. While the image software is 510K approved, WorldCare contracted an independent validation company to guarantee that all analytical and statistical systems are running efficiently. The patient-tracking database has been maintained with small reorganizations designed to more accurately audit patient visit information and data. Previously instituted filing systems, logbooks and binders have been kept current to track both the history and progress of efforts made by all parties in contact with WorldCare.

To accurately reflect the procedural changes made in the NF1 project, WorldCare document control updates and revises standard operating procedure manuals as necessary. These manuals outline procedures for the collection, receiving and analysis of data specific to the study within Good Clinical Practices (GCP) guidelines. The NF1 Collection Center Study Manual and has been distributed to the clinical coordinators at each MRI facility in the study.

g. **Prepare project monitoring flow sheet at Brigham and Women's Hospital**

This was addressed last year and no changes have occurred since this time.

h. **Prepare recruitment letters for study subjects**

This was addressed last year and no changes have occurred since this time. As will be detailed in section 2.b below, we are considering generating a new recruitment letter and disseminating it broadly to the NF patient community to further increase enrollment of study arms for which enrollment is a particular challenge.

i. **Publicize study to NF community**

The study continues to be publicized in newsletters of the National Neurofibromatosis Foundation and of NF, Inc.

Task 2. ***Recruitment of Study Subjects*** **– Months 6-12**

a. **Centers contact prospective study subjects**
b. **Enrollment of study subjects**
c. **First MRI and clinical data received**

Substantial progress has been made in enrollment since last year, although total enrollment remains below the originally projected 300 patients. In part this is due to the delay in recruitment stemming from problems with the IRB approval of many centers, and in part it is attributable to the dropout of some centers due to lack of follow -through with the process of obtaining IRB approval. The increased enrollment during the past two quarters (Figure 2) represents the addition of new centers in Australia, National Cancer Institute, and Massachusetts General Hospital. We also expect additional patients to be enrolled at Massachusetts General Hospital during the next quarter.

The delay in enrollment is also due, in part, to challenges in access to certain types of patients. First, it will be noted (Table 2) that two of the three categories of participants < 18 years of age are filled (actually more than filled, see below), whereas there is a paucity of adults enrolled in all categories. We believe this reflects the fact that most of the NF Clinics tend to see more

children than adults, largely because parents are likely to bring their children to see an NF specialist, but adults tend to seek care only when specific problems are present. Second, the category of "trunk and extremity tumors not externally visible," is least well subscribed in both children and adults. This is likely due to the fact that most individuals with NF1 are only aware of plexiform neurofibromas that are externally visible. The centers are following relatively few individuals who are known to have neurofibromas that are not visible externally, since it is not routine to screen asymptomatic patients for hidden tumors.

A meeting is being set up of the project steering committee to consider how to deal with these issues. Several options are being considered at this point, including:

i) Increase the number of participating centers to increase the overall number of enrolled patients. Although we would be interested in having additional centers join the study, given the complexity of the IRB approval process it is not likely that this will quickly result in increased enrollment.

ii) Increase the publicity about the study to encourage more individuals with NF1 to inquire about participation. Although the study is listed in newsletters of the National Neurofibromatosis Foundation (NNFF) and NF, Inc., and on the NNFF clinical trials website, a letter to individuals on the mailing list of these two organizations will likely increase enrollment. This will require permission of the IRB.

iii) Increase the enrollment in categories for younger participants with externally visible tumors to maximize the likelihood of obtaining meaningful results for these tumors, and accept that for the near future we may have only pilot data for other categories. We have tentatively allowed increased enrollment in some arms of the study, although we will need to obtain approval of the IRB to accept this amendment before these enrolled patients can be formally included in the study.

The steering committee will meet during the next month and consider these options. It is likely that all three will be pursued to some extent, with the goal of completing enrollment as quickly as possible.

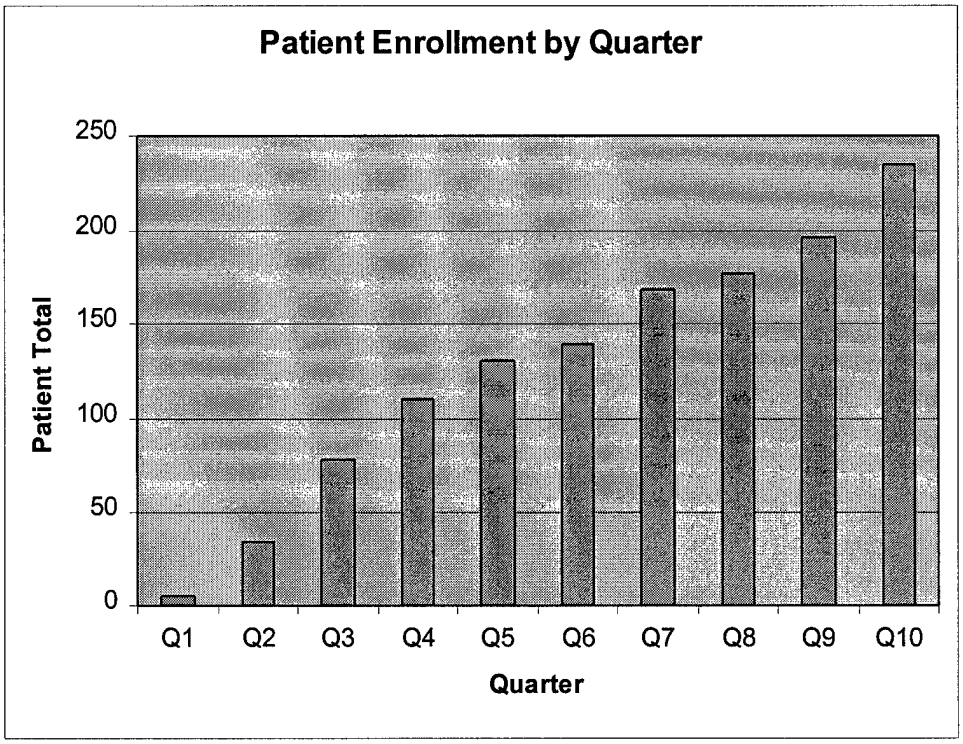

Figure 2. Cumulative subject enrollment by quarter from start of study to present.

Study Category		Number Recruited
Head & Neck	< 18 years old	58
	> 18 years old	16
Trunk & Extremity Externally Visible	< 18 years old	52
	> 18 years old	39
Trunk & Extremity Not Externally Visible	< 18 years old	22
	> 18 years old	16
Total		203

Table 2. Number of subjects recruited by study category.

d. Review of clinical entry criteria

Entry and exclusion criteria were reviewed in a meeting held in February 1999 at the Banbury Center in Cold Spring Harbor, N.Y. A follow-up meeting of the steering committee and participating clinical centers was held in Aspen, CO in June, 2000. No changes were made in the entry criteria at that meeting.

e. Test of inter-observer reproducibility of designation of tumor margins by MRI

The results of the reproducibility study were reported in the progress report last year. A paper has been prepared and submitted for publication to *Radiology*. A copy is appended to this report. We have found excellent inter-observer agreement (correlation coefficient 0.996 for three observers), with reproducibility of volume measurements for almost all scans within 10% and most within 5%.

Task 3. *Data Acquisition and analysis* – Months 13-42

MRIs are sent from individual study sites in batches. In order to facilitate this task, we are encouraging centers to send scans through file transfer protocol. This method benefits all parties since it is faster and less expensive than sending them by optical disk. The current status of MRI receipt in shown in Table 3:

Center	MRI Scans
Children's Hospital	19
Children's Hospital Medical Center	9
Children's Hospital of Oklahoma	8
Children's Memorial Hospital	31
Children's National Medical Center	10
Guy's Hospital	19
Klinikum Nord Ochsenzoll	69
National Cancer Institute	12
Texas Children's Hospital	2
University British Columbia	11
University of Utah	14
Washington University	8
Total	212

Table 3. Number of MRI scans received by site.

Task 4. ***Interpretation of Data*** **– Months 43-48**

MRI Data

It is premature to report on interpretation of longitudinal data, since relatively few patients have been imaged multiple times at this point in the study. We have, however, analyzed data from a sample tumor that has been imaged on three occasions, to serve as an example of the kind of data we anticipate as the study continues. These data are shown in Figure 3.

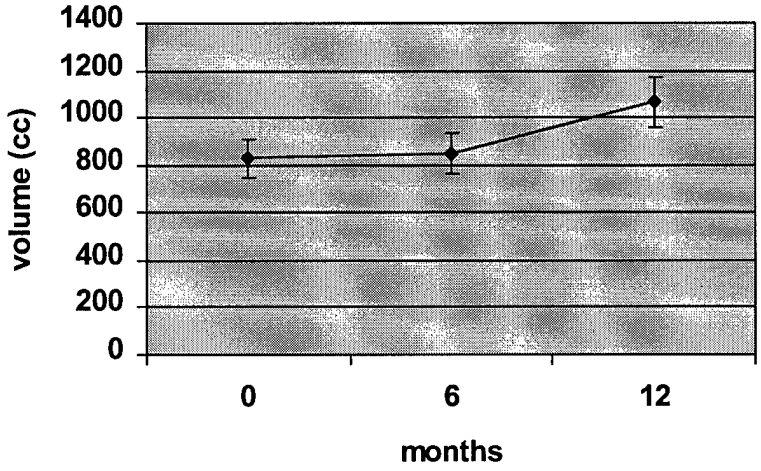

Figure 3. Three successive measurements, each separated by a period of six months, of tumor 160-0471-400. Error bars indicate plus or minus 10%, based on results of reproducibility study. Representative images are shown in Figure 4.

Figure 4. Representative scans of tumor 160-0471-400 imaged at 0, 6, and 12 months, with area of tumor outlined.

Neuropathology Data

The tissue bank and neuropathology review facility has been involved in collection of blood and tumor specimens, and has conducted a study in which

fluorescence *in situ* hybridization has been used along with immunohistochemistry for S100 to examine plexiform neurofibromas and malignant peripheral nerve sheath tumors for loss of *NF1* gene function. Drs. Perry et al. have shown that deletion of one copy of the *NF1* gene occurred in 4/7 plexiform neurofibromas and in all of 8 MPNST's. All cells in the plexiform neurofibromas that harbored deletions displayed S100 staining, indicative of Schwann cell origin, consistent with emerging evidence implicating the Schwann cell as the primary tumor cell of the neurofibroma. In contrast, some MPNST cells with loss of *NF1* did not stain with S100, suggested that these cells had lost (or were derived from cells that never had) this property of differentiated Schwann cells. Copies of two papers resulting from this work are attached.

Development of Infrastructure for Clinical Trials

Dr. Korf has worked closely with Drs. Frank Baylis and Brigitte Wiedemann at National Cancer Institute in the design of their clinical trial of the farnesyl protein transferase R115777 in the treatment of growing plexiform neurofibromas. This clinical trial will also use volumetric MRI as a measurable endpoint, using an imaging protocol adapted from the one used in this natural history study. This clinical trial also shares tissue banking and neuropathology facilities. Finally, participants in the natural history trial who demonstrate tumor growth will be able to cross over into the treatment trial if eligible and if the patient and family are willing. Similarly, a trial of the fibroblast inhibitor Pirfenidone has been proposed (PI: Dr. Roger Packer) which will also use the same tissue bank and similar volumetric MRI protocols.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of reproducibility study showing high inter-rater correlation coefficient, suggesting that volumetric analysis will provide reproducible data on tumor volumes, resulting in paper for publication.
- Substantial increase in study centers that have passed complete IRB approval (15) and steady increase in patient enrollment
- Addition of 3 new sites to study
- Steady acquisition of MRI data and data analysis at WorldCare
- Improvement in imaging database available on website at www.nfstudies.org
- Streamlining of IRB approval process to increase the speed of IRB approval of new sites
- Coordination with NCI farnesyl transferase study and proposed Pirfenidone trial
- Identification of *NF1* gene deletions in plexiform neurofibromas and MPNST as basis for loss of *NF1* function in some PN and most MPNST

REPORTABLE OUTCOMES

1. Manuscripts: attached and see references.

2. Presentations

National Neurofibromatosis Foundation Clinical Trials Workshop – Dr. Korf was a co-organizer and chair of the session on outcomes measures. He presented a paper describing the study of the natural history of plexiform neurofibromas in NF1. A paper describing this meeting has been submitted for publication to the journal Neurology.

Dr. Korf was a co-organizer of an NIH-sponsored meeting on NF1 held in October, 2001 in conjunction with the annual meeting of the Child Neurology Society in Victoria, B.C., Canada. He presented a paper on the natural history study, which will be published in the journal Child's Nervous System.

3. Patents, licenses: not applicable

4. Degrees obtained: not applicable

5. Tissue Repositories: A repository of blood and tumor tissue is now established at Washington University, St. Louis. This repository was initiated as part of this project, but is now being used by treatment protocols for Pirfenidone and farnesyl transferase inhibitor, as well.

6. Informatics: The NF International Database has been modified to accommodate the specialized data collection required for use in this project. This database is open to investigators anywhere in the world (to input their own data, or query the database in a manner that preserves the confidentiality of patients.

