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Skin neurofibromas are the most common tumors of patients with neurofibromatosis type 1 (NF1), yet we know very little about how they develop. We are studying NF1 subjects with microdeletions because they are predisposed to large numbers of neurofibromas. We propose that deletion of NF1 gene and one of the 15 adjacent genes favors the development of skin neurofibromas, and possibly also other tumors. We are currently evaluating NF1 subjects to identify those with deletions and determine when and what kind of tumors and other clinical features they develop. We have developed new competitive PCR assays to rapidly screen for deletion patients. Later in the study we, will look for specific genetic changes in skin neurofibromas of patients with *NF1* deletions to provide insight into how they develop. We propose to employ new and powerful technology from the Human Genome Project to confirm or disprove the presence of a tandem *NF1*-like gene. If present, this gene may contribute in some unknown way to making the disease more or less severe. Therefore, identifying a tumor modifying gene and the possible tandem *NF1*-like gene will help us understand tumorigenesis in these patients.

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Microdeletion, tumor initiation, modifying gene, genotype/phenotype, prognostic utility, paralogs, fosmid mapping, duplications

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Table of Contents

Introduction

About 5-10% of neurofibromatosis type 1 (NF1) patients are heterozygous for a contiguous gene deletion that includes the *NF1* gene. Although limited in scope, previous studies provide compelling evidence that microdeletion patients show early onset and large numbers of cutaneous neurofibromas, and a higher frequency of plexiform neurofibromas, malignant peripheral nerve sheath tumors, and other solid tissue malignancies. We propose to perform systematic, comprehensive clinical and molecular studies of subjects with *NF1* microdeletion. The specific aims of this research are 1) To determine the clinical spectrum, genotype/phenotype correlations, and prognostic utility associated with an *NF1* microdeletion; 2) To determine if cutaneous neurofibromas of *NF1* microdeletion subjects show evidence of genomic instability or homozygous *NF1* microdeletion that may contribute to the early onset of neurofibromagenesis; 3) To investigate the molecular basis for early onset cutaneous neurofibromas in patients/families who are heterozygous at *NF1*; (4) To employ the newly developed FLASH technology to analyze the *NF1* microdeletion region and construct a physical map that will determine, with a high degree of confidence, the sequence of all of the genes, unique noncoding regions, and paralogs (including the putative *NF1* duplicated gene) of the *NF1* microdeletion region. NF1 subjects will be screened to identify those with microdeletions, microdeletions mapped at the sequence level and correlated with comprehensive clinical evaluations to determine the clinical spectrum and genotype/phenotype correlation utility associated with *NF1* microdeletion. Primary neurofibroma tissue from microdeletion patients will be analyzed for evidence of genomic instability or homozygous *NF1* microdeletion that may contribute to early onset of neurofibromagenesis. Somatic mosaicism for an *NF1* microdeletion and inactivating mutations in *JJAZ1*, a strong candidate for causing potentiation of neurofibromas in microdeletion patients, will be evaluated as underlying causes of early onset neurofibromagenesis in subjects who are heterozygous at the *NF1* locus. In collaboration with the University of Washington Genome Center, sequence-tagged-sites (STS) in the *NF1* region will be used to interrogate a fosmid library with 18X haploid genome equivalents constructed from a single individual. STS-content maps will be constructed, fosmids sequenced, and haplotypes determined. Sequence redundancy and perfect match of overlapping fosmids will facilitate correct assembly of regions harboring paralogs.

Body

The original STATEMENT OF WORK

Year 1, Months 1-8:

- Develop a clinical database, train personnel to use.
- Design and test clinic evaluation forms for patient assessment.
- Design STS primers for interrogation of fosmid library. Begin library screen.

Progress:

- Develop a clinical database, train personnel to use. We have developed two databases using FileMaker Pro, a customizable database software package. One database contains patient names, contact information, and coded identifiers. Aaccess to this database is limited by two levels of passwords to researchers who have human subjects training and patient contact. These include Drs. Stephens (Principal Investigator) and Sybert (Co-Investigator) and two new members of our research team Dr. Paula Zook (Dermatologoy fellow) and Mercy Laurino, MS (Genetic Couselor). The second database contains no patient history information. Coded identifiers are used to link subjects with their a summary of their clinical history/manifestations and laboratory data regarding the results of screening for microdeletion. Access to this database is limited by two levels of passwords to the above

reserachs and to Dr. Stephens' laboratory staff. Relevant laboratory personnel have been trained to use this database.

- Design and test clinic evaluation forms for patient assessment. We have designed and are in the process of testing a clinic evaluation form for patient assesment. See Appendix for a copy of the form in current use. Our IRB was not approved by the Army until February, 2003, so testing of this tool is still in progress.
- Design STS primers for interrogation of fosmid library. Begin library screen. We have begun working with Drs. Maynard Olsen, Marcia Paddock, and Raj Kaul at the University of Washington Genome Center to investigate the possibility that the NF1 gene is tandemly duplicated in the genome. They are currently assessing all sequence tracts submitted by the public Human Genome Project and by the whole shotgun sequence (Celera) along with mouse and rat sequences to assess whether there is current data supporting more than one NF1 gene. In addition, Dr. Stephen Forbes, a postdoctoral student in the Stephens' laboratory is also involved in this informatics process. We have chosen 5 lymphoblastoid cell lines from control individuals and purified high quality genomic DNA for the genome center for the construction of the fosmid libraries.

Years 1-2:

- Enroll new patients in the study.
- Screen for NF1 microdeletion patients, map extent of deletions, develop new deletion junction assays as needed.
- Ascertain NF1 subjects that show early onset cutaneous neurofibromas that do not carry microdeletions.
- Determine conditions for immunohistochemistry, test and choose optimal antibodies
- Write manuscript on novel microdeletions and whether they are mediated by paralogous recombination.

Progress:

- Enroll new patients in the study. A major accomplishment during year one was to obtain human subjects approval from the local Institutional Review Board at Children's Hospital and Regional Medical Center/University of Washington (submitted June, 03 and approved September, 03) and also from the Army HSRRB (submitted July 03, approved February, 04). The process to obtain approval by the Army HSRRB has improved since my last grant, but remains a major hurdle that consumes time and resources.

