

AD _____

Award Number: DAMD17-03-1-0203

TITLE: Clinical and Molecular Consequences of NF1 Microdeletion

PRINCIPAL INVESTIGATOR: Karen Stephens, Ph.D.

CONTRACTING ORGANIZATION: University of Washington
Seattle, WA 98195-7720

REPORT DATE: May 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20050927 099

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE May 2005		3. REPORT TYPE AND DATES COVERED Annual (7 Apr 2004 - 6 Apr 2005)	
4. TITLE AND SUBTITLE Clinical and Molecular Consequences of NF1 Microdeletion				5. FUNDING NUMBERS DAMD17-03-1-0203	
6. AUTHOR(S) Karen Stephens, Ph.D.					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Washington Seattle, WA 98195-7720 E-Mail: millie@u.washington.edu				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES					
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited				12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 Words) 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 subjects with microdeletions because they are predisposed to large numbers of neurofibromas. We propose that deletion of NF1 gene and at least one of the 15 adjacent genes favors the development of skin neurofibromas, and probably other NF1-related tumors. We are 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 rapid and sensitive assays for the detection and mapping of NF1 microdeletions. Using these assays, we screened 242 patients and identified 56 carriers of the recurrent 1.4 Mb NF1 microdeletion and 16 carriers of novel deletions. Clinical evaluation of these patients, along with age/sex matched NF1 patients that are not NF1 deletion carriers, is in progress to delineate the clinical manifestations most prevalent in deletion patients. We have collected tumors from NF1 microdeletion carriers, which are now being examined for mutations and genomic instability. We began a new collaboration and generated preliminary data that identified an apparent new function of neurofibromin as a caspase target. Since this work is outside the scope of this grant, we have submitted a new application for investigating the role of caspase in NF1 disease and tumorigenesis. Therefore, our progress on genotype/phenotype correlations in carriers of NF1 microdeletions, along with our new work identifying neurofibromin as a caspase target, will provide clinically applicable information for patient diagnosis and management and basic knowledge about neurofibromin function in disease pathogenesis.					
14. SUBJECT TERMS Microdeletion, tumor initiation, modifying gene, genotype/phenotype, paralogs, duplication, neurofibromas				15. NUMBER OF PAGES 18	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited		

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	15
Reportable Outcomes.....	15
Conclusions.....	16
References.....	16
Appendices.....	18

Introduction

About 5-10% of neurofibromatosis type 1 (NF1) patients are heterozygous for a contiguous gene deletion that includes the entire 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 to examine the gene(s) responsible for the severe tumor phenotype of microdeletion patients. The specific aims of this research are 1) To determine the clinical spectrum, genotype/phenotype correlations associated with heterozygosity for an NF1 microdeletion. Genomic DNA of NF1 subjects will be examined by a multi-step screening protocol to identify germline microdeletion carriers, to map the extent of each deletion. We will correlate molecular data with the results of a comprehensive clinical evaluation of deletion and nondeletional NF1 control subjects. 2) To determine if cutaneous neurofibromas of germline NF1 microdeletion patients show evidence of genomic instability or homozygous NF1 microdeletion that may contribute to the early onset of neurofibromagenesis. Primary neurofibroma tissue from microdeletion patients will be analyzed to determine the presence and nature of 2nd hit mutations and whether these cells exhibit characteristics of genomic instability. 3) To screen candidate modifier genes in the NF1 microdeletion region for mutations in subjects with early onset cutaneous neurofibromas who are not carriers of an NF1 microdeletion. 4) To employ the newly developed FLASH technology to interrogate the NF1 microdeletion region and construct a physical map that will determine, the sequence of all of the genes, unique noncoding regions, and paralogs (including the putative NF1 duplicated gene) of the NF1 microdeletion region.