7. Employment/research opportunities: not applicable

CONCLUSIONS

The study has made significant progress during the past year, in spite of a slow start in recruitment of participating centers and patients due to problems with the IRB approval process. The need for two IRBs to approve each center remains a cumbersome approach, but we have learned to work within the system to expedite the approval of new centers. We will need to make some decisions regarding the recruitment of adults, and patients at any age with tumors that are

not externally visible, but are confident that we can deal with these challenges and accomplish the goals of the study. We are well along on the task of validating the utility of volumetric MRI in measuring plexiform neurofibromas by showing that reproducible measurements can be made in spite of the complexity of the lesions. Accrual of data is also proceeding, and an efficient approach has been set in place to acquire the MRIs and analyze them. Finally, we have developed an infrastructure that is already helping to catalyze clinical trials for promising drugs in the treatment of plexiform neurofibromas. It is our expectation that the data derived from this study will be especially important in future trial design, as we learn about the patterns of neurofibroma growth and how to follow them.

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Perry A, Roth KA, Banerjee R, Fuller CE, Gutmann DH. NF1 Deletions in S-100 Protein-Positive and negative cells of sporadic and neurofibromatosis 1 (NF1)-associated plexiform neurofibromas and malignant peripheral nerve sheath tumors. American Journal of Pathology 2001;159:57-61.

Appendix A

Volumetric Measurement of Plexiform Neurofibromas using MR Imaging

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Volumetric Measurement of Plexiform Neurofibromas using MR Imaging

Abstract

Purpose: To validate MR imaging volumetric measurements for evaluating growth of plexiform neurofibromas in neurofibromatosis type 1(NF1).

Material and Methods: On MR examinations of 12 children with NF-1, lesion volumes were measured on axial STIR images by 3 independent observers.

Results: Overall inter-rater correlation coefficient was 0.996 (excellent agreement). Variation increased with greater lesion size.

Conclusions: Volumetric measurements of NF-1 lesions are reproducible.

Introduction

Neurofibromatosis type 1(NF1) is an autosomal dominant disorder that affects approximately 1 in 4,000 individuals. (1). The hallmark feature is the occurrence of benign nerve sheath tumors, neurofibromas. Other features include café-au-lait macules, skin-fold freckles, optic gliomas, iris hamartomas (Lisch nodules), skeletal dysplasias, and malignant peripheral nerve sheath tumors. Much of the morbidity of the disorder is associated with the neurofibromas. Cutaneous neurofibromas can be present in large numbers, causing cosmetic disfigurement. Plexiform neurofibromas, occurring in 25% of individuals with NF1, are characterized by longitudinal neurofibroma growth along nerves and involving multiple fascicles and branches. They lead to disfigurement, overgrowth, nerve compression, and even malignancy. The only current treatment for plexiform neurofibromas is surgery, but surgical resection is usually difficult because lesions are large and infiltrative. Recurrence is therefore common (2, 3)

The cloning of the NF1 gene has resulted in insights into pathogenesis that may ultimately lead to the development of non-surgical therapies. The gene responsible for NF1 encodes a protein referred to as "neurofibromin," which functions at least in part as a negative regulator of Ras family GTPases (4). Clinical trials that have been undertaken or are contemplated include the use of farnesyl protein transferase inhibitors, angiogenesis inhibitors, cytodifferentiating agents, and hormonal modulators (5, 6).

The high rate of morbidity associated with plexiform neurofibromas makes them a good target for non-surgical therapies. There are major challenges, however, in the determination of outcomes measurements that will complicate these trials. Plexiform neurofibromas may grow erratically, exhibiting periods of rapid growth followed by

spontaneous stabilization. Also, the lesions may be large and irregularly shaped, making it difficult to measure their size and follow changes related to growth or shrinkage in response to treatment.

Given these challenges, and the likelihood that drugs will be available for clinical trial in the near future, we have organized a multicenter trial to determine the natural history of plexiform neurofibromas in NF1 using volumetric MRI. The major goals of this study are, first, to validate volumetric MRI as a means of following the growth of plexiform neurofibromas, and, second, to generate a body of normative data on the growth rate of plexiform neurofibromas from different regions of the body. The validity of the volumetric analysis is critically dependent on the ability of an observer to reproducibly determine the margins of a tumor in an MR image. We have therefore studied inter-observer correlation of three observers analyzing volumetric MRI data to determine a) the degree of interobserver variation in the measurement of plexiform neurofibromas, and b) the factors influencing this variation.

Material and Methods

Study Description

The overall study of the natural history of plexiform neurofibromas involves the recruitment of 300 patients with NF1, half children under 18 years of age and half adults, with plexiform neurofibromas of the head and neck or trunk and extremities. Serial MRI's are done at three time points over a three year period of observation. The MRI data are sent to a central location for volumetric analysis of the plexiform neurofibroma. The participating institutions' human research committees have approved the study.

The initial part of this study was to compare the volumetric measurements of neurofibromas performed by two radiologists and by a technologist experienced in volumetric assessment of the lesions. MR imaging studies of the first 12 consecutive patients recruited were reviewed. All patients had been diagnosed as having neurofibromatosis type 1, with the diagnosis established according to clinical criteria. The lesions involved the head and neck (n = 5), spine (n = 4), and trunk or extremities (n = 3).

MR Imaging

The protocol included coronal and sagittal short tau inversion recovery (STIR) images. The parameters varied according to the area being examined and to the extent of the lesion. Because patients were referred as part of a multicenter study, the MRI units were of several makes and field strengths. In addition, coil and field of view selection varied according to the location of the lesion. In general, the following parameters were used: (150/6000/35 [inversion time/repetition time/echo time]; echo train length=8). The main goal of imaging was to include contiguous axial STIR slices for volumetric measurements. Slice thickness was 4 mm for the head and neck, 5 mm for the spine, and 10 mm for the extremities. The extremities were imaged with a matrix of 512 x 160 so that coverage of the entire lesion could be done in a reasonably short time; the other anatomic areas were imaged with a 256 x 256 matrix.

Volumetric Analysis

The volumetric analysis was performed using Cheshire software (Version4.4, Parexel, Waltham, MA), a desktop visualization and analysis program. The images were imported from the MRI systems or hardcopy images were scanned to a digital form. A

scale value is used to display size and dimensions, calculate operations, and export to another file or report. Measurements are performed manually. An Auto Segmentation Tool determines the best guess edge of a lesion, based on pixel values, and creates a Region of Interest (ROI) around the object. With STIR images, neurofibromas are of high signal intensity, whereas solid structures in the body are of very low signal intensity. The segmentation tool therefore successfully identifies the margins of the neurofibromas in most instances. To use this tool, the user must click in the center of the object, drag until the entire circle lies outside the object, and then release the mouse to create the ROI. After the segmentation, the user may use any of the ROI modifying tools, such as the Nudge Tool, to adjust the ROI to perfectly outline the object. The Volume Statistics function is used to compute the volume for all volumes containing a selected ROI, bounded by the processing range.

The technologist was initially instructed by the radiologists on the MR appearance and signal characteristics of neurofibromas, using examples different from the ones used in the evaluation. To perform the reproducibility study, the technologist measured the volume of the lesion on each slice using the autosegmentation tool. Each radiologist then reviewed the automated measurements and made adjustments using the Nudge Tool, according to her or his clinical assessment. The measurements of the two radiologists were made independently, and blinded to the other radiologist's interpretation.

Statistical Analysis

Inter- and intra-observer reliability was assessed using the kappa statistic. The categorization by Landis and Koch (7) of the range of values for kappa with respect to the level of agreement is as follows: negative kappa values, poor agreement; kappa values of

0.0 to 0.19, slight agreement; kappa values of 0.2 to 0.39, fair agreement; kappa values of 0.4 to 0.74, good agreement; and kappa values of 0.75 to 1.00, excellent agreement.

Results

The results of the measurements are summarized in Table 1 and in Fig. 1. In Fig. 1, the average of the 3 measurements is taken as the standard, and deviations are recorded as percentage of the total volume. As demonstrated on the graph, the variability was usually under 5 %, and in all but two measurements, under 10%. There was greater variability with increasing volume of the lesion. The data were insufficient to assess difference in variability related to anatomic area. On the average, Radiologist 1 measured a higher volume than the average and the technologist measured the lowest volume. Measurements from Radiologist 1 ranged from 3.2% lower to 19.1% higher when compared to measurements from Radiologist 2. The overall inter-rater correlation coefficient (ICC) was 0.996, which shows excellent agreement among raters. Stratified by group, the ICCs for Head-Neck and Spine were both >0.999. In Fig. 2, plexiform neurofibromas in 3 representative locations are demonstrated with the volumetric measurement outline.

Discussion

The study shows that interobserver variability in the volumetric measurement of MR images of plexiform neurofibromas is small. Interobserver variability increases with increasing volume of the lesions, but volume measurements are generally within 10% of the mean of the measurements by three observers. The technique of automatic preliminary volume determination followed by correction by the radiologist thus appears to be a reliable tool.

Determination of the rate of growth of plexiform neurofibromas will become important for selecting tumors appropriate for treatment in clinical trials, and for measurement of the outcomes of treatment. There are, however, numerous difficulties in measuring plexiform neurofibromas. In each slice, the lesion may branch in many directions. Unlike most tumors, the borders of neurofibromas are ill defined, and their extension makes adequate lesion coverage challenging. In some anatomic areas, it may be difficult to differentiate neurofibromas from normal structures. For example, bowel in the abdomen and pelvis, and lymph nodes in the head, neck, and mediastinum may resemble neurofibromas. Finally, neurofibromas may have different MR appearances in various parts of the body. All these difficulties have created a lack of enthusiasm or outright skepticism of the possibility of measuring tumor burden in neurofibromatosis 1.

Our strategy was based on two objectives: a) to maximize contrast between the lesions and the normal tissues, and b) to cover the entire lesion. Contrast maximization was best achieved using the STIR sequence with long TR, which has been used for MR neurography (8, 9). On long TR STIR images, most of the normal structures in the extremities, head and neck, and spine are of low signal intensity, whereas neurofibromas are of very high signal intensity. Slow flowing vessels are usually indistinguishable from tumor, but we feel that they do not contribute substantially to tumor volume. The technique is less optimal when examining abdominal and pelvic structures, as fluid-filled bowel can closely resemble neurofibromas. In order to cover the entire lesion, we used large fields of view, 10 mm slice thickness, and 160 phase encoding steps, all of which allowed for fast imaging, albeit at the expense of optimal image quality.

Our data are limited by a relatively small sample size, and the fact that the measurements of the radiologists were not done blinded to the initial assessment by the technologist. We believe, however, that the major source of disagreement between observers lies with the determination of tumor versus non-tumor on the MR images rather than with the identification of the initial area of interest. We do not have a means of determining the true volume of any of the tumors measured. Our data only address the degree of reproducibility in assessment of tumor volumes as determined by three observers. The data therefore reflect the precision but not the validity of the observations.

It remains to be demonstrated whether the degree of reproducibility will allow detection of growth or shrinkage of plexiform neurofibromas based on serial MRI assessment. The current trial of a farnesyl transferase inhibitor, for example, defines progressive disease as an increase greater than or equal to a 20% in the volume of the lesion. The observed interobserver variability, usually under 5% and generally under 10%, and the high inter-observer correlations suggest that determination of volumetric change is feasible. We thus believe that reproducible computer-assisted volumetric analysis of plexiform neurofibromas can be performed successfully, and that it may allow reliable assessment of changes in lesion volume.

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Table 1- Variation between Observers

The following table shows the total volume data in mm3, for Radiologist 1 (R1), Radiologist 2 (R2), and Technologist. The last 3 columns are the percentage deviance from the average of the three from each rater.

Pt.	Area	R1	R2	Tech	R1	R2	Tech
1	TE	863,955.0	864,606.0	783,899.0	3.2%	3.2%	(6.4%)
2	TE	253,074.0	261,562.0	214,304.0	4.2%	7.6%	(12%)
3	TE	60,483.0	57,234.9	53,504.8	6.0%	0.3%	(6.3%)
4	HN	18,023.0	18,073.2	17,721.7	0.5%	0.7%	(1.2%)
5	HN	30,486.3	28,477.5	28,167.4	5.0%	(1.9%)	(3.0%)
6	S	2,073.8	17,41.4	2,073.8	5.6%	(11%)	5.6%
7	HN	77,669.2	79,521.5	74753.5	0.5%	2.9%	(3.3%)
8	HN	321,513.0	319,903.0	320,177.0	0.3%	(0.2%)	(0.1%)
9	HN	9,271.2	8,905.0	8,728.0	3.4%	(0.7%)	(2.7%)
10	S	2,010.7	1,811.5	1,660.8	10%	(0.9%)	(9.1%)
11	S	34,266.4	34,628.9	342,66.4	0.4%	0.7%	(0.4%)
12	S	27,351.4	26,435.9	26,298.5	2.5%	(1.0%)	(1.5%)

TE = trunk or extremities; HN = head and neck; S = Spine. Negative values are in parenthesis

Illustrations

Figure 1

Graph illustrating variation in volumetric measurements from the mean amongst the 3 observers. Most variation is under 5% of the mean volume.

Figure 2

A. Axial FSEIR image demonstrates lobulated scalp plexiform neurofibroma involving right temporalis muscle and suboccipital soft tissues. The volumetric measurements are outlined in red.

B. Axial FSEIR image demonstrates right brachial plexus lesion at level of lower cervical spine. The volumetric measurements are outlined in red.

C. Axial FSEIR image obtained in the prone position demonstrates a large lesion involving the subcutaneous tissues of the gluteal region. The volumetric measurements are outlined in red.