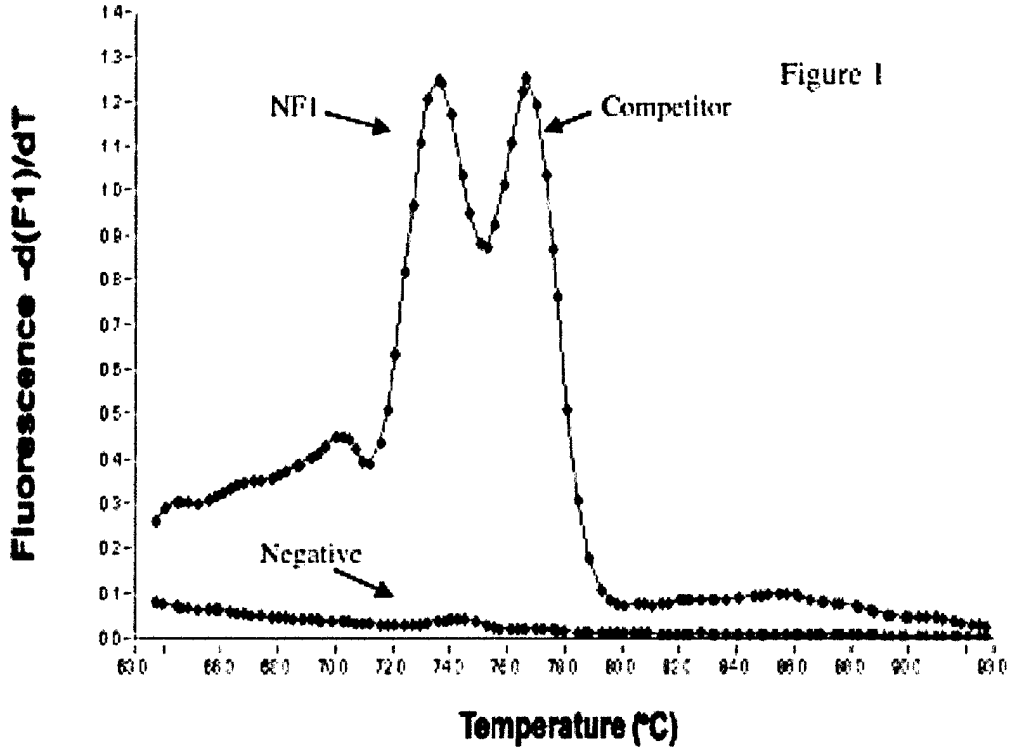
  In the six weeks since approval of our study we have enrolled 16 NF1 subjects in the study.

- Screen for NF1 microdeletion patients, map extent of deletions, develop new deletion junction assays as needed. We are currently screening the 16 newly enrolled subjects and about 300 existing subjects for microdeletion using a multiple assays. Two assays include deletion-specific PCR assays that generate 7 kb amplicons only when deletions occur at either PRS1 or PRS2 (paralogous recombination sites 1 and 2), which are hotspots for strand exchange in the flanking NF1REP repeats and account for the recurrent 1.5 Mb microdeletions (~70% of all NF1 microdeletions) (1-3). In addition, we developed new deletion junction assays for two novel sites of recombination that we mapped within the NF1REPs. Samples that are negative for these four assays are evaluated by quantitative PCR to detect deletions that may occur at novel sites. We have developed five competitive PCR assays that assess gene dosage at five loci including AH1 (centromeric to NF1 gene), NF1 exon 5, NF1 intron 32, WI-12393, which are all loci within the recurrent 1.5 Mb deletion, and D17S250, a conrol locus that is distal on chromosome 17 and not deleted in any cases to date.

These assays required the capability and sensitivity to determine if a locus or gene has no deletion (2 copies) or one gene deletion (1 copy). The difficulty is in reliably differentiating one versus two copies of a gene. Therefore, we have been focusing on developing the best assay to detect a one gene deletion. We developed assays that are precise, sensitive, and specific and involve SYBR green for detection, competitive quantitative PCR, and melting curve analysis as detailed below for the NF1 exon 5 assay as an example.

We chose to employ SYBR green for detection in combination with competitive PCR, which is the most suitable method of quantification when highly accurate determinations are required. We adapted and modified a method published by Ruiz-Ponte et al (4). In this method, a known copy number of a competitor is introduced directly in the PCR mixture along with the target DNA of the patient/tumor. The competitor, which is almost identical to the target DNA but distinguishable by product length, is amplified with the same set of primers so that efficiency of amplification for the two amplicons is the same. Calibration curves of different competitor concentrations determines the optimal concentration that equals that of the target DNA. Figure 1 below shows the melting curves of a normal control DNA samples (2 copies of NF1), where competitor and NF1 exon 5 are co-amplified with equal efficiency and the area under the curves are equal (roughly equivalent to peak height in this example). We constructed the competitor such that it would be amplified with the NF1 exon 5 primers, but have a different melting curve by replacing an internal TTT sequence with a CCC sequence. As expected, negative samples lacking human DNA did not amplify.

Figure 1. Melting curve analysis after competitive, quantitative PCR at NF1 exon 5 in genomic DNA of a normal control individual. The peaks representing the melting curve of the amplicon of the competitor and the amplicon of the patient's target DNA are indicated. The negative control without DNA shows no evidence of amplificiation.



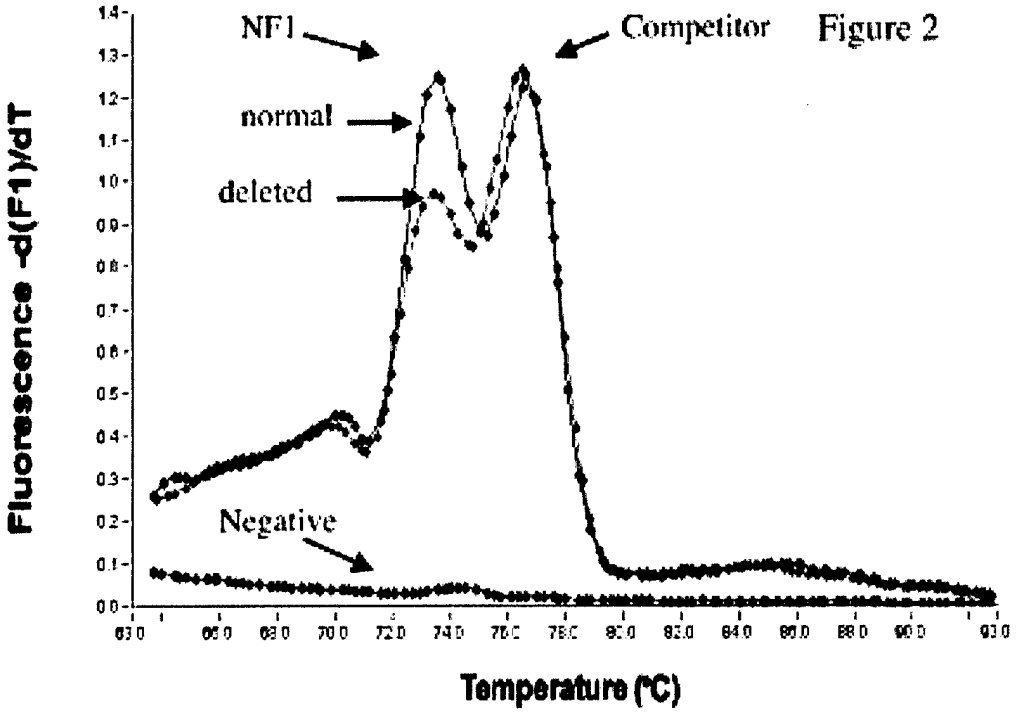


Figure 2. Melting curve analysis after competitive, quantitative PCR at NF1 exon 5 can differentiate one gene copy versus two gene copies. The results of two reactions are shown, closed circles represent target DNA from a normal control individual and closed squares represent target DNA from an NF1 patient with a deletion of one gene. The peaks representing the melting curve of the amplicon of the competitor and the amplicon of the patient's target DNA are indicated. The negative control without DNA shows evidence of amplification.