Body

The original STATEMENT OF WORK

Year 1, Months 1-8 Statement of Work:

- 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. This objective has been completed. We developed 2 databases using FileMaker Pro database software package. One contains confidential patient history information, while the second one employs only coded patient identifiers, clinical, and molecular data. Both databases comply with the new requirements of HIPAA regarding password and firewall protection and training of personnel.
- Design and test clinic evaluation forms for patient assessment. This objective has been completed. Forms for use in the clinic to record patient manifestations and history were approved by the Army IRB in February, 2003, and are now in routine use.
- Design STS primers for interrogation of fosmid library. Begin fosmid library screen. In conjunction with the University of Washington Genome Center, we decided to delay the fosmid library screen until years 3 and 4. This was based on recent data from Hildegard Kehrer-Sawatski and collaborators arguing against the present of a duplicated NF1 gene. We are currently evaluating all the sequences from the shotgun (Celera) cloning of the human genome, which we expect to provide evidence to help us decide if a duplication is possible. In addition, we are discussing the sequencing of selected parents of de novo NF1 microdeletion patients that may carry polymorphisms in the NF1REP elements that could predispose to recombination and subsequent deletion.

Years 1-2 Statement of Work:

- 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. Enrollment of patients was significantly delayed by the time required for the Army HSRRB to approve our application (submitted July 2003, approved February, 2004). In the roughly one year since approval, we have enrolled 38 new subjects into this study. In addition, we have received 35 DNAs from Dr. Victor Mautner, Hamburg, Germany to screen for NF1 microdeletions.
- Screen for NF1 microdeletion patients, map extent of deletions, develop new deletion junction assays as needed. This objective has been the major focus of year 2. Three types of assays were employed. They are described below:
 - 1) Assays to detect the recurrent 1.4 Mb deletions at PRS1 and PRS2 recombination hotspots. An estimated 70% of NF1 germline microdeletions occur by homologous recombination between NF1REP-P1 and NF1REP-M, 51 kb direct repeats that share 97.5% sequence identity (Figure 1; (1-3). We have previously shown that these deletions map to two distinct paralogous recombination hotspots within the NF1REP sequences, which are denoted PRS1 and PRS2 (paralogous recombination sequences) (2-4). We previously developed two deletion-specific PCR assays that each generate a 7kb product only if a deletion is present that was generated by recombination between either the two PRS1 or two PRS2 sites in NF1REP-P1 and NF1REP-M.
 - 2) Assays to detect microdeletions that did not occur at PRS1 or PRS2. These assays employed real-time competitive PCR to measure gene dosage. Assays were developed for four loci within the region, AH1, NF1 exon 5, NF1 exon 49, and WI-9521 (Figure 1). In addition a gene dosage assay was developed for a control locus D17S250, which is distal and never observed to be co-deleted along with NF1 (Figure 1).