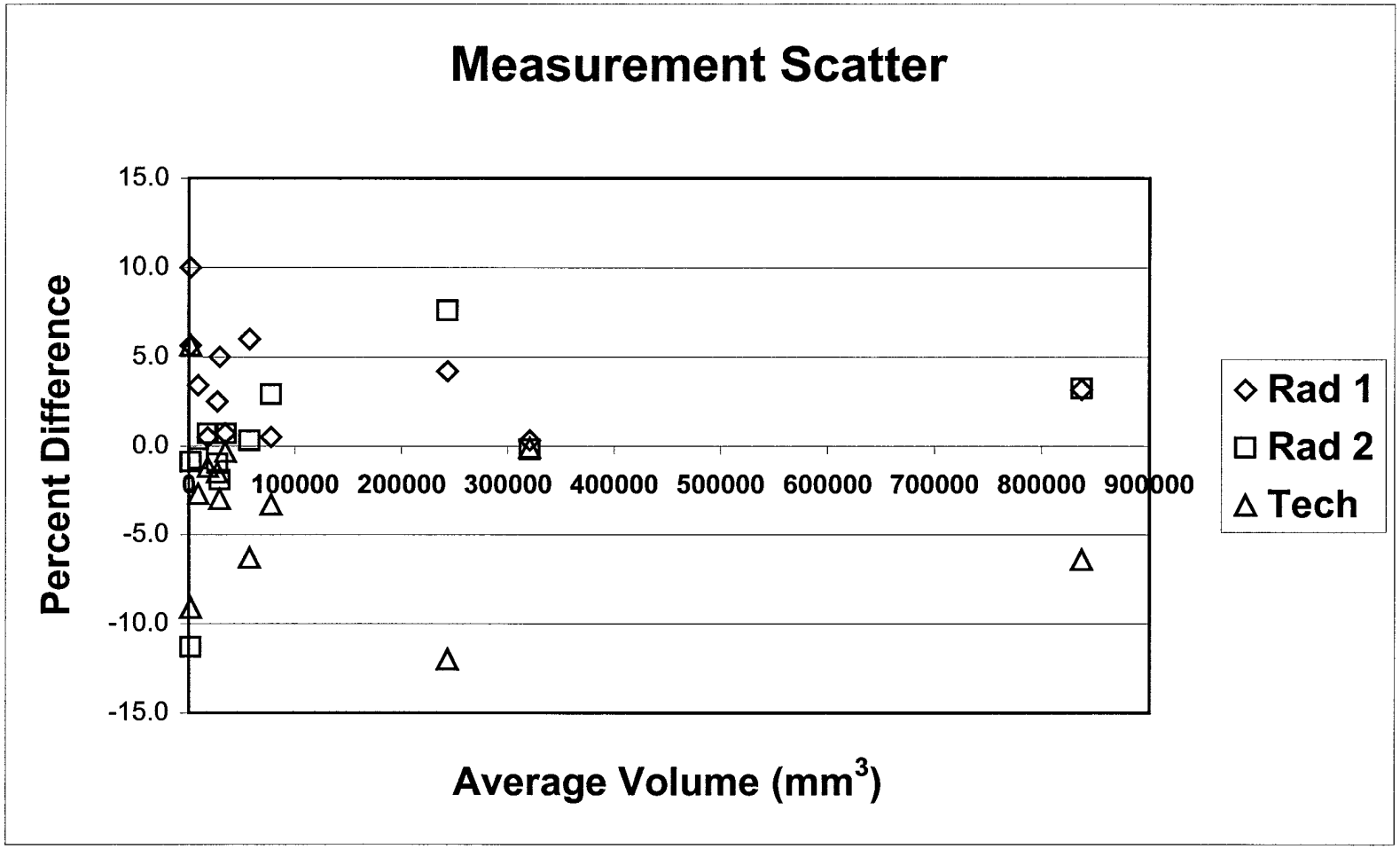
Measurement Scatter
Percent Difference
15.0
10.0
5.0
0.0
-5.0
-10.0
-15.0
100000
200000
300000
400000
500000
600000
700000
800000
900000
Average Volume (mm3)
Rad 1
Rad 2
Tech

Fig. 1

Appendix B

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Short Communication

NF1 Deletions in S-100 Protein-Positive and Negative Cells of Sporadic and Neurofibromatosis 1 (NF1)-Associated Plexiform Neurofibromas and Malignant Peripheral Nerve Sheath Tumors

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Although plexiform neurofibroma (PN) is thought to represent a benign neoplasm with the potential for malignant transformation (malignant peripheral nerve sheath tumor; MPNST), its neoplastic nature has been difficult to prove due to cellular heterogeneity, which hampers standard molecular genetic analysis. Its mixed composition typically includes Schwann cells, fibroblasts, perineurial-like cells, and mast cells. Although *NF1* loss of heterozygosity has been reported in subsets of PNs, it remains uncertain which cell type(s) harbor these alterations. Using a dual-color fluorescence *in situ* hybridization and immunohistochemistry technique, we studied *NF1* gene status in S-100 protein-positive and -negative cell subpopulations in archival paraffin-embedded specimens from seven PNs, two atypical PNs, one cellular/atypical PN, and eight MPNSTs derived from 13 patients, seven of which had neurofibromatosis type 1 (NF1). *NF1* loss was detected in four of seven PNs and one atypical PN, with deletions entirely restricted to S-100 protein-immunoreactive Schwann cells. In contrast, all eight MPNSTs harbored *NF1* deletions, regardless of S-100 protein expression or NF1 clinical status. Our results suggest that the Schwann cell is the primary neoplastic component in PNs and that S-100 protein-negative cells in MPNST represent dedifferentiated Schwann cells, which harbor *NF1* deletions in both NF1-associated and sporadic tumors. *(Am J Pathol 2001, 159:57–61)*

Neurofibroma is defined as a benign nerve sheath tumor composed of a variable mixture of Schwann, perineurial-like, and fibroblastic cells, as well as ones with features intermediate between these various cells.[1] Additional elements that may be encountered include mast cells, CD34-immunoreactive cells, melanocytic cells, heterologous epithelial elements, entrapped axons, ganglion cells or other native neural, dermal, or soft tissue components.[1–3] This cellular heterogeneity has made it difficult to determine whether neurofibromas are neoplastic or hyperplastic in nature and, if the former, which cell type(s) are primarily neoplastic. Recognized variants of neurofibroma include localized cutaneous, diffuse cutaneous, localized intraneural, plexiform, and massive soft tissue forms.[1] Also, mitotically inactive examples with increased cellularity and/or pleomorphism are referred to as cellular and/or atypical neurofibromas or plexiform neurofibromas, and such cases may be difficult to distinguish from low-grade malignant peripheral nerve sheath tumor (MPNST). The plexiform neurofibroma (PN) is the only neurofibroma subtype with a significant rate of malignant transformation (~5%) into MPNST.[1] Because PN is encountered most commonly in the setting of neurofibromatosis type 1 (NF1), *NF1* is a logical candidate tumor suppressor gene for involvement in PN and MPNST tumorigenesis. Recent studies have demonstrated that ~63% of MPNSTs have *NF1* or 17q loss of heterozygosity (LOH); however, estimates of those genetic alterations in neurofibromas have ranged from 0 to 57% of cases[4–12] (Table 1). Because most studies have not specified the growth patterns of their neurofibromas, these widely differing results likely reflect not only the complex cellular composition of individual tumors, but also varying subtypes of neurofibroma being analyzed. For example, those that have specified neurofibroma subtype have

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Table 1. Repeated Loss of Heterozygosity Studies for *NF1* in Neurofibromas and Malignant Peripheral Nerve Sheath Tumors

Report (year)	All neurofibromas with *NF1* LOH	PNs with *NF1* LOH	MPNSTs with *NF1* LOH
Skuse GR[4] (1989)	0/11	NS	6/11 (55%)
Legius E[5] (1993)	ND	ND	1/1 (100%)
Lothe RA[6] (1993)	0/5	NS	1/1 (100%)
Lothe RA[7] (1995)	0/8	NS	4/6 (67%)
Colman SD[8] (1995)	5/22 (23%)	NS	ND
Däschner K[9] (1997)	1/38 (3%)	1/5 (20%)	ND
Serra E[10] (1997)	15/60 (25%)	NS	ND
Kluwe L[11] (1999)	8/14 (57%)	8/14 (57%)	ND
Rasmussen SA[12] (2000)	6/25 (24%)	4/10 (40%)	3/5 (60%)
Total	35/183 (19%)	13/29 (45%)	15/24 (63%)

NS, not specified; ND, not done.

reported high rates of LOH in PNs, with only rare LOH in cutaneous examples.[9,11,12] In contrast to neurofibromas, MPNSTs are obviously neoplastic and often demonstrate some degree of Schwann cell differentiation. Given that some MPNSTs arise from PNs, the Schwann cell is thought to represent the most likely neoplastic component in PNs as well. However, a small minority of MPNSTs demonstrate perineurial differentiation[13] suggesting that other cell types may be occasionally implicated. Interestingly, none of the perineurial MPNSTs reported thus far have been associated with either an underlying neurofibroma or the NF1 syndrome.[13] Most recently, Schwann cells have been further implicated in studies finding cytogenetic alterations[14] and lack of neurofibromin expression[15] in cultured Schwann cells from neurofibromas, with no detectable alterations from cultured fibroblasts obtained from the same specimens. However, it is not clear from these *in vitro* experiments what selection biases were introduced by expansion of these cell populations in culture. In this study, we have performed the first *in situ* evaluation of *NF1* deletions within intact PNs and MPNSTs.

Materials and Methods

Eighteen cases of PN, atypical PN, and MPNST were retrieved from the archives of the Lauren V. Ackerman Surgical Pathology Laboratory at the Washington University Medical Center in St. Louis. All available slides were reviewed, and diagnoses confirmed using current criteria.[1] Atypical PNs were defined by the presence of nuclear atypia in the absence of significant mitotic activity, whereas, cellular PNs were defined by hypercellularity in the absence of significant mitotic activity. A representative formalin-fixed paraffin-embedded block was selected per case for further study with dual-color immunohistochemistry/fluorescence *in situ* hybridization (FISH). Sporadic schwannomas were used as disomic (ie, normal 2 copies) *NF1* controls because they contain S-100 protein-positive Schwann cells of similar size and shape to those typically encountered in neurofibromas and would not be expected to harbor *NF1* deletions. Clinical records were reviewed, and the diagnosis of neurofibromatosis 1 (NF1) was rendered in patients fulfilling National Institute of Health (NIH) guidelines.[16] Most of these patients have been carefully examined and followed in the Neurofibromatosis Clinic at Washington University.

Unstained 5-μm thick sections were cut onto superfrost/plus, precleaned glass slides from each paraffin block. The sections were deparaffinized in CitriSolv (Fisher, Pittsburgh, PA) and rehydrated in isopropanol and water. Endogenous peroxidase activity was inhibited by incubation in 3% hydrogen peroxide in phosphate-buffered saline (PBS; 10 mmol/L; pH = 7.2) for 5 minutes. Non-specific antibody binding was inhibited by incubation in PBS-blocking buffer (PBS with 1% BSA, 0.2% powdered milk and 0.3% Triton X-100) for 20 minutes at room temperature and polyclonal rabbit anti-S-100 protein antiserum (Z311, Dako, Carpinteria, CA; 1:50,000 in PBS blocking buffer) was added to the sections overnight at 4°C. Sections were then washed in 1× PBS (3 × 5 minutes each) and incubated with horseradish peroxidase conjugated donkey anti-rabbit secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA; diluted 1:1000 in PBS-blocking buffer) for one hour at room temperature. Antigen-antibody complexes were subsequently detected by direct tyramide signal amplification (Perkin Elmer Life Sciences, Boston, MA) using cyanine-3 conjugated tyramide (tyramide signal aplification plus cyanine 3) for 20 minutes at room temperature according to the manufacturer's instructions. Slides were washed in PBS and 2× SSC for 5 minutes each.