Figure 2 shows the melting curve of a patient with an NF1 deletion (1 copy of NF1) versus that of the normal control individual. Note that the amplitude of the melting curve of the NF1 amplicon for the deletion patient is less that of the competitor amplicon because there are fewer targets in the deleted patient's DNA. For precision, we use the peak areas of each melting curves for quantitation rather than peak height. A ratio of peak area of normal control (2 copies) over the peak are of the patient target DNA is calculated, see below.

Once the concentration of competitor is determined for a certain concentration of the normal control DNA, it is essential that all subsequent reactions with unknown patient DNA samples contain exactly the same concentration of target DNA. Prior to the competitive quantitative PCR assay, we determine the exact concentration of each patient sample using real-time quantitative PCR at a different locus. We amplify the TPA (tissue plasminogen activator) gene on chromosome 12 in each patient and compare that to a standard curve using the normal control DNA. From this reaction, we can calculate exactly what volume of patient DNA must be

added to the competitive quantitative PCR assay. An example of the TPA real-time PCR and standard curve is shown in Figure 3.

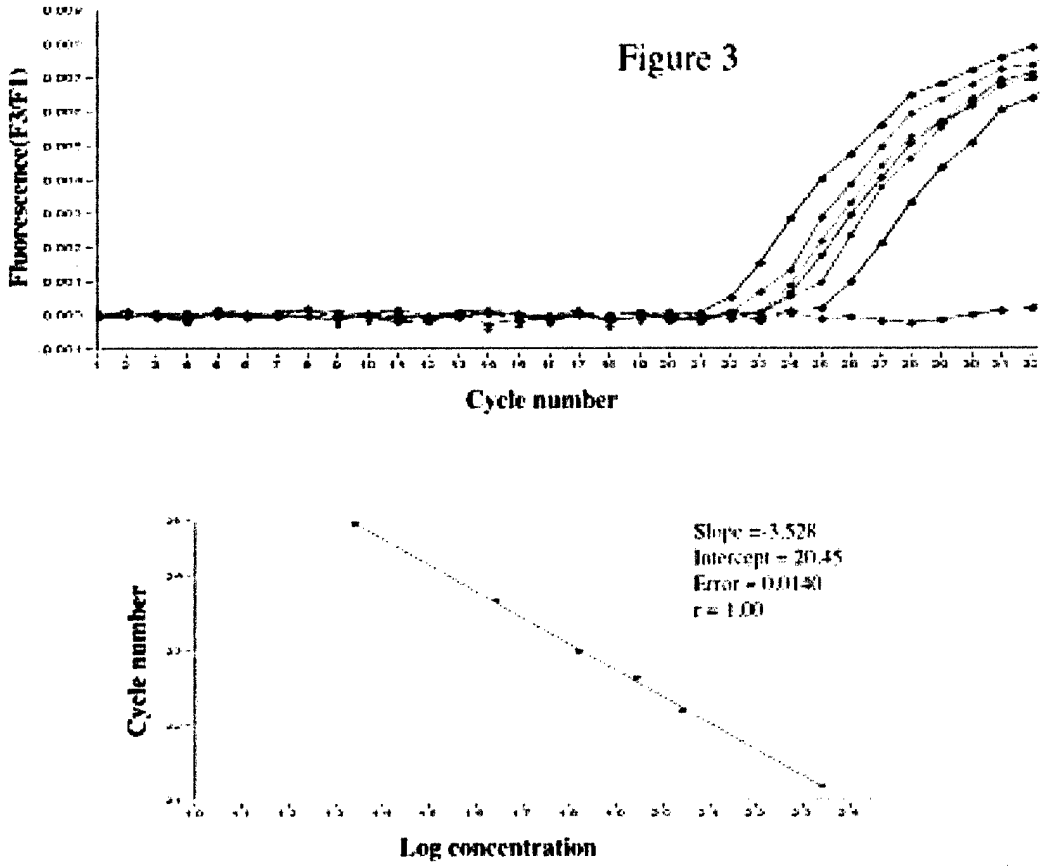


Figure 3. LightCycler real-time PCR at TPA locus showing standard curve. The upper panel shows the results of real-time PCR of the TPA locus of a dilution series of a normal control individual. The reaction consists of unlabeled primers and uses an internal labeled (fluorescence resonance energy transfer (FRET) probe for detection of product. The crossing point (Ct) is defined as the fractional cycle at which fluorescence begins to increase exponentially and is calculated by the LightCycler. Ct becomes larger as the number of TPA targets decreases. The lower panel shows the standard curve calculated from the above data. Note the low error and high correlation coefficient.

The competitive quantitative gene dosage assays we have developed for AH1, NF1 exon 5, NF1 intron 31, and WI-12393, and the D17S250 control locus are very precise and shows minimal variability. These assays are currently being employed to screen ~300 patients for deletions. Each sample is in various stages of the screening process, but it is clear that we have identified new deletion patients amoung our cohort. We expect to have this screen completed by mid summer, 2004 for all patients collected by that time.

One such deletion is NF46-3, that is deleted for each of the 4 loci in the NF1 region but has a novel deletion since the breakpoints are not at PRS1 or PRS2. We are currently constructing a somatic cell

hybrid line that carries only the chromosome 17 with the deletion in order to map the breakpoints on the sequence level.

These competitive PCR assays for detecting NF1 microdeletions were developed in my laboratory by Dr. Bingbing Wang who is currently writing a manuscript for publication.

Year 2:

- Screen subjects with early onset cutaneous neurofibromas that are heterozygous at NF1 for somatic mosaicism for an NF1 microdeletion.
- Construct STS-content maps, sequence fosmids, and construct haplotypes.
- Obtain cutaneous neurofibromas from NF1 microdeletion adults.
- Perform immunohistochemistry and nucleic acid extraction of neurofibromas
- Assemble data on clinical spectrum of NF1 microdeletion patients; write manuscript.
- Write manuscript on novel microdeletions and whether they are mediated by paralogous recombination

Progress:

Perform immunohistochemistry and nucleic acid extraction of neurofibromas. Dr. Paula Zook, fellow in Dermatology has experience in tissue immunohistorchemistry/immunofluorescence and is just beginning these studies. We have established contacts with Dr. Michele Kliot (UW neurosurgeon) and Dr. Zsolt Argenyi (UW neuropathologist) and will meet next week to design a protocol for tumor excision, dissection, fixation, and immunohistochemistry. Dr. Argenyi can provide anonymous unused neurofibroma tissue to assess the optimal fixation process.

Year 3:

- Screen JJAZ1 gene for inactivating mutations in subjects with early onset cutaneous neurofibromas that are heterozygous at NF1.
- Clinically evaluate age-matched NF1 patients without microdeletions and control patients for comparison to determine prognostic utility of a microdeletion.
- Obtain cutaneous neurofibromas from NF1 microdeletion adults.
- Perform immunohistochemistry and nucleic acid extraction of neurofibromas
- Perform microsatellite instability studies on neurofibromas tissue
- Identify 2nd hit NF1 mutations in neurofibroma
- Write manuscript on results of neurofibroma studies.
- Continue fosmid analysis of NF1 region; construct new libraries if needed.

Year 4

- Submit clinical information on NF1 microdeletion patients to the National Neurofibromatosis Foundation International Database.
- Analyze data for phenotype/genotype correlations and prognostic utility.
- Analyze the complete sequence of the NF1 microdeletion region for new genes and paralogs.
- Perform comparative mapping of final human sequence with that of the mouse.