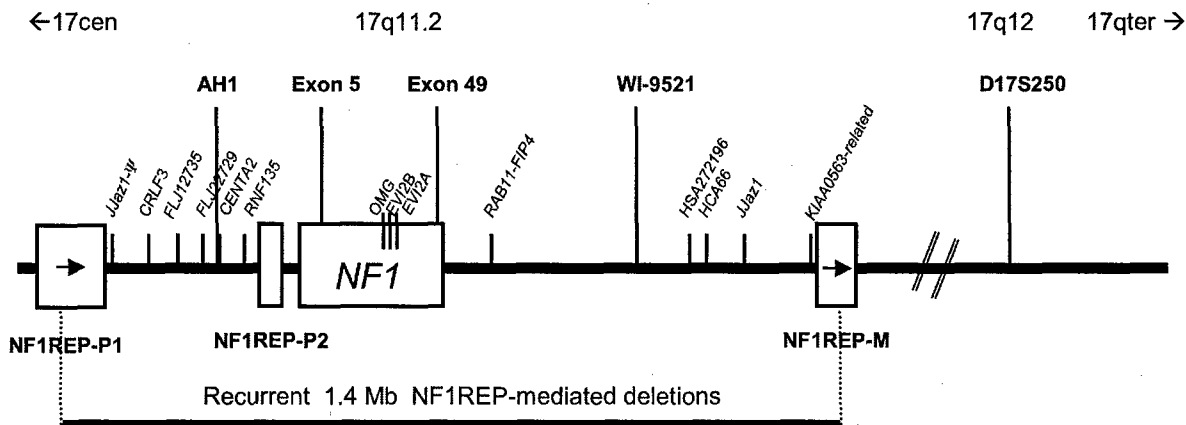


Figure 1. Schematic of the NF1 microdeletion region. The NF1REP low copy repeat elements in the NF1 region are shown, including NF1REP-P1 and -M which mediate the 1.4 Mb recurrent germline microdeletions. Genes in the region are shown above the line (*italics*). In bold are shown five loci for which we developed real-time competitive PCR assays to determine the gene dosage at these sequences.

The assays are adapted from a method by Riz-Ponte et al (5) in which a known copy number of a competitor is introduced directly in the PCR along with the target patient DNA. The competitor is a mutated PCR product that is synthesized and cloned. The competitor is amplified by the same primers as the target, but differs slightly in DNA composition such that it will melt at a different temperature. PCR of genomic and competitor DNAs are performed in a capillary in the LightCycler instrument, followed by a melting curve. If the same number of target and competitor molecules are present the result will be as shown in Figure 2.

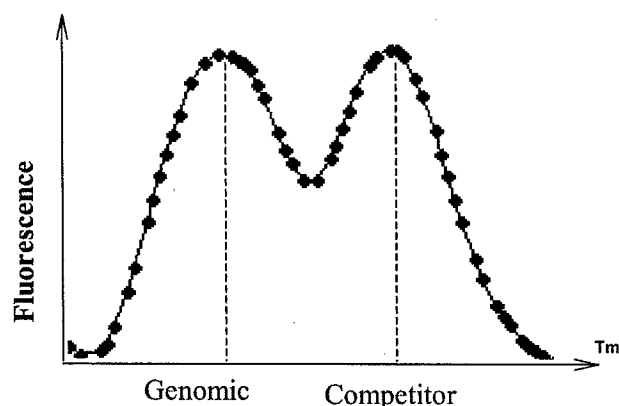


Figure 2. Idealized result of real-time competitive PCR melting curves where the number of targets is the same in genomic and competitor DNA samples. The ratio of the areas under the genomic amplicons peak to that under the competitor determines the dosage at the locus. If the genomic target is deleted, the area under the curve will be approximately one-half that of the area under the melting peak of the competitor.

Once the concentration of competitor is determined by a certain concentration of 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 PCR assays, we determined the exact concentration of the genomic DNA of each patient to be screened using real-time quantitative PCR at TPA (tissue plasminogen activator, chromosome 12). The TPA gene is amplified in each patient and compared to a standard curve using normal control DNA. This is used to calculate exactly what volume of patient DNA must be added to the competitive PCR assay (Figures 3 and 4).