Subsequent FISH was performed on the S-100 protein immunolabeled slides using our previously published protocol[17] and a fluorescein isothiocyanate (FITC)-labeled P1 artificial chromosome DNA probe targeting the exon 28 to 3′ region of the *NF1* gene on chromosome 17q11.2 (donated by Dr. Eric Legius, Belgium). The probe was diluted 1:50 in DenHyb buffer (Insitus, Albuquerque, NM) and 10 microliters was directly applied to each tissue section. Probe and target DNA were co-denatured at 90°C for 13 minutes, followed by overnight hybridization at 37°C in a humidified oven. The slides were then washed for 5 minutes with 50% formamide in 1x SSC followed by two more washes of 2× SSC for 5 minutes each. The nuclei were counterstained with DAPI/Antifade (Insitus). Fluorescent signals were enumerated under an Olympus B ×60 fluorescent microscope with appropriate filters. Because cytoplasmic borders were

Table 2. Summary of Clinical Cases and FISH Results

Case no.	Age/sex	*NF1* status	Diagnosis	Tumor location	*NF1* in S-100$^+$ cells	*NF1* in S-100$^-$ cells
681	3 M	Yes	PN	Eye	Deleted	Normal
957-A	16 F	Yes	PN	Buttock	Deleted	Normal
957-B	16 F	Yes	PN	Flank	Normal	Normal
957-C	16 F	Yes	PN	Scalp	Normal	Normal
017-A	2 F	No	PN	Neck	Deleted	Normal
017-B	5 F	No	PN	Neck	Normal	Normal
482	8 F	No	PN	Ulnar nerve	Deleted	Normal
795	3 F	Yes	At-PN	Perineum	Deleted	Normal
882-A	24 M	Yes	At-PN	Scalp	Gains	Normal
147-A	11 F	Yes	Cell-At-PN	Thigh	Gains	Normal
501	13F	Yes	MPNST	Paraspinal	Insufficient cells*	Deleted
882-B	24 M	Yes	MPNST	Neck	Deleted	Deleted
147-B	13 F	Yes	MPNST	Thigh	Deleted, gains	Deleted
215	33 M	Yes	MPNST	Retroperitoneum	Insufficient cells*	Deleted
459	62 F	No	MPNST	Brachial plexus	Deleted	Deleted
566	36 F	No	MPNST	Brachial plexus	Deleted	Deleted
286	42 F	No	MPNST	Brachial plexus	Insufficient cells*	Deleted
883	47 M	No	MPNST	Mediastinum	Insufficient cells*	Deleted

At-PN, atypical plexiform neurofibroma.
* MPNST with too few S-100$^+$ cells to enumerate.

often indistinct under fluorescence microscopy, only cells with immunopositive nuclei (ie, some red fluorescence over the nucleus) were scored for *NF1* signals in the evaluation of S-100 protein-positive cellular subsets. However, because cytoplasmic staining was also frequently observed, only immunonegative (ie, blue) nuclei with no surrounding red fluorescence were scored for *NF1* signals in the evaluation of S-100 protein-negative cellular populations. Because the S-100 protein staining sometimes obscured the underlying *NF1* signals when the colors were viewed simultaneously, signal enumeration required the consecutive viewing of individual nuclei under each single-pass filter (ie, blue, red, and green).

Given the truncation artifact (ie, fewer signals in sectioned nuclei with incomplete DNA complement) associated with thin tissue FISH, cutoffs for genetic alterations were based on results from four control hybridizations (see above). The cutoff for *NF1* gene deletion was based on the mean percentage of nuclei with one signal in controls plus two standard deviations. Because nuclei with >2 signals were never seen in these controls, *NF1* (17q) polysomy (gain) was arbitrarily defined as >5% nuclei with three or more FISH signals.

Results

The clinical features, tumor diagnoses, and FISH results are summarized in Table 2. There were 18 tumors obtained from 13 patients, 7 of which had diagnostic features of NF1. The seven PNs, two atypical PNs, and one cellular/atypical PN came from five female and two male patients ranging in age from 2 to 24 years of age (median 8 years). All but two (017 and 482) fulfilled criteria for NF1. Given the young ages of these two patients, however, it seems likely that they either represent mosaic forms of NF1 or as of yet undiagnosed NF1 in young individuals with insufficient clinical criteria to warrant a definitive diagnosis. The NF1-associated MPNST patients consisted of two males and two females ranging in age from 13 to 33 years (median 18.5 years). The sporadic MPNSTs were derived from one male and three female patients ranging in age from 36 to 62 years (median 44.5 years). One of the NF1-associated and one of the sporadic MPNSTs had rhabdomyoblasts (ie, Triton tumor). Three of the sporadic MPNSTs were probably radiation-induced sarcomas based on clinical history (radiation for prior breast cancer or Hodgkin's disease).

Representative examples of dual immunohistochemistry/FISH results are illustrated in Figure 1. Control sections demonstrated 1 *NF1* signal in 27 to 40% of nuclei. Based on the mean (35%) plus 2 standard deviations (12%), a cutoff of >47% nuclei with one signal was established for *NF1* deletion. These results are similar to those obtained using other DNA FISH probes in our laboratory on thin paraffin sections from non-neoplastic controls (data not shown). The fraction of cells with one *NF1* signal ranged from 50 to 93% (median 67%) in populations interpreted as deleted *versus* 14 to 39% (median 27%) in populations interpreted as nondeleted. *NF1* deletion was detected within the S-100 protein-positive cellular populations of four (57%) PN and one (33%) atypical PN (Table 2). The S-100 protein-negative populations from these same tumors were disomic (normal 2 copies). Four of the MPNSTs had too few S-100 protein-positive cells to determine *NF1* status within this subset of tumor cells. However, *NF1* deletion was found in the S-100 protein-negative cells of these same cases (Table 2). The remaining four MPNSTs demonstrated *NF1* deletion in both the S-100 protein-positive and -negative components. Polysomies (gains with 3 to 4 signals per nucleus) of *NF1* were identified in subpopulations of S-100 protein-positive cells of one atypical PN, one cellular/atypical PN and one MPNST. These cells likely represent polyploid or aneuploid clones within these tumors.

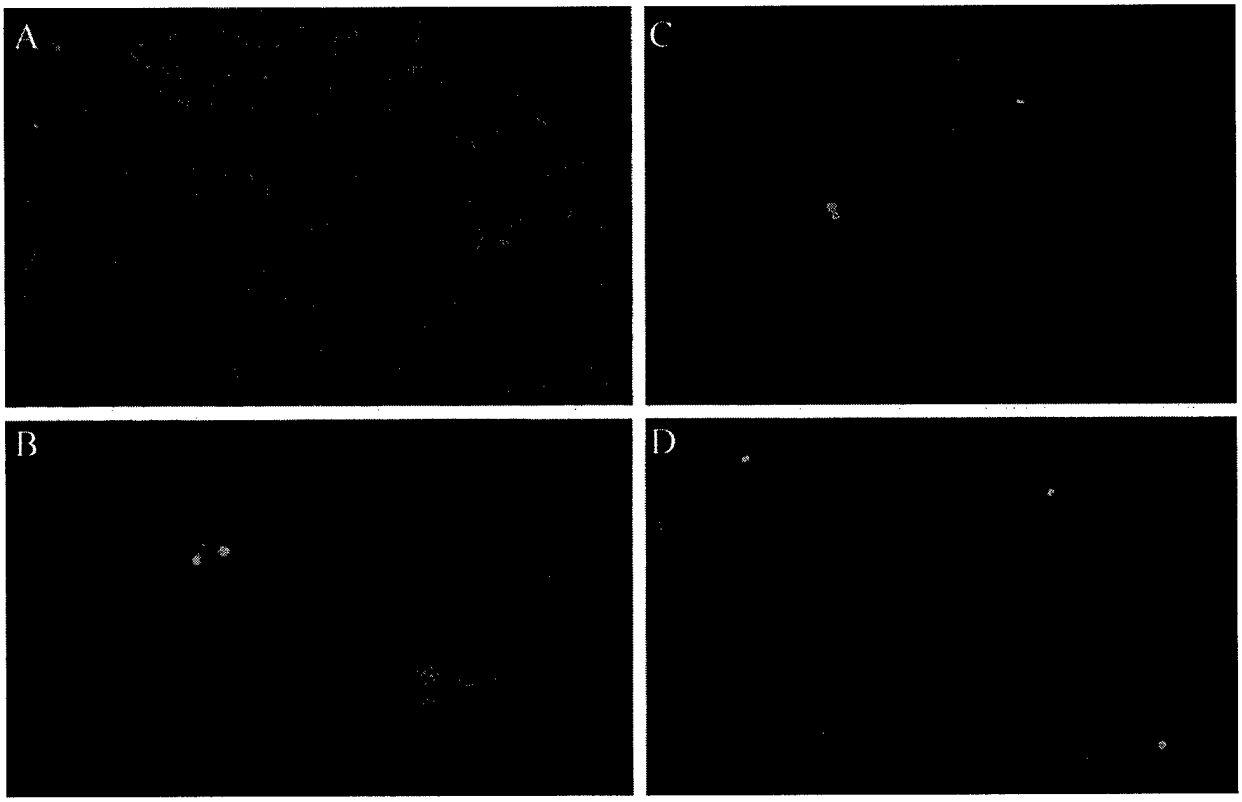

Figure 1. Representative examples of dual S-100 protein immunohistochemistry and *NF1* FISH hybridization. **A:** Low-power image from a control schwannoma, demonstrating relatively diffuse S-100 protein immunoreactivity (red). As is typical of this antibody, some of the staining is cytoplasmic and some is nuclear. **B:** At higher magnification, two S-100 protein-positive cells demonstrate the normal disomic state, with two copies of *NF1* (green signals) per nucleus. **C:** Two adjacent nuclei from a representative plexiform neurofibroma (case 957-A) are shown. The nucleus on the right demonstrates S-100 protein immunoreactivity and only a single *NF1* signal, whereas the nucleus on the left is S-100 protein-negative with the normal disomic *NF1* dosage. Hybridization counts from this case revealed one *NF1* signal in 67% of S-100 protein-positive *versus* 27% of S-100 protein-negative nuclei. This is consistent with a gene deletion that is restricted to the S-100 protein-positive population of cells. **D:** This S-100 protein-negative region of an MPNST (case 566) demonstrated one *NF1* signal in 93% of nuclei, consistent with deletion. S-100 protein-positive regions of the same tumor similarly showed evidence of deletion (not illustrated).

Discussion

Using a dual-color FISH-immunohistochemical method, we have demonstrated, for the first time, *NF1* gene copy numbers in S-100 protein-positive *versus* -negative cellular populations in PNs and MPNSTs. One of the primary advantages of this technique is that it is applied *in situ* with preserved tissue morphology. In this fashion, some entrapped native tissue elements, such as uninvolved nerve fascicles and infiltrated fat, skeletal muscle, sweat glands, etc can easily be excluded from genetic analysis. Our results provide the most conclusive evidence thus far that the S-100 protein-positive Schwann cell is the primary target for *NF1* deletions in PNs, both typical and atypical subsets. Furthermore, it adds support to the growing body of literature suggesting that most, if not all PNs are neoplastic, rather than hyperplastic in nature[9,11,12,14,15]. Because some of our cases, and many of those in the literature, harbor no detectable genetic alterations, however, we cannot exclude the possibility that a subset of PNs, and perhaps most cutaneous neurofibromas, are in fact, hyperplastic or hamartomatous. Alternatively, these cases may harbor inactivating mutations beyond the resolution of FISH or LOH, involve alterations of other genes besides *NF1*, or consist of tumors with minute neoplastic clones that induce an overshadowing reactive process including non-neoplastic fibroblasts, perineurial-like cells, native intraneural Schwann cells, etc. Further resolution of these issues will likely require sophisticated screening techniques capable of detecting genetic alterations within individual cells.

Another interesting finding in our study was the prevalence of *NF1* deletion in MPNSTs, regardless of S-100 protein expression or NF1 status. The simplest interpretation is that S-100 protein-negative tumor cells within MPNSTs represent dedifferentiated Schwann cells that still harbor *NF1* deletion. In other words, the loss of *NF1* represents an early tumorigenic event that is still detectable in high-grade neoplastic clones no longer manifesting immunohistochemical evidence of Schwann cell differentiation. The finding of divergent epithelial and/or mesenchymal differentiation in some MPNSTs (eg, Triton tumors) and complete lack of S-100 protein expression in others would further support this dedifferentiation hypothesis. In any case, only a few MPNSTs have been genetically characterized in terms of *NF1*. Reported LOH studies have been largely limited to examples from NF1 patients (Table 1),[4–7,12] where *NF1* loss has been common. In a small cytogenetic study by Rao and colleagues,[17] monosomy was identified in one of four sporadic MPNSTs, suggesting that *NF1* may be implicated in

some of these cases as well.[18] Gómez and colleagues found no mutations in nine sporadic MPNSTs within the GAP-related domain by polymerase chain reaction/single-strand conformational polymorphism.[19] However, this was a fairly limited screening and additional studies are obviously needed with larger numbers of both sporadic and NF1-associated examples.

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Appendix C

Fluorescence In Situ Hybridization (FISH) in Diagnostic and Investigative Neuropathology

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Over the last decade, fluorescence in situ hybridization (FISH) has emerged as a powerful clinical and research tool for the assessment of target DNA dosages within interphase nuclei. Detectable alterations include aneusomies, deletions, gene amplifications, and translocations, with primary advantages to the pathologist including its basis in morphology, its applicability to archival, formalin-fixed paraffin-embedded (FFPE) material, and its similarities to immunohistochemistry. Recent technical advances such as improved hybridization protocols, markedly expanded probe availability resulting from the human genome sequencing initiative, and the advent of high-throughput assays such as gene chip and tissue microarrays have greatly enhanced the applicability of FISH. In our lab, we currently utilize only a limited battery of DNA probes for routine diagnostic purposes, with determination of chromosome 1p and 19q dosage in oligodendroglial neoplasms representing the most common application. However, research applications are numerous and will likely translate into a growing list of clinically useful markers in the near future. In this review, we highlight the advantages and disadvantages of FISH and familiarize the reader with current applications in diagnostic and investigative neuropathology.

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Introduction

Although in situ hybridization (ISH) has been around for over 30 years (53, 84), its application to the study of DNA alterations in solid tissue has only recently become popular. Unique among molecular techniques due to its morphologic basis, it involves direct microscopic visualization of probe-specific, intranuclear signals utilizing either chromogenic (CISH) or fluorescence (FISH) detection. Given that nuclei in any phase of the cell cycle may be analyzed and metaphase chromosomes are not required for interpretation, this technique has also been referred to as interphase cytogenetics. In clinical laboratories, it is currently utilized most often for either prenatal detection of germline alterations (eg, aneusomy or microdeletion syndromes) or the detection of somatic cancer-associated alterations that have known diagnostic, prognostic, or therapeutic implications. In neuropathology, the oncology-associated application has predominated, though the former may also be useful, since a number of cytogenetic disorders are associated with CNS manifestations. In essence, FISH provides data on intranuclear target DNA localization and copy number. Therefore, with the exception of some sex-chromosome determinations, two signals per nucleus would normally be expected and four common alterations are amenable to detection: aneusomy (gain or loss of a chromosome or chromosomal region), gene deletion, amplification, and translocation. The objective of this review is to provide the reader with detailed background into the FISH assay, its appropriate applications, and its utility in diagnostic and investigative neuropathology. The first portion covers technical considerations, while the second demonstrates specific applications, focusing primarily on CNS tumors.