Key Research Accomplishments

- Developed a database of NF1 subjects and their clinical manifestations and NF1 microdeletion study results.
- Initiated a screen of ~300 NF1 subjects to identify those with NF1 microdeletions at the breakpoint hotspots (PRS1 and PRS2).
- Developed new quantitative gene dosage assays for rapid screening of NF1 subjects for novel microdeletions that do not occur at PRS1 or PRS2. We have developed competitive PCR assays for 5 loci, including AH1, NF1 exon 5, NF1 intron 31, and WI-12393, and the D17S250 control locus.
- Designed a clinical evaluation form for assessment of NF1 subjects.

Reportable Outcomes

- Developed immortalized lymphoblastoid cell lines from 16 newly enrolled NF1 subjects
- Developed a database of NF1 subjects to track clinical manifestations and NF1 microdeletion data
- Manuscript: Stephens K. Neurofibromatosis. In *Molecular Pathology in Clinical Practice.* D.G.B. Leonard, Ed. New York: Springer –Verlag, in press. [See Appendix]
- Abstract: Bingbing Wang, Shinny Vong, and Karen Stephens. Identification and Mapping of NF1 Contiguous Gene Deletions Using Real-Time Competitive PCR. The NNFF International Consortium for the Molecular and Cell Biology of NF1 and NF2, Aspen, Colorado, May, 2004. [See Appendix]
- Training of Dr. Paula Zook, a dermatology fellow, in the field of NF1
- National seminar: National Neurofibromatosis Foundation International Consortium on Gene Cloning and Gene Function of NF1 and NF2. "Megabase Deletions", Aspen, CO, June, 2003.
- International Invited Seminar: Neurocutaneous Syndromes in the Developmental Age, "Molecular Genetics of NF1 and NF12", Fondazione Mariani, Lucca, Italy, March 3-5, 2004.
- Guest lectures in graduate level classes at the University of Washington:
  - PATH516 Molecular Basis of Human Genetic Disease, "Large Scale Intra-Chromosomal Rearrangements & Contiguous Gene Disorders", April 9, 2004
  - PATH516 Molecular Basis of Human Genetic Disease, "Mechanisms of large scale intra-chromosomal rearrangements", April 12, 2004.
  - PATH516 Molecular Basis of Human Genetic Disease, "Trinucleotide repeat disorders and Genetic Instability – Fragile X", April 16, 2004.
  - PATH516 Molecular Basis of Human Genetic Disease, "Genetic Testing", May 7, 2004.
  - GENOME 531 Genetics of Human Disease, "Neurofibromatosis", May, 2004

Conclusions

We have developed rapid and sensitive assays for the detection and mapping of *NF1* microdeletions. These assays are important for identification of cohorts of NF1 subjects with microdeletions for genotype/phenotype studies. These studies should determine if microdeletion subjects would benefit from increased surveillance for neoplasia or other complications. We have developed a database for NF1 patients that will facilitate genotype/phenotype studies and transfer of data to the National Neurofibromatosis Foundation International Database upon completion of the study. These accomplishments will provide a strong foundation for the remaining years of our research study.

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2. Dorschner, M.O., Sybert, V.P., Weaver, M., Pletcher, B.A. and Stephens, K. (2000) NF1 microdeletion breakpoints are clustered at flanking repetitive sequences. *Hum Mol Genet,* **9,** 35-46.
3. Lopez-Correa, C., Dorschner, M., Brems, H., Lazaro, C., Clementi, M., Upadhyaya, M., Dooijes, D., Moog, U., Kehrer-Sawatzki, H., Rutkowski, J.L. *et al.* (2001) Recombination hotspot in NF1 microdeletion patients. *Hum Mol Genet,* **10,** 1387-1392.
4. Ruiz-Ponte, C., Loidi, L., Vega, A., Carracedo, A. and Barros, F. (2000) Rapid real-time fluorescent PCR gene dosage test for the diagnosis of DNA duplications and deletions. *Clinical Chemistry.,* **46,** 1574-82.

Appendices

Appendix A. Data Sheet for NF1 Subjects

Appendix B. Manuscript: Stephens K. Neurofibromatosis. In Molecular Pathology in Clinical Practice. D.G.B. Leonard, Ed. New York: Springer –Verlag, in press.

Appendix C. Abstract: Bingbing Wang, Shinny Vong, and Karen Stephens. Identification and Mapping of NF1 Contiguous Gene Deletions Using Real-Time Competitive PCR. The NNFF International Consortium for the Molecular and Cell Biology of NF1 and NF2, Aspen, Colorado, May, 2004.

APPENDIX A

Name: ______________________________ Study No. ______

Address: ______________________________

Telephone Number: ________________ e-mail: ________________

Perforations --

DATA SHEET FOR NF1 SUBJECTS

Study Number: ____________ Date: ____________
Age: ____________ BD ____________ Sex: M F

<u>Age/sex Matched Pair</u>
Study # Deletion subject ________
Study # Nondel. Subject ________

Family history, may include drawing of pedigree.

History:

Age at diagnosis or 1st memory of disease: ______________________

Age of onset first neurofibroma: __________

OR qualitative description ________________ _____prepubertal ____postpubertal

Age at puberty: menarche (for females) ______________

Pregnancy history (females only) ______________________

Surgeries: type ____________ date ________ outcome ____________
type ____________ date ________ outcome ____________
type ____________ date ________ outcome ____________

Malignancies:

type ____________ date ______ treatment ____________ outcome ____________
type ____________ date ______ treatment ____________ outcome ____________

Schooling: Highest grade achieved: __________
Need for assistance: yes no Descriptive:
Special education: yes no Descriptive:

Mental retardation: yes no IQ scores: ______________________

Employment history:

Medications:

Psychiatric diagnoses: Type ____________ Date _____ Outcome ____________

Cardiac lesions: yes no Treatment ______________________ age ______
Hypertension: yes no Treatment ______________________ age ______
Renal involvement: yes no Treatment ______________________ age ______
Pulmonary disease: yes no Treatment ______________________ age ______
Pheochromocytoma: yes no Treatment ______________________ age ______

Eye exam:
Lisch nodules: yes no Age of appearance _____
Optic glioma: yes no Age of occurrence_____Treatment_____Outcome_____

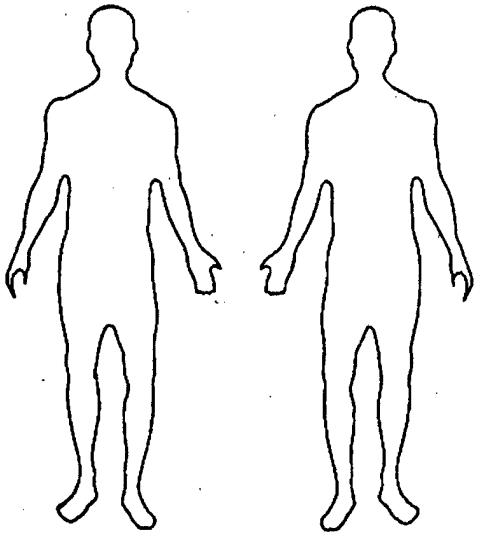
MRI:

Date of examination_____ UBOs: yes no Hamartomas: yes no Other:_____

Radiologic studies:

Abdominal Ultrasound:

Physical examination:



Café au Lait spots : yes no <15 >15
Giant spots: yes no location: _____
Congenital nevoid changes: yes no location
Axillary freckling: yes no
Inguinal freckling: yes no DISTRIBUTION :
Generalized hyperpigmentation: yes no NEUROFIBROMAS
General Impression of neurofibromas:
Neurofibromas: yes no

|-------|----------|----------|----------|
0 10 50 100 500

Atypical distribution, explain: _____
_____
Locations: generalized regional
Lesions > 4 cm: _____#
Neurofibroma count: Left forearm: ____#typical _____#pseudoatrophic blue macules
height:_____weight: _____ BSA(calc): _____
Neurofibroma score: Total # neurofibromas forearm/BSA = _____

Pseudoatrophic blue macules: yes no <10 >10<50 >50<100 >100
locations: generalized regional
Giant Plexiform neurofibromas (>4cm):_____#
Location(s):_____
Other tumor (explain):

Facies; normal abnormal
Ptosis short neck low hairline small jaw telecanthus
Other: _____
Scoliosis: yes no severity treatment description

Miscellaneous information:

APPENDIX B

Chapter 20.

Neurofibromatosis

Karen Stephens, PhD

Research Professor

Departments of Medicine (Medical Genetics) and of Laboratory Medicine

University of Washington

Co-Director, Genetics Laboratory, University of Washington Medical Center

Neurofibromatosis 1 and neurofibromatosis 2 are two distinct genetic disorders that predispose to the development of tumors primarily of the nervous system. A comparision of some features of these disorders is given in Table 20-1.

NEUROFIBROMATOSIS (NF1)

MOLECULAR BASIS OF DISEASE

NF1 is an autosomal dominant progressive disorder with high penetrance but extremely variable expressivity (reviewed in Ref.[1,2] and http://www.geneclinics.org/). The cardinal features are café au lait macules, intertrigenous freckling, Lisch nodules, and multiple neurofibromas. The National Institutes of Health diagnostic criteria for a diagnosis of NF1 established in 1987 are still appropriate and are widely used by clinicians and research investigators. Neurofibromas are benign nerve sheath tumors that arise on peripheral nerves. Dermal neurofibromas develop in virtually all cases of NF1, appear in late childhood, grow slowly, increase in number with age, and are at low risk for transformation to malignant peripheral nerve sheath tumors (MPNST; previously termed neurofibrosarcoma). However, diffuse plexiform neurofibromas and deep nodular plexiform neurofibromas can give rise to MPNST. Individuals affected with NF1 have a 10% lifetime risk for MPNST. Other neoplasms epidemiologically-associated with NF1 include medulloblastoma, pheochromocytoma, astrocytoma, adenocarcinoma of the ampulla of Vater. Primarily children affected with NF1 are at increased risk for optic pathway gliomas and brainstem gliomas, rhabdomyosarcomas, and malignant myeloid leukemias. NF1 patients are also at increased risk for a second malignancy, some of which may be treatment-related.

NF1 is caused by haploinsufficiency for the tumor suppressor neurofibromin, the protein product of the *NF1* gene (Table 20-1). Most constitutional mutations are private, predict the truncation of neurofibromin, and occur throughout the gene, although some exons are more mutation-rich than others

(see below). Less frequent are missense mutations and large *NF1* deletions that involve contiguous genes. About one-half of cases are familial (inherited from an affected parent) and one-half are sporadic resulting from a *de novo NF1* mutation. Neurofibromin normally functions as a negative regulator of the ras oncogene by stimulating the conversion of active GTP-bound Ras to the inactive GDP-bound form by hydrolyzing GTP. Biochemical, cell culture, and genetic studies in both NF1 patients and mouse models are consistent with a model whereby a somatic mutation inactivates the remaining functional *NF1* gene in a progenitor Schwann cell as an early, probably initiating, event in the development of neurofibromas (reviewed in [3]). Biallelic inactivation of *NF1* also occurs in progenitor cells of NF1-associated tumors other than those of the nerve sheath. Homozygous inactivation of neurofibromin leads to increased activated ras, resulting in aberrant mitogenic signaling, and consequent loss of growth control.

CLINICAL UTILITY OF TESTING

A diagnosis to NF1 can nearly always be made based on clinical findings, particularly after 8 years of age. Clinical DNA-based testing is available (http://www.geneclinics.org/). Testing is not typically used for diagnostic purposes, but can be useful for confirming a clinical diagnosis, reproductive counseling, and prenatal diagnosis. Blanket recommendations for diagnostic testing for NF1 cannot be made because the sensitivity of making a clinical diagnosis is very high and the sensitivity of molecular testing is not 100% (see below). Furthermore, benefits of diagnostic testing are subjective and may differ from family to family. Prenatal diagnosis of amniocytes or chorionic villus tissue is available only in cases where the pathogenic germline mutation has been identified previously in an affected parent.

The primary genetic counseling issue related to molecular testing of NF1 is the inability to predict the severity or course of the disorder in a patient or fetus. Even among family members who carry the same *NF1* mutation, there can be considerable variation in clinical manifestations and complications. For the majority of cases, there is no correlation between genotype and phenotype. For the ~5-10% of cases that are hemizygous for *NF1* due to a submicroscopic deletion (most commonly 1.5 Mb) there is a predisposition to an early age of onset and excessive numbers of dermal neurofibromas (Ref.[4] and

references therein). *NF1* testing may be useful to confirm a diagnosis in a patient with equivocal findings, such as child who has a few café au lait macules and carries a presumptive diagnosis of NF1. In this instance it is important to realize that the sensitivity and specificity of testing patients that do not fulfill the NIH diagnostic criteria for a diagnosis of *NF1* is unknown. For unaffected parents of a child with sporadic NF1, recurrence risk is a concern. Because germline mosaicism has been reported in an asymptomatic parent of a child with sporadic NF1[5], there is a small but unknown increased risk of recurrence even if the child's pathogenic mutation is not detected in the genomic DNA of parental lymphoblasts. Sporadic NF1 cases with post-zygotic mutations resulting in somatic mosaicism have been reported, but the frequency is unknown. Mosaic individuals, who carry the *NF1* mutation in only a fraction of their cells, may have mutation-negative test results due low signal to noise ratio, i.e., low level of mutant allele in a background of two normal *NF1* alleles. Genetic counseling regarding the clinical and reproductive implications of NF1 mosaicism is recommended.[6]