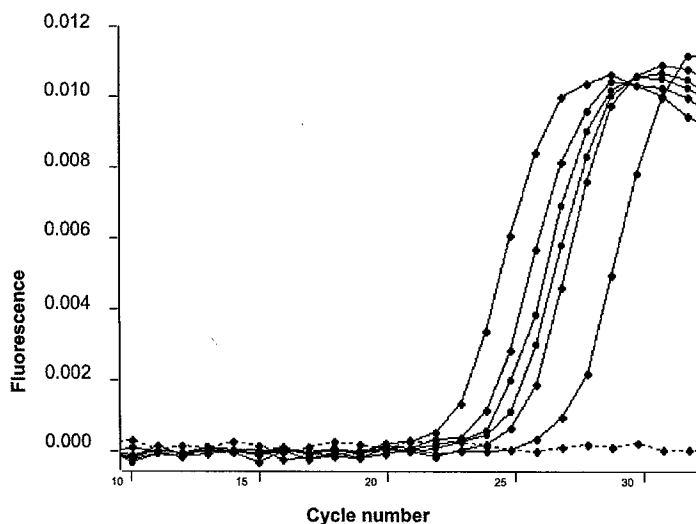


Figure 3. LightCycler real-time PCR at TPA locus showing standard curve. Shown is a dilution series of genomic DNA of a control individual. The reaction consists of unlabeled primers and uses fluorescence resonance energy transfer probe for detection of the product.

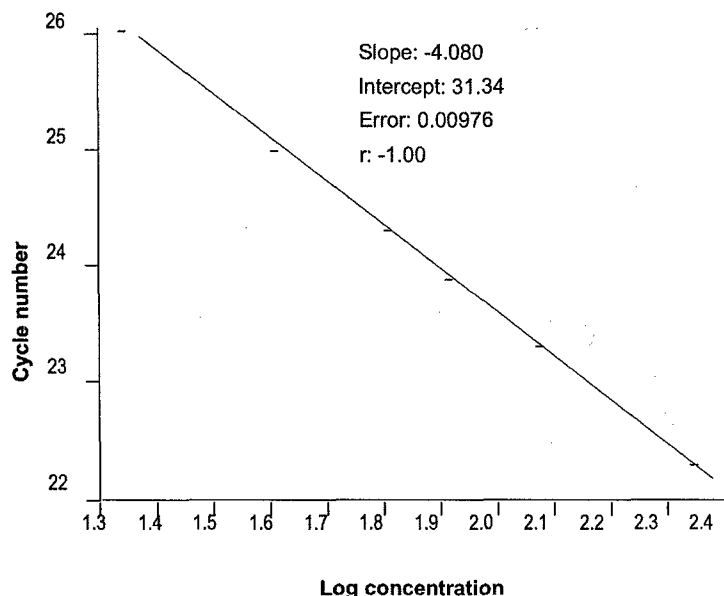


Figure 4. Standard curve calculated from the real-time data in Figure 3. Note the low error and high correlation coefficient.

Real-time competitive PCR assays were developed for AH1, NF1 exon 5, NF1 exon 49, WI-9521 in the NF1 region and for D17S250 as a control assay. Samples of assay results for each locus are shown in Figure 5.

To validate the real-time competitive PCR assays, we tested 24 bona fide NF1 microdeletion patients previously confirmed to carry the 1.4 Mb recurrent NF1 microdeletion and 22 unaffected control individuals. The results of the intra- and inter-assay variation are shown in Table 1. Importantly, the approximated 95% confidence limits (measured as ± 2 S.D.) for normal and deleted values do not overlap. Inter-assay variations still demonstrate distinct value ranges for normal and deleted (or disomy and monosomy). Note that values for D17S250 are the same for both cohorts of patients, as this locus lies distal to the deleted region. Although the assays could theoretically detect at least a fraction of mosaic deletions, we have insufficient patients mosaic for a microdeletion to test this possibility.

Table 1. Variation of real-time competitive PCR assays.

Intra-assay variations (Mean $\pm$ SD)					
	AH1	Exon 5	Exon 49	WI-9521	D17S250
Unaffected Control	0.97 $\pm$ 0.18	1.21 $\pm$ 0.13	1.03 $\pm$ 0.10	1.10 $\pm$ 0.15	1.11 $\pm$ 0.16
NF1 Deletion Pt.	0.33 $\pm$ 0.05	0.52 $\pm$ 0.13	0.51 $\pm$ 0.11	0.44 $\pm$ 0.13	1.16 $\pm$ 0.18
Inter-assay variations (Mean $\pm$ SD)					
	AH1	Exon 5	Exon 49	WI-9521	D17S250
Unaffected Control	0.95 $\pm$ 0.13	1.23 $\pm$ 0.26	0.97 $\pm$ 0.16	0.98 $\pm$ 0.17	1.07 $\pm$ 0.15
NF1 Deletion Pt.	0.32 $\pm$ 0.14	0.53 $\pm$ 0.14	0.48 $\pm$ 0.06	0.36 $\pm$ 0.10	1.12 $\pm$ 0.14

± 1 S.D.