Advantages	Disadvantages
Morphology-based	Not a genomic screening tool
Simple, IHC-like protocol	Limited to detecting large alterations
Applicable to FFPE tissue	Signal fading
Dual or multicolor analyses possible	Limited number of commercial probes*
No culture or proliferation requirements	Tissue sample variability
Patient's normal cells not required	Cytologic artifacts
High sensitivity and specificity	
Quantitative	

IHC = immunohistochemistry, FFPE = formalin-fixed, paraffin-embedded, *though the ability to obtain homemade probes is no longer limited (see discussion in text).

Table 1. Advantages and disadvantages of FISH analysis.

Advantages, limitations, and artifacts of FISH analysis. The major pros and cons of FISH analysis are summarized in Table 1. FISH is applicable to a variety of specimen types, including fresh or frozen tissue, cytologic preparations, and formalin-fixed paraffin-embedded (FFPE) tissue. The latter provides a particularly rich source of archival material and may be performed using either thin (4-6 μm) sections, such as those cut for immunohistochemistry or intact nuclei

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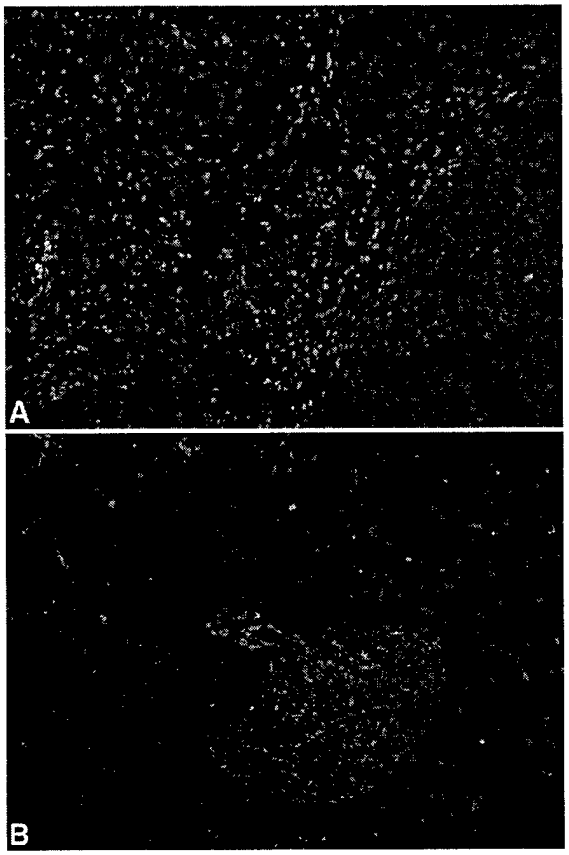

Figure 1. Morphologic preservation is evident in these low-power images from a case of combined meningioma/meningioangiomatosis (MA) studied by FISH analysis. The meningioma is evident in the right lower corner of (**A**), whereas the spindled, perivascular cells of MA are easily distinguishable from the intervening neuroglial tissue in (**B**).

extracted from thick sections (eg, 50 μm), such as those normally prepared for flow cytometry. Although adjustments must be made for nuclear truncation (see below), we prefer the thin sections because it preserves architecture, is simpler to prepare, and wastes less tissue.

For pathologists, morphologic preservation is a distinct advantage, particularly attractive for studies on heterogeneous tissue samples without the need for microdissection. One example is illustrated in Figure 1, where FISH was performed on a case of meningioangiomatosis (MA) in association with meningioma. Due to the morphologic preservation, it was possible to separately enumerate signals from meningioma cells, perivascular MA cells, and intervening neuroglial cells, all on a single slide. The detection of identical genetic alterations in the meningioma and MA suggested that both elements were neoplastic and arose from a single clone (165). The lack of any alterations in the intervening neuroglial cells further suggested that these represent entrapped non-neoplastic elements, thus providing a useful internal control. Such analyses would be substantially more difficult to achieve utilizing any other molecular techniques. Another morphologically heterogeneous tumor where FISH has been similarly applied is the gliosarcoma (23, 122). The finding of identical genetic alterations in both components refuted the notion of a collision tumor and supported the hypothesis that both elements originate from a single clone, with the mesenchymal component arising from metaplasia.

An extension of this morphologic advantage comes from the possibility of combining FISH with immunohistochemistry, wherein separate counts are rendered in immunopositive and negative cells. For example, this approach was required to demonstrate numerical chromosomal alterations in the CD30-positive Reed-Sternberg cells of Hodgkin's lymphoma (118). Because these neoplastic cells typically constitute only a minor fraction of the lymphoid population, clonal alterations are not amenable to detection by "averaging" techniques such as flow cytometry and PCR. Using this dual FISH-immunohistochemistry approach, we similarly demonstrated that *NF1* deletions are restricted to the S-100 protein positive, Schwann cell elements of the cellularly and immunophenotypically heterogeneous plexiform neurofibroma (130).

Another distinct advantage to neuropathologists is the similarity of FISH to immunohistochemistry, which is already familiar and widely applied in pathology laboratories. In many ways, the techniques are analogous, except that FISH utilizes DNA probes, rather than antibodies. Unlike the typical qualitative or semiquantitative immunohistochemical interpretation schemes (eg, +/- or 0-3+), FISH provides quantitative results and is therefore more objective. Such comparisons have become particularly evident in recent assessments of Her-2/*neu* status in breast cancer (121, 183).

In comparison to classic metaphase cytogenetics (ie, karyotyping), FISH has several advantages, most importantly, the lack of requirements for mitotically active cells and culturing. Given that only the cells capable of proliferating in vitro are assessable on karyotype, there can be significant growth selection biases, including overgrowth of non-neoplastic elements, particularly when analyzing benign or low-grade neoplasms. An example of this drawback was the virtual absence of any 1p or 19q deletions reported in early karyotypes of oligodendrogliomas (16, 61, 82, 180), compared with 60 to 70% losses of these chromosomal arms by later stud-

ies utilizing loss of heterozygosity (LOH) or FISH (10, 90, 143, 167). On the other hand, FISH is not a genomic screening tool, only providing a more targeted approach for alterations that have been initially identified by more global assessments, such as conventional cytogenetics, comparative genomic hybridization (CGH), or gene expression microarray chips.

In terms of sensitivity and resolution, FISH is better than karyotyping and CGH, but worse than PCR-based assays for detecting small alterations. The former is limited to alterations of several Mb in size, whereas the latter can be designed to detect even single base mutations. Since FISH probes are typically at least 30 Kb in size, alterations need to be equally large for reliable detection. For this reason, FISH cannot detect small intragenic mutations and this technique is best reserved for alterations that occur at the "cytogenetic" level. PCR is also more sensitive than FISH for the detection of abnormal fusion transcripts resulting from translocations, picking up as few as one per million cells. This is particularly useful when attempting to detect "minimal residual disease" or early recurrence, though the biologic relevance of such small fractions is not always clear and it is possible that in certain situations, PCR is "too sensitive". In contrast, FISH is more sensitive than PCR at identifying gene deletions or amplifications from samples of mixed cellularity, such as neoplasms with clonal heterogeneity or contaminating non-neoplastic elements (129). It is estimated that sample purity must reach at least 70% tumor for such quantitative PCR-based assays and this is sometimes difficult to achieve in highly infiltrative neoplasms, such as gliomas. FISH, on the other hand, can identify amplification in as few as 5% and deletion in 15 to 30% of cells within a sample.

In comparison to LOH studies using microsatellite markers, FISH results are often similar, but not identical and each has its advantages and disadvantages. A common misconception is to equate the two techniques, stating that "FISH demonstrated LOH" for a region of interest. Since FISH measures absolute copy number rather than allele status, such a statement is inaccurate. Although LOH most often results from simple deletion, this is not always the case. For example, mitotic recombination of chromosome 17p may lead to loss of the wild type *p53* allele and duplication of the mutated allele. Although one "allele" (maternal or paternal) would be lost in this scenario (ie, LOH), there would still be two copies of the *p53* gene, simulating the normal situation on FISH analysis. This was in fact, found to be the most common mechanism for *p53* inactivation in gliomas (79) and therefore, FISH is not a suitable assay for detecting this type of loss (125). Another advantage of the LOH studies is the ease of evaluating large numbers of markers spanning the entire length of a chromosome or chromosomal arm. However, as emphasized above, morphologic correlation is not possible unless regions of interest are microdissected first. LOH also requires matching germline DNA from the patient's leukocytes or microdissected normal tissue and this is not always available.

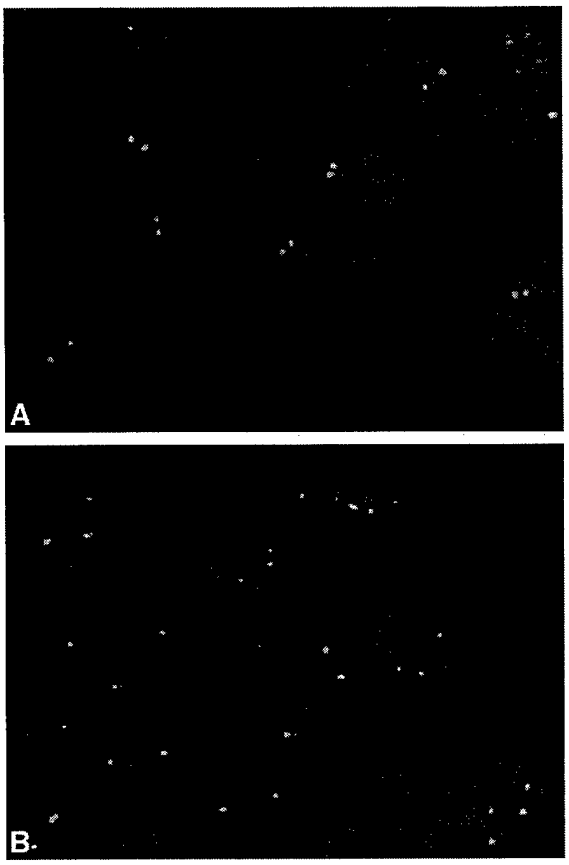

Figure 2. Examples of common deletions detected by dual-color FISH of a high-grade astrocytoma tissue microarray (TMA). Monosomy 10 was diagnosed in (**A**) with only one PTEN (green) and one DMBT1 (red) signal evident in most nuclei. The case in (**B**) was interpreted as having polysomy 9 (green) and homozygous p16 deletion (red), since >2 CEP9 and no red signals were seen per nucleus, except within endothelial cells that provided an internal control (not shown).

Another recent application of FISH is high-throughput analysis via tissue microarray (TMA). This technology takes advantage of multispecimen paraffin blocks constructed from up to 1000 0.6-mm neoplastic, non-neoplastic, and control tissue cores of interest. Therefore, hundreds of specimens can be simultaneously

evaluated on a single slide using TMA-FISH, markedly increasing efficiency and reducing data acquisition time, probe, reagent, and storage space requirements. A recently popularized approach is to initially screen a small number of tumors with gene expression profiling and then verify the resulting candidate genes in a large number of tumors, utilizing TMA-immunohistochemistry and TMA-FISH (116, 149). Recent TMA studies have shown excellent morphologic, antigenic, and genomic preservation with high levels of concordance compared to the traditional whole slide approach (34, 72, 88, 116, 157). Since each core is quite small, sampling bias is a real concern for heterogeneous tumors, such as gliomas. However, this problem is minimized if a neuropathologist carefully selects 2 to 3 representative regions per donor block. Figure 2 illustrates sample hybridizations from a glioma TMA, constructed at M.D. Anderson and recently analyzed by FISH in our lab (51). For gene amplifications, TMA-FISH is particularly appealing, since interpretations are rapid, typically requiring only seconds per tissue core. For aneusomies and deletions, manual signal counts still remain tedious and time consuming, though automated spot counting software is currently under development and promises to further increase the efficiency of this technique.

As already discussed in part, recent technical advances have greatly enhanced the applicability of FISH. However, a number of limitations remain. One of the main disadvantages of FISH as a clinical tool is signal fading. By storing hybridized slides in a freezer and avoiding prolonged exposure to light, hybridization signals remain visible for up to a year. However, a permanent record is not currently possible, unless chromogenic detection is used. Unfortunately, multicolor CISH is not as simple as multicolor FISH and currently available chromogens lack the spectral versatility, sensitivity and spatial resolution attainable with fluorochromes. Some are currently working on alternatives by developing non-fading fluorochromes (Bobrow MN and Roth KA; US patent pending) or improved protocols for multicolor CISH (173). Alternatively, software solutions now make it possible to archive high volume FISH results through digital imaging.