AVAILABLE ASSAYS

NF1 gene mutation is the only known cause of the disorder. Efficient detection of *NF1* gene mutations requires a multipronged approach due to the large number of exons and size of the gene (Table 20-1), variation in type and distribution of mutations, and large fraction of private mutations. About 70-80% of mutations result in a premature translation termination codon with nonsense and splicing defects being the most common.[7] These can be detected by the protein truncation test (PTT) (see Chapter 2), which identifies truncated neurofibromin polypeptides synthesized by *in vitro* translation of multiple overlapping segments of the *NF1* cDNA isolated from primary lymphoblasts or Epstein-Barr virus (EBV)-transformed lymphoblasts. A detection rate of about 80% can be attained with an optimized PTT protocol (see below). The vast majority of these mutations are private to each individual/family, although there are recurrent mutations that may account for, at most, a few percent of cases (Figure 20-1). About 10% of *NF1* mutations are missense or in-frame insertions/deletions of a few nucleotides,[7,8] some of which show clustering (Figure 20-1). Direct sequencing of cDNA or scanning of *NF1* exons and splice

junctions in genomic DNA by denaturing high performance liquid chromatography (DHPLC), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP), and/or heteroduplex analysis (HA) (see Chapter 2) have been used to detect missense and other subtle mutations in primary lymphoblasts. DHPLC detected both truncating, missense, and small in-frame deletions/insertions with a detection rate of 97% in a retrospective study[9] and of 72% in a prospective study.[10] For mutation scanning testing protocols, the detection rate will be laboratory-specific due to the degree of optimization of the specific technique; a survey of testing laboratories is recommended. DHLPC has the advantages of using genomic DNA and high throughput capability compared to the cDNA/gel-based PTT. However, a recently reported high-throughput PTT may be available for clinical testing in the future.[11] Fluorescent in situ hybridization (FISH; see Chapter 2) with *NF1* probes is used to detect the estimated 5-10% of cases due to submicroscopic *NF1* microdeletion[12] (Figure 20-1), although a recently reported *NF1* deletion junction-specific PCR assay may be clinically applicable in the near future.[13] Routine cytogenetic analysis is used to detect rare cases due to translocation or chromosomal rearrangement. Linkage analysis is an indirect test that tracks the inheritance of a disease allele in members of a family. Linkage testing is clinically available for at-risk individuals with multiple family members carrying a definitive diagnosis of NF1. The availability of *NF1* intragenic markers increases the informativeness of this methodology. This may be the quickest, most economical NF1 test for individuals of families that fulfill the testing criteria.

INTERPETATION OF TEST RESULTS

The detection of a truncated neurofibromin polypeptide by PTT is the most sensitive method (~80%), but can result in false positives.[7] High specificity requires identifying the underlying mutation at the genomic DNA and cDNA levels since false positives can arise during handling of the peripheral blood sample (see below). The interpretation of missense and subtle in-frame alterations as pathogenic mutations versus neutral polymorphisms is complicated by the lack of a functional assay for neurofibromin. Apparent recurrence of a putative mutation based on prior literature reports must be

interpreted with caution, as the majority of *NF1* mutational analyses did not sequence the entire gene. No comprehensive *NF1* mutation database is available, however some mutations have been submitted to the Human Gene Mutation Database (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html) and the National Neurofibromatosis Foundation International *NF1* Genetic Mutation Analysis Consortium Web Site (http://www.nf.org/nf1gene/nf1gene.home.html). Although most likely rare, families with different independent *NF1* inactivating mutations in affected individuals have been reported,[14] presumably a reflection of the high mutation rate of the gene (~10^5/gamete/generation). The interpretation of FISH with *NF1* probes can be complicated by mosaicism for an *NF1* microdeletion.[15] Furthermore, until the recent report of an apparent tandem duplication of the *NF1* gene[16] is confirmed or disproven, the interpretation of negative FISH-based test results should be made with caution. The frequency of mosaicism for an *NF1* mutation is not known, however this is likely the underlying mechanism for patients with segmental or localized signs of the disorder.[2]

LABORATORY ISSUES

Optimal detection of mutations that predict a truncated neurofibromin polypeptide occurs when the nonsense-mediated decay pathway is at least partially inhibited, thereby increasing the ratio of mutant transcripts with a premature termination codon to normal transcripts. A protein synthesis inhibitor, such as puromycin, in the culture medium is effective for EBV-transformed lymphoblasts or phytohemaglutinin-stimulated primary lymphocytes.[7, 17] Furthermore, blood handling and shipping protocols must be used to reduce false positives in PTT resulting from environmental effects such as cold shock[18] or delay in mRNA isolation.[7, 17] There is no approved program of interlaboratory comparison for NF1 testing; performance assessment must be conducted by participation in ungraded proficiency survey programs, split sample analysis with other laboratories, or other suitable and documented means. There are no commercially available NF1 testing kits, probes, or controls. Intronic primers for amplification of individual *NF1* exons that apparently do not co-amplify the *NF1* pseudogene fragments located at

multiple chromosomal sites have been published.[19] Some issues related to NF1 testing have been reviewed recently.[20]

NEUROFIBROMATOSIS 2 (NF2)

MOLECULAR BASIS OF DISEASE

The development of bilateral vestibular schwanomas is a hallmark of NF2. Other commonly-associated tumors include schwannomas of other central, spinal, and peripheral nerves and meningiomas (reviewed in Ref[2, 21, 22] and http://www.geneclinics.org/). Although these tumors are not malignant but their location, along with the propensity to develop multiple tumors, make this a life-threatening disorder. Most patients become completely deaf and can have poor balance, vision and weakness. The mean age of onset is 18-24 years and the mean age of death is 36 years. Ependymomas and astrocytomas occur less frequently and are usually indolent CNS tumors. Patients affected with NF2 are at minimal increased risk for malignancy. Juvenile posterior subcapsular cataract is a common nontumor manifestation. The disorder may be under-diagnosed in children who present with ocular and skin manifestations. Early diagnosis improves management, which is primarily surgical and radiological. Modifications to the criteria for a diagnosis of NF2, initially established by the 1987/1991 National Institute of Health Consensus Conference, have been proposed to increase the specificity.[23]

NF2 is caused by haploinsufficiency for the tumor suppressor merlin (or schwannomin), the protein product of the *NF2* gene (Table 20-1). About one-half of patients are the first case of NF2 in the family. These sporadic cases result from *de novo* mutation of the *NF2* gene, a significant fraction of which are postzyogotic mutations that result in mosaicism (see below). The majority of constitutional mutations are private, predict the truncation of merlin, and occur throughout the gene (see below). Vestibular schwannomas develop from a progenitor Schwann cell that does not express merlin due to a somatic mutation that inactivated the single remaining *NF2* gene. Merlin is a protein of the cytoskeleton (reviewed in Ref.[3, 22]). Merline associates with transmembrane proteins important in adhesion, proteins

involved in signaling pathways, and cytoskeletal proteins. Merlin deficiency alters cell adhesion, motility, and spreading. The normal function of merlin remains to be determined but likely involves control of adhesion, proliferation, and the Rho signaling pathway.