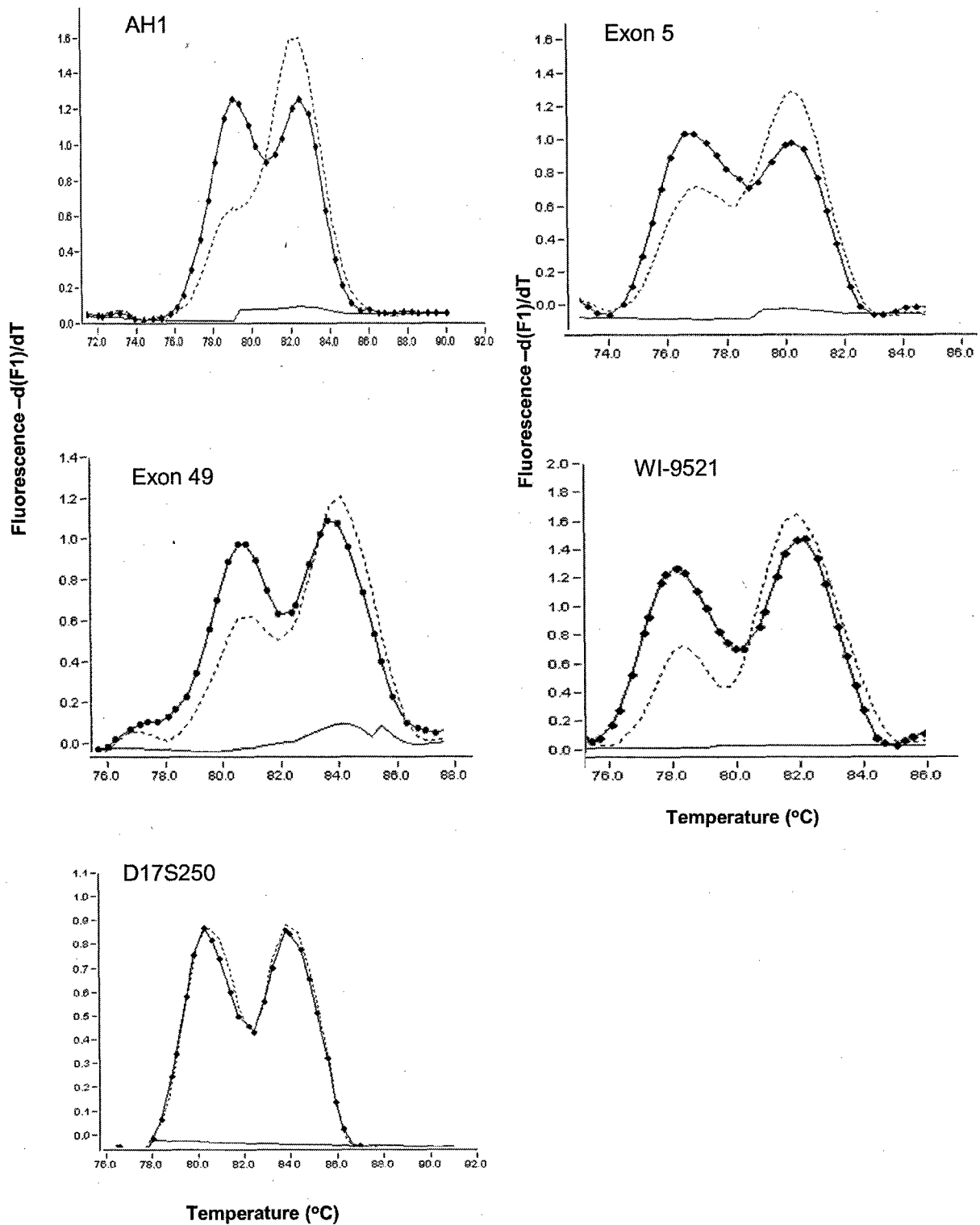


Figure 5. Melting curves of real-time competitive PCR assays for 5 loci. The genomic DNAs of known NF1 microdeletion subjects (---) and control subjects (---●---) were amplified at each locus and melting curves were generated, with amplicons detected by SYBR green fluorescent dye. Negative controls (____) are shown. D17S250 give the same results for microdeletion and control subjects, as this locus lies far outside of the deleted region (see Figure 1).

3) The third type of assays employed were fluorescent in situ hybridization (FISH) and polymorphic marker analysis to confirm selected real-time competitive PCR assay results and to assay for mosaicism.

Results of screening NF1 patients for microdeletions using the three types of assays.

Screen #1. To date we have screened 242 NF1 probands for the common 1.4 Mb NF1REP-mediated microdeletion using the PRS1 and PRS2 deletion-specific PCR assays. As detailed in Table 2 below, we have identified 65 new probands with the recurrent 1.4 Mb microdeletion.

Table 2. Newly identified NF1 patients carrying the 1.4 Mb recurrent microdeletion as determined by deletion-specific PCR assays.

	Total Screened	PRS1-mediated deletion	PRS2-mediated deletion	Total
All of our patients	207	3	6	9
Known deletion patients from Hamburg	35	0	21	56
Total	242	3	27	65

Screen #2. Among our patients at the University of Washington, we had 198 probands negative for NF1 deletions after the first screen, which identified 9 patients with the recurrent 1.4 Mb microdeletion. To identify deletions at novel locations, we screened these 198 probands with real-time competitive PCR assays at four loci spanning the NF1 region. As shown in Table 3, we have identified 16 probands with deletions. Eight had deletions spanning the NF1 gene, but the exact breakpoints of their deletions are yet to be determined. Six appear to be deleted for the 5' end of the NF1 gene and centromeric contiguous sequences, while one was deleted for the 3' end of the NF1 gene and telomeric contiguous sequences. The breakpoints and phenotypes of these patients will be important to determine, as they are novel deletions that may serve to narrow the critical region of the putative modifier gene that predisposes to tumorigenesis and malignancy in deletion patients. One patient was deleted for WI-9521, but it is unknown if this deletion involves the very 3' end of the NF1 gene.