Other limitations include a variety of artifacts, particularly common in brain tissue sections. It is for this reason that while the FISH protocol itself is often mastered quickly, interpretation requires significantly more experience. Most troublesome are truncation artifacts, aneuploidy, autofluorescence, and partial hybridization failure. Truncation artifact refers to the underestimation of target copy numbers due to incomplete DNA complements in transected nuclei. Therefore, it is important to include controls cut at the same thickness. In our lab, we usually set cutoffs for deletion based on median percentages of control nuclei with <2 signals plus 3 standard deviations. However, a number of other approaches have also been applied and are acceptable.

Aneuploidy and polyploidy are particularly common in malignant neoplasms and can result in confusing signal counts. The assessment of multiple targets is most informative in such situations. Although the simplest approach is to interpret absolute losses (<2 copies) and gains (>2 copies), one may opt to delineate "relative" losses and gains compared with a reference ploidy, obtained either by flow cytometry or the assessment of multiple chromosomes by FISH. For example, the finding of 3 copies would be considered a relative gain in a diploid tumor, normal in a triploid tumor, and a relative loss in a tetraploid tumor. Lastly, one may combine a centromere and locus-specific probe from a single chromosome and determine their ratios. For example, cells with 4 chromosome 9 centromeres and 2 copies of the *p16* region on 9p21 would be interpreted as having polysomy 9 and a hemizygous *p16* deletion. A similar tumor with 4 centromere and no *p16* signals would be interpreted as polysomy 9 with homozygous *p16* deletion. Similarly, cells with 6 copies of *EGFR* might be interpreted as low-level amplification if there were only 2 chromosome 7 centromeres, but would represent polysomy 7 without gene amplification if there were 6 centromeres.

Autofluorescence is a particularly pesky problem in the brain, where it is often encountered in abundance. Since autofluorescent tissue fragments are typically larger and more irregular than true signals, they can often be disregarded. However, some fragments present at just the right size to simulate signals. In this case, the use of multiple filters is helpful, since autofluorescence will typically appear on both green and red filters, whereas true signals only fluoresce under one or the other. The problem of partial hybridization failure can be minimized by counting only in regions where the majority of cells have clear signals.

FISH assays. A number of FISH protocols have been published and vary depending on individual preferences and specimen type. In general, the simpler protocols are preferable, since they require less hands on time, have fewer steps in which errors may be introduced, and are easier to troubleshoot. The basic steps are similar to those of immunohistochemistry and include deparaffinization, pretreatment / target retrieval, denaturation of

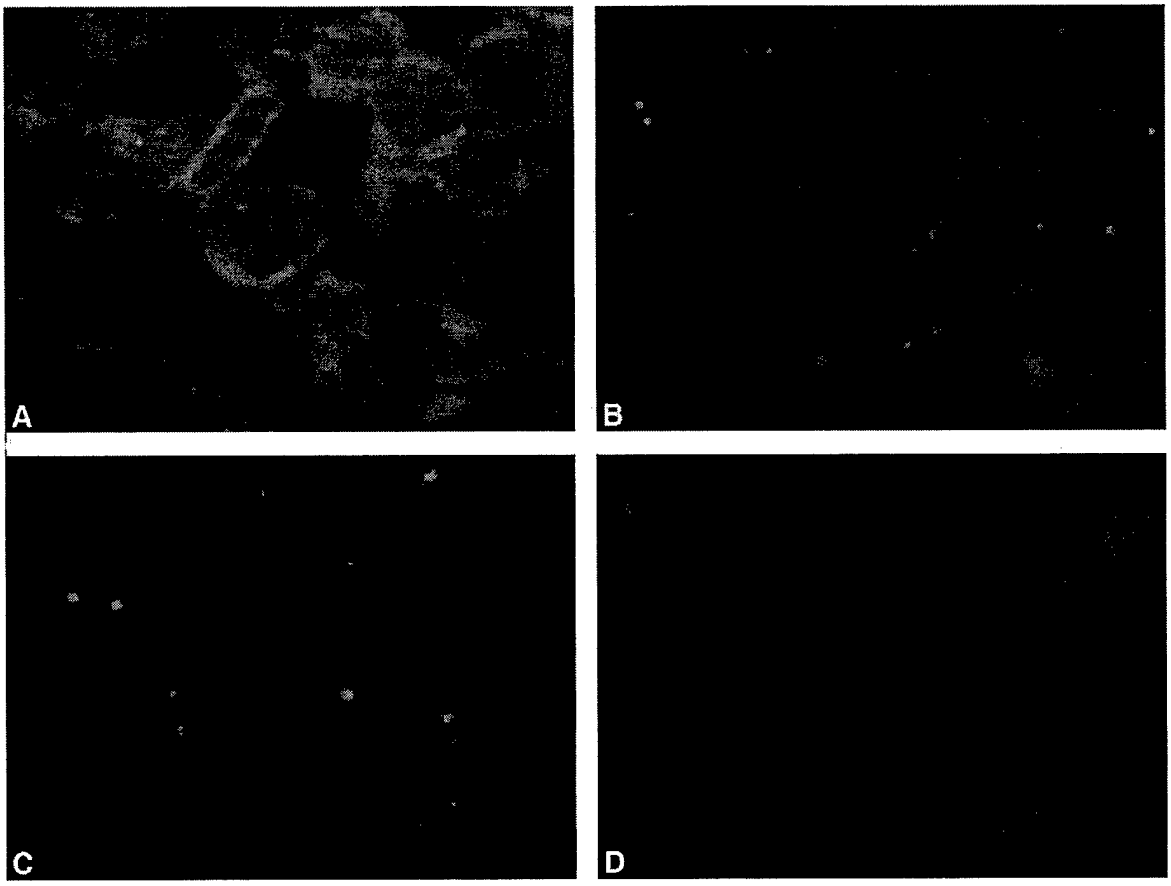

Figure 3. Example of tyramide signal amplification (TSA)-FISH. Routine FISH analysis for a FITC-labeled bcr probe at 1:50 dilution yielded small, weak signals in this medulloblastoma (**A**). TSA-FISH at 1:200 probe dilution using HRP-conjugated anti-FITC antibody and cyanine 3-tyramide converted the green signals into larger, brighter red signals (**B**). The TSA+ amplification kit with a 1:800 probe dilution yielded similar results with less background (**C**). A control slide with omission of the bcr probe yielded no signals and little background staining (**D**).

probe and target DNA, hybridization (usually overnight), post-hybridization washes, detection, and microscopy/imaging. This is typically a 2-day assay, which requires roughly 3 to 4 hours the first day and 30 minutes the second day. Alternatively, same day protocols are possible with robust probes and automated systems are now available to reduce the required tech time to a minimum (eg, *http://www.vysis.com*, *http://www.ventanamed.com*).

A few technical caveats should be kept in mind. Similar to immunohistochemistry, microwave or heat-induced target retrieval often works better than chemical forms of pretreatment and significantly improves hybridization efficiency (69, 129, 162). When this step is included, protein digestion may often be reduced or eliminated altogether. Nevertheless, optimal pretreatment and digestion varies from one specimen to another, depending on methods of fixation/processing. We have also found that some hybridization buffers are significantly more efficient than others and therefore work with lower probe concentration requirements (eg, DenHyb from Insitus, *http://www.insitus.com*). This is particularly useful when utilizing expensive commercial probes, because they may last 5 to 20 times as long as they would when using the manufacturers recommended dilutions. Lastly, a variety of amplification steps are available for cases with weak signals. However, we have rarely found this necessary and in our lab, we prefer the simpler protocol and cleaner background associated with directly labeled fluorochrome probes (eg, FITC, rhodamine), in contrast to indirectly labeled probes (eg, digoxigenin, biotin) that require an addition-

al step (eg, fluorochrome-labeled secondary antibody) with or without further amplification. Nevertheless, dramatic levels of signal amplification are now achievable, particularly with tyramide signal amplification (TSA) or catalyzed reporter deposition (CARD) (108, 153, 173, 174, 188). This technique takes advantage of peroxidase-mediated deposition of haptenized tyramine molecules, not only in the precise site of hybridization, but also in the nearby vicinity. This results in increases of signal size and up to 1000-fold or greater amplification (Figure 3). Although one possible application is marked reductions of probe concentration requirements, the more exciting potential is the use smaller probes, perhaps down to the level of 1 Kb or less (158). Therefore, TSA could potentially increase the sensitivity for small alterations, such as those detectable by PCR, while maintaining the morphologic advantage of FISH.

Types of FISH probes and probe development. A number of different probe types are currently available for FISH. Centromere enumerating probes (CEPs) were among the first to be developed and are ideal for detecting whole chromosome gains and losses, such as monosomy, trisomy, and other polysomies. Because they target highly repetitive 171 bp sequences of α-satellite DNA, they are associated with excellent hybridization efficiencies and typically yield large, bright signals. However, sequence similarities in some pericentromeric regions result in cross-hybridization artifacts. Because of the inevitable cross-hybridization between centromeres 13 and 21 or between centromeres 14 and 22, these CEPs have been previously utilized as probes with 4 expected signals rather than 2. Today, a better solution is to use locus-specific or painting probes (see below) to enumerate these individual chromosomes. Anecdotally, we have also encountered cross-hybridization problems with CEP9, though the non-specific signals are usually dimmer and the utilization of either more stringent washes or lower probe concentrations sometimes resolves this problem. Also, an interesting phenomenon in non-neoplastic brain is that certain chromosomes are packaged into interphase nuclei with paired centromeres in close proximity, a concept referred to as somatic pairing (3, 5, 39, 125). This is most dramatic with CEP17, but may be encountered to a lesser extent with other centromeres as well, including CEP1 and CEP8. Because of this close proximity, FISH yields an unexpected fraction of cells harboring a single large signal rather than 2 smaller ones, an artifact that could potentially lead to overinterpretation of monosomy. This somatic pairing is typically not encountered in brain neoplasms, such as gliomas. Therefore, interpretations with these CEPs are more difficult, if utilizing normal brain controls to establish cutoffs for monosomy. Despite these technical limitations, CEPs remain extremely useful for detecting aneusomies and are still among the best FISH probes available. The presence of similarly repetitive DNA sequences in subtelomeric regions has now led to the development of commercially available telomere probes for each chromosomal arm as well (eg, *http://www.vysis.com*).

Another chromosome-specific probe is the whole chromosome paint (WCP), in which a cocktail of DNA fragments is created to target all the non-repetitive DNA sequences in an entire chromosome. Because they cover such a large region of DNA, they yield more diffuse signals in interphase nuclei and are primarily utilized on metaphase spreads for resolving complex structural alterations. However, some of the smaller, acrocentric chromosomes yield sufficiently discrete signals that enumeration is possible in interphase nuclei. The WCPs also form the basis for advanced applications such as spectral karyotyping (SKY) and M-FISH, where each chromosome is painted with its own unique mixture of fluorescent colors. In contrast, another advanced application, comparative genomic hybridization (CGH), utilizes entire genomes as the "probe." Genomic tumor DNA is labeled in one color, normal DNA is labeled in another color, and equal quantities of both are competitively hybridized to a normal human metaphase in order to screen for regions of relative tumoral losses and gains. These techniques are beyond the scope of this paper and the interested reader is referred to recent reviews (9, 80, 120, 197).

Today, some of the most versatile and commonly used FISH probes are the locus-specific (LSI) or gene-specific probes. As the names imply, these probes target specific regions of interest and utilize single copy rather than repetitive DNA sequences. Therefore, in order to yield signals large enough to be detected in tissue sections, the probe typically needs to be at least 30 Kb in size. The largest FISH probes are often >1 Mb and most fall into the 100 to 300 Kb range. Until recently, commercially available LSI probes have been extremely limited in scope. Therefore, cloning vectors have been exploited for creating homemade FISH probes, including cosmids, bacterial artificial chromosomes (BACs), P1 artificial chromosomes (PACs), and yeast artificial chromosomes (YACs). Whereas in the past, this required a rather lengthy and tedious screening of vector libraries with PCR primers, the recent human genome initiative and mapping of entire BAC libraries

has enabled rapid internet screening, utilizing DNA sequences of interest, gene names, or physical maps of chromosomes (eg, *http://genome.ucsc.edu*, *http://gdb-www.gdb.org*). Similarly, mapped BAC clones spread throughout the human genome at 1-Mb intervals have also become available (*http://www.resgen.com*). Therefore, it is now relatively simple to obtain a BAC clone localizing to virtually any region of interest, label the DNA with commercially available kits, and utilize it as a FISH probe. This recent development should greatly enhance the applicability of FISH to investigative neuropathology.

Utility of FISH in Tumor Neuropathology

To our knowledge, the first studies utilizing FISH in normal and neoplastic brain specimens were those of Arnoldus and colleagues (3-6). They showed that FISH was a sensitive method for detecting the aneusomies commonly reported by karyotyping and described the unusual phenomenon of somatic pairing for the chromosome 1 and 17 centromeres. Although we are still in the relatively early stages of genetically characterizing CNS and PNS tumors, subsequent studies have greatly expanded these initial findings, disclosing a number of relatively tumor-specific, progression-associated, and/or prognostically relevant alterations. Individual tumor markers are discussed below, according to diagnostic category.