CLINICAL UTILITY OF TESTING

DNA-based clinical testing for NF2 is available (http://www.geneclinics.org/) and used primarily for presymptomatic testing of at-risk individuals, typically young children of an affected parent. An early diagnosis of NF2 may improve outcome and at-risk children who did not inherit the *NF2* mutation can be spared costly brain imaging and audiologic screening. Genetic counseling is recommended prior to testing presymptomatic at-risk children. Testing is also useful to confirm a clinical diagnosis, which may be most helpful in sporadic cases of NF2, particularly children who present with ocular or skin manifestations or adults with equivocal findings or mild disease. Some of these cases may be mosaic for an *NF2* mutation, as the frequency of mosaicism is estimated at 16.7 – 24.8% of sporadic cases.[24] Genetic counseling regarding the clinical and reproductive implications of NF2 mosaicism is recommended.[6] Testing is also useful for reproductive counseling and prenatal diagnosis. Prenatal diagnosis of NF2 using amniocytes or chorionic villus tissue is available only in cases where the pathogenic germline mutation has been identified previously in an affected parent. Preimplantation genetic diagnosis of NF2 has been reported.[25]

The primary genetic counseling issues regard predicting the course of the disorder and recurrence risks. There are genotype/phenotype correlations, but they cannot predict the age of onset or the course of disease for an individual patient. Typically, constitutional frameshift and nonsense mutations are associated with severe NF2, defined by earlier age at onset and higher frequency and mean number of tumors.[26, 27] Constitutional missense and small in-frame mutations are thought to be associated with mild disease[27] and mutations in splice donor and acceptor sites result in variable clinical outcomes.[28] Recurrence risks for asymptomatic parents of an affected child are unknown, but are somewhat greater than the population risk due to the possibility of germline mosaicism in a parent.[29] For mosaic patients,

the risk of transmitting NF2 to offspring is ≤50%, depending upon the proportion of gametes that carry the *NF2* mutation.[6] Offspring that do inherit the mutation however, will have a constitutional *NF2* mutation and may have more severe disease than their mosaic parent.

AVAILABLE ASSAYS

NF2 gene mutation is the only known cause of the disorder. About 66% of mutations are nonsense or frameshifts that predict the premature truncation of merlin.[30, 31] About 10% of mutations are missense (although most have yet to be tested in functional assays) and the remaining are at splice donor/acceptor sites or due to submicroscopic rearrangements. A multipronged approach to testing is optimal due to the high frequency of private constitutional mutations, the high frequency of post-zygotic mutations, and the distribution of mutations throughout the gene. Direct sequencing of *NF2* in genomic DNA is clinically available and has a detection rate of about 60% in familial NF2 cases. The exon scanning techniques of SSCP, TGGE, and HA (see Chapter 2), followed by direct sequencing to identify the underlying *NF2* mutation, detected 54-81% of mutations in familial NF2 cases.[26, 27, 32, 33] The detection rate of either sequencing or exon scanning methods is significantly lower (34-51%) in sporadic cases due in part to the high frequency of post-zygotic *NF2* mutations, which can be masked by the presence of normal alleles.[27-29, 32] For mutation scanning tests, the detection rate will be laboratory-specific due to the varying degrees of optimization of the technique; a survey of testing laboratories is recommended.

Because schwannomas are clonal tumors with minimal cellular admixture, *NF2* mutations can be detected at high frequency in tumor tissue. Testing of tumor tissue is available clinically (http://www.geneclinics.org/) and is most useful in cases where a mutation is not detected in primary lymphoblasts, clinical manifestations are suggestive of somatic mosaicism, or constitutional tissue is unavailable. Different exon scanning methods have detected from 60-75% of mutations, primarily in vestibular schwannomas.[24, 34] Mutations are likely to be germline (rather than somatic) if the identical mutation is detected in two or more pathologically or anatomically distinct tumors or if a tumor shows

loss of heterozygosity for *NF2* intragenic/flanking loci, while constitutional tissue is heterozygous at these loci. Mutational analysis of tumors is expected to have the greatest sensitivity for *NF2* somatic mosaic mutations[24] and sporadic cases with negative results from mutation scanning tests.[35] Routine cytogenetic analysis and/or FISH with *NF2* probes will identify chromosomal rearrangements. One study detected submicroscopic rearrangements in 70% of patients in whom mutation scanning test results were negative.[36] Rearrangements (primarily deletions) were intragenic or involved all, or nearly all, of the *NF2* gene. It is not known if submicroscopic whole gene deletions extend into flanking contiguous genes that may contribute to the phenotype as in NF1 microdeletions (see above). Linkage analysis is clinically available for at-risk individuals and fetuses with multiple family members carrying a definitive diagnosis of NF2 who are willing to participate in the testing process. The availability of highly informative *NF2* intragenic markers increases the specificity of this methodology. For certain families, linkage analysis will be the most cost effective, fastest, and definitive test. It can sometimes be an option when mutation scanning test results are negative.

INTERPRETATION OF TEST RESULTS

Interpretation of the results of exon scanning tests requires identifying the underlying *NF2* mutation at the genomic DNA and/or cDNA levels to avoid false positives. Functional assays for merlin have been developed that can provide insight into the interpretation of missense and subtle in-frame alterations as pathogenic mutations versus neutral polymorphisms.[37, 38] No comprehensive *NF2* mutation database is available, however some mutations have been submitted to the Human Gene Mutation Database (http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html) and to an *NF2* Mutation Information site (http://neuro-trials1.mgh.harvard.edu/nf2/). Somatic mosaicism or *NF2* gene deletions must be considered in patients that have a negative mutation scanning test result, regardless of the severity of their manifestations. It is optimal for NF2 linkage testing not to include the first affected member in a family, since this individual may be mosaic for an NF2 mutation, which can lead to misinterpretation of test results.[39]

LABORATORY ISSUES

There is no approved program of interlaboratory comparison for NF2 testing; performance assessment must be conducted by participation in ungraded proficiency survey programs, split sample analysis with other laboratories, or other suitable and documented means. There are no commercially available NF2 testing kits, probes, or controls. Direct gene sequencing is not the optimal test to detect mosaicism for an NF2 mutation in lymphoblasts, as reliable detection of a low level point mutation will be difficult. Exon scanning techniques that are semi-quantitative, such as TGGE, will detect relative intensity differences between heteroduplexes and homoduplexes that suggest possible mosaicism.[32] Depending upon age, fixation, and storage conditions, some tumors may not yield nucleic acid of sufficient quality for mutational analysis.

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Table 20-1. Comparison of NF1 and NF2 disorders.

Feature	Neurofibromatosis 1 (NF1)	Neurofibromatosis 2 (NF2)
Alternate name	peripheral neurofibromatosis; von Recklinghausen neurofibromatosis	central neurofibromatosis; bilateral acoustic neuroma
OMIM accession number[1]	162200	101000
Mode of inheritance	autosomal dominant	autosomal dominant
Frequency of disorder	1/3000–1/4000 worldwide	1/33,000–1/40,000 worldwide
Gene symbol	*NF1*	*NF2*
Chromosomal location	17q11.2	22q12.2
Gene size; transcript size	~350 kb; ~11-13 kb[2]	~110 kb; 2 kb[2]
Genbank accession no. (gene;cDNA)[3]	NT_010799; NM_000267	Y18000; NM_000268
Number of exons	60	17
Tissue expression pattern	Widely expressed	Widely expressed
Protein product (size (kD); # residues)	Neurofibromin (>220 kD; 2818)	merlin, also known as schwannomin (65 kD; 595)
Normal functions of protein	tumor suppressor; negative regulator of ras oncogene	tumor suppressor; associates with proteins of the cytoskeleton
Common associated tumors	Neurofibroma, MPNST, optic pathway and brainstem gliomas	Bilateral vestibular schwannomas, schwannomas of other central and peripheral nerves, meningiomas
Animal models	mouse, *Drosophila*	mouse, *Drosophila*