Table 3. Newly identified NF1 patients carrying novel NF1 deletions as determined by real-time competitive PCR assays.

Marker in <i>NF1</i> microdeletion region				Total
AH1	NF11-Exon 5	NF1-Exon 49	WI-9521	
				8
				6
				1
				1
			Total	16 (8%)

Total number of patients screened = 198.



= Indicates one allele deleted for marker

Screen #3. We are currently employing FISH and polymorphic marker analysis to confirm the results of the competitive PCR assays and to assess the patients with novel deletions for mosaicism. These experiments are in progress.

Year 1-2 Progress continued:

- Ascertain NF1 subjects that show early onset cutaneous neurofibromas that do not carry microdeletions. To date we have identified 5 such patients, including one family that appears to have early onset cutaneous neurofibromas. Assessment of other patients is ongoing.
- Determine conditions for immunohistochemistry, test and choose optimal antibodies. These experiments are in progress by Dr. Paula Zook, a dermatology fellow who joined our research project last fall. She is obtaining antibodies and determining optimal conditions for use on neurofibromas from microdeletion patients. In addition, she is assessing antibodies for centrosomal proteins to determine whether tumors from NF1 patients (with or without microdeletions) exhibit a form of genomic instability characterized by abnormal numbers of centrosomes and abnormal multipolar mitoses.
- Write manuscript on novel microdeletions. We are currently writing a manuscript of the real-time competitive PCR assays and the results of our patient screens. In addition, the Co-investigator on this grant, Dr. Sybert, along with Dr. Lombillo (University of Washington) are writing a clinical manuscript of their work on mosaicism in cutaneous pigmentation, which in large part, is a result of evaluating patients for possible enrollment in this study.