In terms of clinical utility, FISH is currently incorporated into only a small number of diagnostic neuropathology labs, though there has been a growing interest as the awareness of this technique's potential increases. For example, over the past 2 years, we have performed FISH on well over 100 in-house and consultation cases in our own lab and the increasing volume is evident in the fact that nearly half of these have been performed over the last 6 months. In our surgical neuropathology practice, we have utilized FISH mostly for chromosome 1p and 19q assessment in oligodendroglial tumors (see below and Table 2). However, other clinically useful markers thus far have included EGFR for small cell astrocytoma versus oligodendroglioma (Figure 4), 22q assessment for medulloblastoma/PNET versus AT/RT and various meningioma-associated markers (eg, NF2, DAL-1) for either anaplastic dural-based neoplasms of uncertain histogenesis or small meningothelial proliferations of reactive versus neoplastic nature.

Astrocytomas. Of all the CNS tumor types, astrocytomas have been the most thoroughly studied in terms of molecular pathogenesis and the reasons for this interest

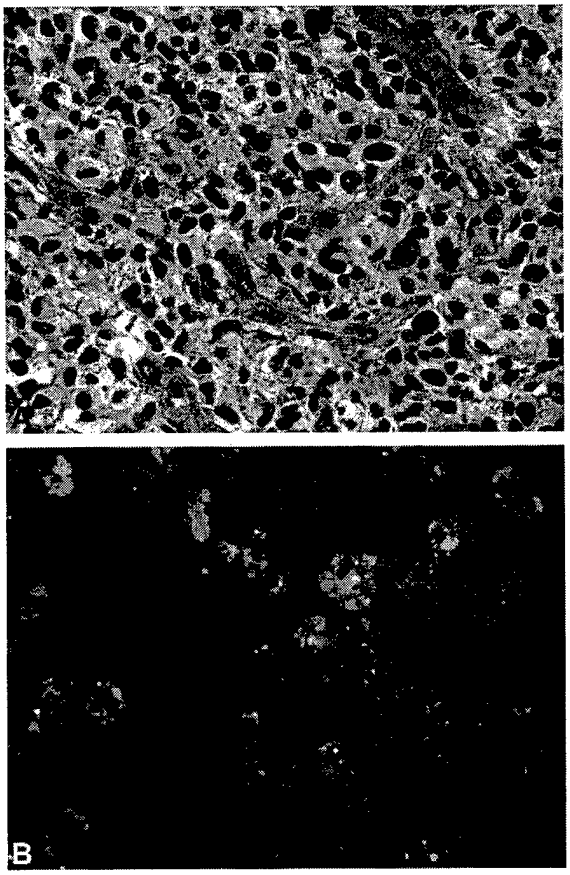

Figure 4. This recently encountered high-grade glioma harbored small, relatively uniform cells with a rich capillary network, microcalcifications, and a high mitotic index (**A**). FISH analysis revealed polysomy 7 (green) with EGFR amplification (red) (**B**). This finding and the lack of 1p and 19q deletions supported the diagnosis of small cell astrocytoma rather than anaplastic oligodendroglioma.

Diagnosis	-1p and -19q	+1p or +19q	-19q only
Oligodendroglioma II-IV (N=47)	62%	17%	2%
MOA or Glioma, NOS II-IV (N=48)	17%	48%	13%
Astrocytoma III-IV (N=8)	0%	25%	25%

MOA = mixed oligoastrocytoma, NOS = not otherwise specified

Table 2. Summary of FISH results for 1p/19q status in 103 recently diagnosed gliomas.

are readily apparent to neuropathologists. Astrocytomas are among the most common and most lethal CNS primaries, with adequate therapies generally lacking. Furthermore, although they are stratified largely based on histologic grade and patient age, these parameters do not

fully account for the wide biologic variability encountered clinically. Therefore, a genetic classification scheme could provide the basis for targeted molecular therapeutic approaches and serve as an ancillary diagnostic and prognostic tool. Since most currently known astrocytoma-associated alterations are readily detectable by FISH (eg, Figures 2, 4), this technique may play an important role as such assessments become incorporated into routine practice.

It is now clear that there are at least two clinical and molecular subsets of the grade IV astrocytoma (193, 195). De novo (primary) glioblastomas are clinically aggressive tumors associated with older age of onset and epidermal growth factor receptor (EGFR) amplification/overexpression. Secondary glioblastomas develop from lower grade precursors, tend to have a more protracted course, and frequently harbor mutations of the *p53* gene (195). *EGFR* amplification in glioblastomas may in fact have differing biologic significance based on patient age, being associated with worse survival in young patients (age<60) and prolonged survival in those over 60 (164, 171). Interestingly, pediatric high-grade astrocytomas lack *EGFR* amplifications (35, 137, 177) and there is conflicting evidence as to whether or not p53 alterations correlate with patient survival (136, 137, 177). Although FISH is unreliable for p53 assessment due to mitotic recombination (125), several labs have successfully utilized locus-specific EGFR probes and have found it to be a sensitive technique for detecting amplification (51, 171). Lastly, *EGFR* amplification has been found in the majority of small cell glioblastomas, a tumor that may mimic anaplastic oligodendroglioma, due to its relatively uniform, round to oval nuclei (27). Therefore, FISH analyses for 1p, 19q and EGFR may be especially useful for this diagnostic consideration (Figure 4).

Losses involving chromosome 10 are also frequent in high-grade astrocytomas and several groups have utilized a CEP10 probe to detect such losses in paraffin-embedded cases (2, 23, 37, 40, 73, 122, 132, 175). Whereas some report this loss as an independent negative prognosticator (7, 37, 132), others found a significant association only on univariate analysis, since monosomy 10 is so tightly linked with GBM histopathology (54, 73). Several candidate tumor suppressor genes have been mapped to the long arm, including *PTEN* (10q23) and *DMBT1* (10q25.3-26.1). LSI probes have now been used successfully to detect both deletions (51, 171). Mutations of *PTEN* appear to be restricted to high-grade astrocytomas, but not to either the primary or secondary GBM variant (138, 154, 202). Similar to the FISH reports of monosomy 10, several reports suggest that *PTEN* alterations are predictive of short survival, both in adult and pediatric patients with high-grade astrocytoma (105, 137, 171). Therefore, the detection of 10q losses, by FISH or other techniques may eventually become incorporated into routine practice.

Alterations of the cell cycle regulatory cascade that include *p16/CDKN2*, cyclin-dependent kinase 4 (*CDK4*), and retinoblastoma (*RB*) genes are also involved in the malignant progression (68, 155, 185). The most frequent mechanism of inactivating this pathway in gliomas is through homozygous deletion of *p16* (125, 185). Several studies have suggested a correlation between p16 loss and poor survival (85, 115). However, in a large FISH study of diffuse gliomas, we were unable to verify this on multivariate analysis, since this alteration was tightly linked to high-grade astrocytic pathology (125). Importantly, Iwadate and colleagues found that mutations and homozygous deletions of *p16* were correlated with increased sensitivity to antimetabolite chemotherapeutic agents, suggesting that *p16* status may predict chemosensitivity for individual brain tumors (78). The recent release of a commercially available p16/CEP9 FISH probe cocktail (Vysis, Downer's Grove, Ill) may further facilitate investigational efforts.

In comparison to diffuse astrocytomas, little is known about the genetics of pilocytic astrocytomas (PA). The majority of tumors studied have shown no detectable chromosomal abnormalities, although gains of chromosomes 5 to 9 have been observed by cytogenetic techniques, including FISH (1, 36, 150, 200). As PAs occur frequently in patients with neurofibromatosis type 1, it is not surprising that the *NF1* gene has been scrutinized as a potentially important tumor suppressor (191). Indeed, loss of neurofibromin expression has been detected in the majority of NF1-associated, but not sporadic PAs (63, 104, 134). However, the method for this inactivation is unknown and does not seem to involve FISH-detectable deletions (104).

Oligodendrogliomas and the -1p/19q genetic variant. In no other area of brain tumor pathology has FISH proved more clinically valuable than in the genetic profiling of oligodendroglial tumors. Comprising approximately 20 to 25% of adult gliomas, oligodendrogliomas tend to progress more slowly than astrocytomas, and are associated with longer patient survival (50, 86, 161). The diagnosis of these tumors is particularly critical given that many anaplastic oligodendrogliomas respond

favorably to chemotherapy, especially the PCV regimen (procarbazine, lomustine (CCNU), and vincristine) (32, 50, 60, 107). Unfortunately, the histologic classification of oligodendroglial neoplasms remains subject to considerable interobserver variability and the oligodendroglial phenotype has expanded over time to include "minigemistocytes" and "gliofibrillary oligodendrocytes" (41, 92, 124).

LOH, CGH, and FISH studies have shown that 60 to 70% of oligodendrogliomas are characterized by a distinctive genetic pattern, consisting of combined deletions of the entire chromosome 1p and 19q arms (10, 17, 90, 143, 144, 167). These molecular alterations have potential diagnostic, prognostic, and even therapeutic relevance. For instance, using 1p36 and 19q13.3 LSI FISH probes, Smith et al showed that 1p and particularly combined 1p/19q deletions were highly associated with the oligodendroglial phenotype (167). They further established that such deletions were associated with prolonged patient survival in pure oligodendrogliomas, irrespective of grade (169). This favorable association was not detected in astrocytomas or mixed oligoastrocytomas (MOAs) with this molecular signature, though a relatively small number of MOAs were analyzed and this issue remains unresolved (75, 91, 169). Losses of 19q have also been associated with malignant progression in astrocytomas, though the deletions are often smaller in such cases and 1p is typically not codeleted (144, 167, 170, 190, 192). Perhaps of greatest clinical relevance, Cairncross et al have shown that allelic loss of 1p was a statistically significant predictor of PCV chemosensitivity, and that combined 1p and 19q loss was associated with both chemosensitivity and longer recurrence-free survival (33). The mechanism for this enhanced therapeutic responsiveness is poorly understood, though additional studies have shown enhanced radiosensitivity as well (8). Therefore, it is possible that the combined 1p/19q-losing oligodendrogliomas are also more sensitive to other drugs, such as temozolamide, a currently popular chemotherapeutic agent with less patient toxicity than the PCV regimen. In other words this oligodendroglioma variant may be a better behaving tumor, almost regardless of the therapeutic approach that is chosen. Since this is still speculative, clinical trials are needed to address this issue. Nevertheless, sufficient data now exists to support ancillary 1p or 1p/19q testing and a number of labs currently offer this by LOH, FISH, or both. For the past 2 years, we have been prospectively assessing our oligodendroglial neoplasms and the FISH results are summarized in Table 2. To some extent, MOAs and other morphologically ambiguous diffuse gliomas are over-represented due to consultation biases (ie, classic cases are less likely to be sent out). The high-grade astrocytomas analyzed represent cases with either vague oligodendroglial features or

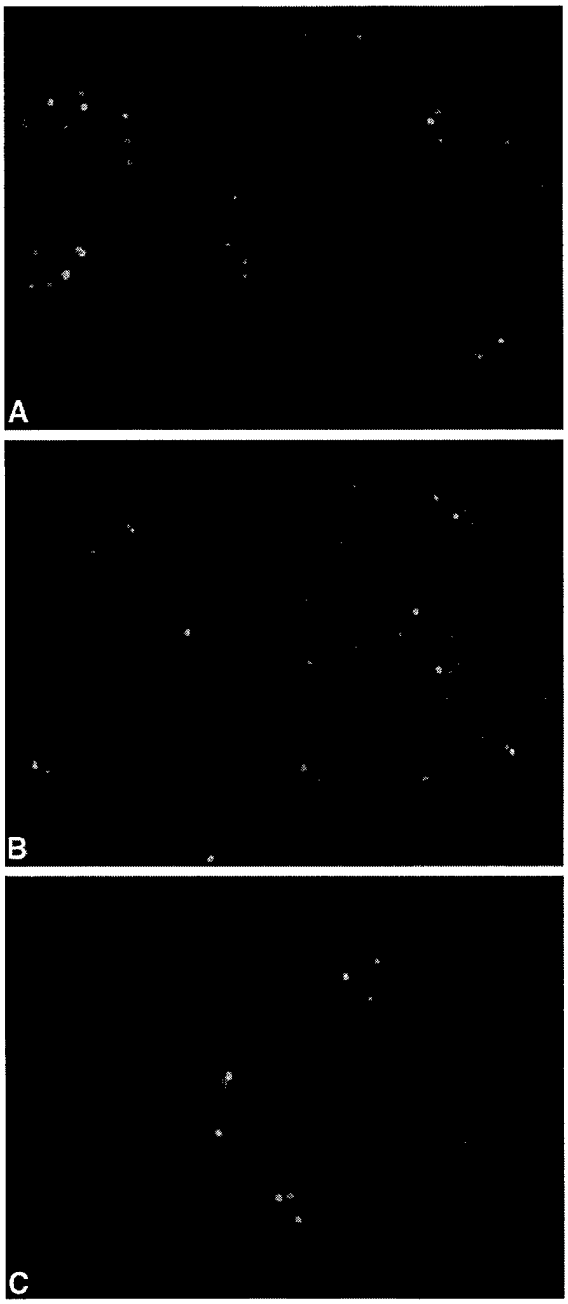

Figure 5. Common patterns of 1p (green) and 19q (red) FISH analysis include the normal, disomic state (**A**), codeletion of both markers (**B**), and loss of 19q only (**C**).

those with unexpectedly long survival. In any case, the four common patterns seen by FISH are illustrated in Figure 5 and include normal, 1p/19q codeletion, 19q loss only, and polysomies (gains), likely reflecting a state of aneuploidy. As in prior retrospective series, the 1p/19q codeletion was most commonly encountered in classic oligodendrogliomas. Polysomies have been particularly common in our morphologically equivocal gliomas, though the significance of this finding, if any, is currently unclear. Another phenomenon we have experienced is the "astrocytoma" that recurs after more than 10 years. Nearly invariably, re-review has demonstrated oligodendroglial rather than astrocytic features and FISH often reveals combined 1p and 19q deletions.