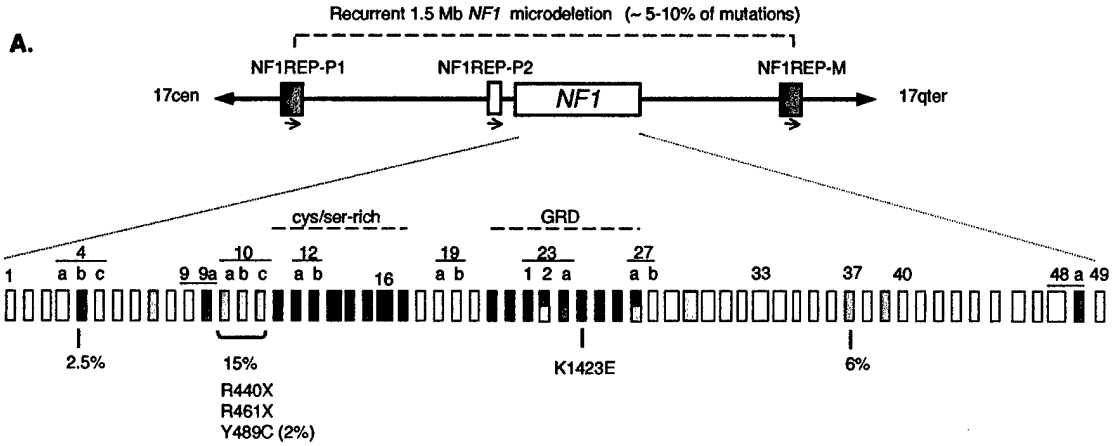
[1]Online Mendelian Inheritance in Man (http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=OMIM).

[2]Alternative splicing produces transcripts of varying lengths.

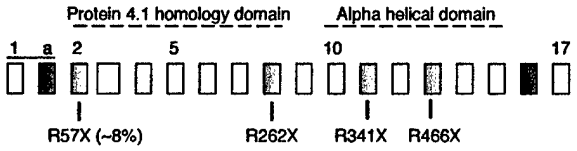
[3]See Gene Lynx Human (http://www.genelynx.org/) for a compilation of, and hyperlinks to, gene, protein structure, and genomic resources.

FIGURE LEGENDS

Figure 20-1. Genomic structure and mutations in *NF1* and *NF2* genes. A). At the top is a schematic of the *NF1* gene region at chromosome segment 17q11.2. The 350 kb NF1 gene is flanked by 51 kb paralogs NF1REP-P1 (previously termed NF1REP-P) and NF1REP-M, which are in direct orientation (orange boxes). Homologous recombination between these NF1REP elements results in the recurrent 1.5 Mb *NF1* microdeletion.[4] NF1REP-P2 is a partial element with a limited role in mediating *NF1* microdeletions. The 60 exons of the *NF1* are represented by boxes (not to scale) and exon numbering is sequential except as indicated. The GRD (exons 21-27a) and a cystein/serine-rich domain with 3 cysteine pairs suggestive of ATP binding (exons 11-17) are indicated. Grey boxes indicated alternatively spliced exons that vary in abundance in different tissues. Mutations have been identified in virtually every exon. Exons where mutations are apparently in greater abundance than expected are indicated (green boxes).[7, 8, 10] In one study, exons 7, 10a,b,c, 23-2, 27a, 29, 37 and accounted for 30% of mutations, 15 of which were in exons 10a,b,c, which harbor 3 recurrent mutations, including Y489C, which alone may account for ~2% of mutations.[7] Blue boxes indicate exons that had clusters of missense and/or single codon deletion mutations.[8] Some of the recurrent, although still infrequent, mutations are given below the exons. B) The 17 exons of the *NF2* gene are represented by boxes (not to scale) and exon numbering is sequential. The Protein 4.1-homology domain thought to mediate binding to cell surface glycoproteins (exons 2-8), the α-helical domain (exons 10-15), and the unique C-terminus (exons 16-17) are indicated. Grey boxes indicated alternatively spliced exons; the inclusion of exon 16 creates a alternate termination codon resulting in a slightly truncated protein. Mutations have been identified involving each exon except for 16. Exons containing recurrent mutations indicated (green boxes). In several studies, R57X occurred in 8% of familial constitutional mutations. Other recurrent nonsense codons are shown.


A.
Recurrent 1.5 Mb NF1 microdeletion (~ 5-10% of mutations)
NF1REP-P1
NF1REP-P2
NF1REP-M
17cen
NF1
17qter
cys/ser-rich
GRD
1
4
a b c
9 9a
10
a b c
12
a b
16
19
a b
23
1 2 a
27
a b
33
37
40
48 a 49
2.5%
15%
R440X
R461X
Y489C (2%)
K1423E
6%

B.


Protein 4.1 homology domain
Alpha helical domain
1 a
2
5
10
17
R57X (~8%)
R262X
R341X
R466X

APPENDIX C

The NNFF International Consortium for the Molecular and Cell Biology of NF1 and NF2

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Identification and Mapping of *NF1* Contiguous Gene Deletions Using Real-Time Competitive PCR

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Neurofibromatosis 1 (NF1) predisposes to benign neurofibromas and malignant peripheral nerve sheath tumors (MPNST) due, at least in part, to loss of neurofibromin tumor suppressor function. About 5% of NF1 patients carry an allele with an *NF1* contiguous gene deletion that commonly spans 1.5 Mb of DNA including the entire *NF1* gene. This subset of patients are predisposed to developing early onset/increased numbers of cutaneous neurofibromas and MPNST, presumably due to the co-deletion of *NF1* and an unknown linked modifier gene. To facilitate ascertaining new deletion patients and mapping the extent of their deletion, we developed a novel strategy based on real-time competitive PCR. A segment of the target gene, such as *NF1*, is co-amplified with an exogenous competitor of identical sequence, except for several internal nucleotides that are altered to differentiate the melting curves of the two amplicons. Double stranded amplicons are detected by SyberGreen and the ratio obtained between the areas under the *NF1* and competitor melting curves determines their relative concentrations, thereby specifying the gene copy number of the target locus. To ensure the addition of a precise quantity of genomic DNA from different patient samples to the competitive PCR assay, the concentration was determined at a reference gene (tissue plasminogen activator at chromosome 8p12) by real-time PCR using the hybridization probe system. In this study, genomic DNAs from 24 NF1 patients previously determined to carry an *NF1* microdeletion and 22 control individuals were analyzed. Primers were selected and competitors were constructed for assaying four different loci residing within the common deletion region of *NF1*: *AH1* 144 kb upstream, *NF1* exon 5 and intron 31, and *WI-9521*, which is 157 kb downstream of *NF1*. An assay was also developed for *D17S250*, a locus at chromosome 17q12 that is not involved in *NF1* microdeletion, to serve as an external disomic control. As expected, there were no differences found in the relative ratio of *D17S250* to competitor for *NF1* microdeletion patients and control individuals. However, the mean relative ratio between target locus and competitor for *NF1* microdeletion patients was 0.34, 0.52, 0.33, and 0.44 for AH1, *NF1* exon 5, *NF1* intron 31, and *WI-9521*, respectively. In contarst, the mean relative ratio between target locus and competitor for control individuals at these same loci were 0.97, 1.21, 1.22, and 1.12, respectively. These data validate real-time competitive PCR as a rapid, direct, and precise method for identifying *NF1* microdeletion patients and mapping the extent of their deletions. We will present the results of ongoing experiments that apply the 5 gene dosage assays described above in a collection of several hundred NF1 patients.