Similar to astrocytomas, alterations involving chromosomes 9 and 10 have been identified in anaplastic oligodendrogliomas, though at a lower frequency (17, 32, 105, 151). Mutations involving both *PTEN* and *DMBT1* have been detected, although to date only loss of PTEN has been implicated as a potentially useful clinical marker (105, 151). Sasaki et al found that allelic loss of chromosome 10q was negatively associated with 1p loss and that *PTEN* gene alterations were independently predictive of poor survival in patients with anaplastic oligodendrogliomas, even those with initially favorable chemotherapeutic response (151). Similarly, *p16/CDKN2A* deletions have also been associated with anaplasia and worsened survival, occurring preferentially in tumors with retained 1p and 19q (17, 33).

Mixed oligoastrocytomas (MOAs). MOAs and other morphologically ambiguous gliomas are a histologically and clinically diverse group of tumors (86). As with pure oligodendrogliomas, some MOAs respond favorably to PCV chemotherapy (60). Unfortunately, objective diagnostic criteria are lacking and neuropathologist concordance rates remain low (124). As a group, survival rates fall somewhere between those of pure astrocytic and oligodendroglial tumors of similar grade, but there is wide individual patient variability (124, 160, 168). Thus, an obvious hope is that molecular profiling will provide a more clinically useful stratification. In this regard, Maintz et al proposed two genetic subsets of MOA, one exhibiting p53 gene mutations as seen in astrocytomas and another with 1p and 19q deletions, more typical of oligodendrogliomas (110). In a more recent CGH study, a 4-category system was proposed (83). In this scheme, two groups similar to pure astrocytomas (+7/-10) and oligodendrogliomas (-1p/-19q) respectively were identified. An additional "intermediate" category with -1p/-19q and +7 and/or -10 was included and tumors lacking any of these alterations were defined as "other." Since genetic studies have typically revealed either a more astrocytoma-like or oligodendroglioma-like pattern, it is still debated whether MOAs represent truly mixed tumors or simply pure gliomas, wherein the histogenesis is less obvious. Whether or not potential "astrocytoma-associated" or "oligodendroglioma-associated" markers will prove to be of clinical utility in such cases remains to be determined and a FISH study is currently ongoing.

Ependymomas. Ependymomas represent a subset of gliomas that occur both sporadically and rarely, in association with neurofibromatosis type 2 (NF2). Thus far, histologic classification has been an unreliable predictor of clinical behavior, and extent of resection remains the most meaningful prognostic determinant (49, 135). In contrast to the diffuse gliomas, little is known regarding tumorigenic and progression-mediated events in ependymoma.

Much interest has centered on *NF2* (22q12) and its gene product merlin (schwannomin), since alterations involving chromosome 22 have been well documented in ependymomas (20, 46, 67, 71, 74, 82, 96, 111, 176, 187, 194, 198, 199, 201). Given the increased frequency of intramedullary ependymomas in NF2 patients, it is interesting that several groups have reported a spinal association for ependymomas with *NF2* mutation (21, 46, 67, 96, 97, 100). Using LSI probes for *NF2* and a related protein 4.1 family member, *DAL-1*, we detected a similar trend, whereas those with *DAL-1* deletion were associated with intracranial localization (manuscript submitted). Age-associated genetic alterations are also suspected, though the data is conflicting (67, 89, 111, 140, 194). In a recent CGH study, chromosome 7 gains were seen primarily in spinal, while gains of 1q and losses of 6q, 9 and 13 occurred preferentially in intracranial ependymomas (71). Therefore, there are currently multiple sites of interest, amenable to further clinicopathologic FISH studies.

Embryonal neoplasms. The generic term "embryonal tumor" is used to describe a diverse group of primitive round cell neoplasms occurring in the CNS and PNS. They include both central and peripheral forms of primitive neuroectodermal tumor (PNET), as well as atypical teratoid/rhabdoid tumor (AT/RT). Although nearly all have "small blue cells" in common, each has defining clinical, morphologic, immunohistochemical, and genetic features. Furthermore, despite the designation of central PNET for both medulloblastomas and

their supratentorial counterparts, it is clear that the latter behave in a more aggressive fashion and differ genetically (86). Whereas isochromosome 17q formation is typical of medulloblastoma (13, 14, 15, 18, 58, 142), losses of 14q and 19q are more common in the supratentorial PNETs (29, 146). Even within the category of medulloblastoma, not all are created equal, with both favorable and unfavorable variants recently recognized. Subsets of aggressive medulloblastomas harbor amplifications of C-*myc* (11, 19, 25, 70, 101, 152) and less frequently N-*myc* (25, 141, 181) oncogenes. These amplifications have recently been associated with the large cell/anaplastic variant, a highly aggressive tumor that characteristically presents with CSF dissemination (25, 101). In contrast, medulloblastomas with hypernodularity, extensive neuronal maturation, and/or TrkC expression reportedly have a more favorable prognosis (45, 55, 56, 62, 172).

AT/RT is an uncommon, primarily infant-associated CNS tumor, notable for its extremely aggressive behavior, its diverse histomorphologic spectrum, and its resistance to standard medulloblastoma therapeutic regimens (28, 86, 145). These polyphenotypic neoplasms are characterized by varying numbers of "rhabdoid cells" with eccentric nuclei, prominent nucleoli, and eosinophilic paranuclear inclusions composed of whorled bundles of intermediate filaments. Epithelial, mesenchymal, and/or immature round cell elements can also be encountered, and when the latter predominate, they are often misdiagnosed as medulloblastoma or PNET (28, 145). However, unlike the latter, most AT/RTs are characterized by monosomy 22 or 22q deletions (11, 28, 145). Recently, deletions and mutations of the *hSNF5/INI1* gene on 22q11.2 have been identified in AT/RTs, as well as the majority of extracranial rhabdoid tumors (12, 14, 26, 52, 189). A commercially available LSI probe directed against the nearby *bcr* gene has both excellent hybridization efficiency and sensitivity for detecting these 22q11.2 deletions (26, 52). It is therefore useful for distinguishing AT/RT from medulloblastoma or PNET, particularly in younger patients with inconclusive morphologic and immunophenotypic findings.

Ewing's sarcoma and peripheral PNET (pPNET), malignant round cell neoplasms with overlapping features are now considered two ends of a single diagnostic spectrum. Characterized by translocations involving the *EWS* gene on chromosome 22q, they may be encountered by neuropathologists, due to their frequent paraspinal localization (182, 184). The t(11;22)(q24;q12) translocation resulting in an aberrant EWS-FLI1 fusion, has been detected in 80 to 90% of cases,

Figure 6. FISH analysis utilizing EWS (red) and FLI (green) probes demonstrate several overlapping, yellow fusion signals in this case of peripheral PNET.

although several variant translocations have also been described (59, 133, 184). Since central PNETs, medulloblastomas, neuroblastomas, and other differential diagnostic considerations do not harbor these translocations, it represents a diagnostically useful finding (77, 81). Similar to RT-PCR and Southern blot analyses, FISH has been shown to be a reliable method for detecting this *EWS-FLI1* fusion (Figure 6) (42, 77, 81, 93, 94, 114, 117). Lastly, neuroblastoma is a genetically distinct embryonal tumor of the autonomic nervous system. A subset are characterized by 1p deletions and/or N-*myc* amplification, both considered negative prognostic indicators that are commonly detected by FISH analysis (66, 178, 179). In summary, i(17q), 22q deletions, *myc* amplifications, and EWS-FLI translocations are readily detectable alterations by FISH and represent useful ancillary diagnostic and prognostic markers for selected forms of embryonal CNS and PNS tumors.

Meningiomas. Although the genotyping of meningiomas has lagged behind similar studies of gliomas, these were among the first neoplasms to be characterized by a cytogenetic alteration, namely monosomy 22. It is now known that the *NF2* gene on 22q12 is a common primary target of inactivation, both in NF2-associated and sporadic tumors (43, 65, 112, 127, 147, 186). Loss of DAL-1, another protein 4.1/ERM family member, has also been implicated recently, both by FISH and other techniques (64, 126, 127). Many progression-associated sites of chromosomal loss and gain have similarly been identified, though the responsible genes remain unknown (24, 30, 31, 95, 102, 128, 156, 163, 196). Nevertheless, these alterations are amenable to

FISH detection. Similar to others, we have found a significant association between 1p and/or 14q deletions and high grade (WHO II or III) (30). Although these alterations have not been independently associated with prognosis thus far, 14q deletions were common in our histologically benign meningiomas that recurred unexpectedly (ie, despite gross total resection). This suggests that despite the lack of a candidate gene, 14q assessment by FISH may eventually supplement routine histology, perhaps in association with other genetic markers.

Utilizing CGH, Weber et al identified a 17q23 amplicon associated with anaplastic (grade III) meningiomas (196). In roughly 15% of anaplastic meningiomas, we subsequently found FISH detectable amplification of the *PS6K* gene in this region, suggesting that this either represents an uncommon, likely late progression-associated genetic event (31). Whether or not this gene is the primary target of this amplicon has yet to be determined. Similarly, in a comprehensive CGH, LOH, PCR, mutation, and hypermethylation study by Bostrom et al, the *p16* gene was frequently inactivated in anaplastic meningiomas, with loss of one or both alleles in the majority of cases (24). We similarly found deletions by FISH in roughly 70% of our anaplastic meningiomas and these cases had a statistically significant reduction in overall survival (unpublished data). Further studies are ongoing in hopes of developing a comprehensive and clinically useful molecular classification scheme for meningiomas.

Peripheral nerve sheath tumors. A number of benign and malignant nerve sheath tumors are now known to harbor distinct genetic aberrations. Given the high frequency of neurofibromas and schwannomas in NF1 and NF2 respectively, it is not surprising that their corresponding genes have been implicated in both the familial and sporadic forms of these common nerve sheath tumors (38, 47, 65, 76, 87, 103, 139, 148, 159). Schwannomas are relatively pure Schwann cell proliferations with loss of merlin expression in the majority of cases (65, 148). The mechanisms of inactivation have not been entirely resolved, though both LOH and cytogenetic studies suggest monosomy 22 in a subset (103, 113). By comparison, the study of neurofibromas has been partially hampered by its heterogeneous composition, including Schwann cells, fibroblasts, perineurial-like cells, and entrapped non-neoplastic elements. Both LOH and FISH have found loss of the *NF1* region in up to half of plexiform neurofibromas, the variant with the greatest potential for malignant transformation (87, 130, 139). Combined FISH and S-100 protein immunohistochemistry further illustrated that this loss is restricted to the Schwann cell component (130). Another rare form of benign nerve sheath tumor recently characterized by FISH is the perineurioma. Similar to schwannomas and meningiomas, monosomy 22 is common in both intraneural and soft tissue examples (48, 57). Whether or not the *NF2* gene is the target of this genetic loss remains unclear at this time (98, 99).

There has been great interest in genetically characterizing malignant peripheral nerve sheath tumors (MPNSTs), given that these tumors are often diagnostically challenging and there are currently no specific immunohistochemical markers. Cytogenetic studies have revealed highly complex, aneuploid or near-triploid karyotypes in most and a specific genetic marker has not been identified (113). However, LOH of the *NF1* gene has been found in roughly two thirds of MPNSTs from NF1 patients (106, 139, 166). By FISH, this region is deleted in both familial and sporadic examples (106, 130) and larger studies to assess the specificity of this finding are currently underway. Synovial sarcoma, one of the main differential diagnostic considerations, harbors a characteristic X;18 translocation, which is detectable by a number of techniques, including FISH (22, 203). Although the fusion transcript has recently been detected by RT-PCR in MPNST as well (119), this alteration is still considered more characteristic of synovial sarcoma. It is unknown whether these RT-PCR-positive MPNSTs may have harbored only rare cells with the fusion, in which case FISH and other less sensitive techniques might have been considered negative. Nevertheless, additional studies of specificity for the t(X;18) in these histologically similar neoplasms may be warranted, including those with FISH.

Utility of FISH in Non-neoplastic Neuropathology

The role of FISH in non-neoplastic neuropathology has barely been explored. Nevertheless, there are a number of potential applications. For example, Down syndrome brains are often studied as a model of early onset Alzheimer's disease. However, they are often received with little available clinical information and lack of cytogenetic confirmation. Since trisomy 21 is present in every cell of a Down syndrome patient (except in mosaic forms), it is a relatively simple matter to confirm this diagnosis with FISH analysis on submitted brain tissue (131). One could envision a similar approach for malformed perinatal autopsy brains. For example, a formalin-fixed holoprosencephalic brain sent in consultation without karyotyping could be evaluated for trisomy 13 or 18. LSI probes have similarly been applied to cases

of lissencephaly or other migrational disorders, where microdeletions have been recently appreciated (44, 123). Such information would obviously be useful for subsequent genetic counseling.

Summary

FISH has become a useful clinical and research tool, which is still relatively underutilized. As with any technique, it has distinct advantages and disadvantages, though its main attraction for neuropathologists lies in its morphologic preservation and applicability to archival FFPE tissue. Given recent technical advances and a rapidly growing body of molecular cytogenetics literature, its role in diagnostic and investigative neuropathology laboratories will likely continue to grow in the near future.

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