Year 2 Statement of Work:

- 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.

Progress:

- Screen subjects with early onset cutaneous neurofibromas that are heterozygous at NF1 for somatic mosaicism for an NF1 microdeletion. The cells are ready and FISH experiments will be performed in year 3.
- Construct STS-content maps, sequence fosmids, and construct haplotypes. No progress.
- Obtain cutaneous neurofibromas from NF1 microdeletion adults. To date we have obtained neurofibromas from two microdeletion patients, including multiple neurofibromas from one of the patients. In addition, we have obtained MPNST from one of these patients. Samples are frozen in OCT.
- Perform immunohistochemistry and nucleic acid extraction of neurofibromas. This is in progress for the neurofibromas and MPNST we have collected to date.
- Assemble data on clinical spectrum of NF1 microdeletion patients; write manuscript. Data is being assembled. Age and sex matched NF1 subjects that do not have microdeletions are being evaluated at this time. These data will be used to determine the clinical spectrum and severity of microdeletion versus non-deletional NF1 subjects.

Year 3 Statement of Work:

- Screen JAZ1 gene for inactivating mutations in subjects with early onset cutaneous neurofibromas that are heterozygous at NF1.

- Continue to clinically evaluate age-matched NF1 patients without microdeletions and control patients for comparison to determine prognostic utility of a microdeletion.
- Continue to obtain cutaneous neurofibromas from NF1 microdeletion adults.
- Continue to 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 Statement of Work:

- 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.
- Write manuscripts

Progress on new and important research outside of the original Statement of Work

1. In collaboration with Drs. Melanie Kuechle and Michi Shinohara (University of Washington) we have had the opportunity to extend and expand upon some earlier observations.

Caspases are a family of highly-conserved cysteinyl aspartate specific proteases of at least 14 enzymes that are intimately identified with apoptosis. Caspases are expressed widely in mammalian tissues as inactive zymogens which are activated by limited proteolysis to form an active tetramer upon receipt of appropriate signals. A significant number of caspase targets that have been identified are involved in signal transduction. The GAP-related domain homologue of neurofibromin, p120 RAS-GTPase activating protein (p120-GAP), offers an example of the level of control that caspase cleavage can have on protein function. Low caspase activity generates an N-terminal fragment of p120-GAP that is antiapoptotic by activating the PI3 kinase pathway (6). In situations of high caspase activity, p120-GAP is cleaved into two proapoptotic fragments (7) which amplify the apoptotic cascade. It is tempting to speculate that neurofibromin is similarly regulated.

Caspases and Cancer: Failure of apoptosis is a hallmark of cancer. To that end, a growing body of work describes deregulation and/or mutations of caspases in human cancers. Inactivating mutations in caspase 8 and caspase 10, both initiator caspases, are reported in a subset of colorectal carcinomas, gastric carcinomas, lung carcinomas, and pediatric tumors (8-11). Somatic mutations in the caspase 3 gene have been described in a subset of colon carcinomas, lung cancers, non-Hodgkin lymphomas, stomach carcinomas, and hepatocellular carcinoma (12). Apaf 1, an essential component of caspase 9 activity, is absent in a subset of aggressive melanomas (13). These studies highlight that in the absence of caspase activity, and hence the absence of caspase cleavage products, neoplastic growth can occur. Activating the cell's own caspase machinery is an area of active research for cancer therapy (14,15).

One unique hallmark of caspases is that their substrates are limited to a restricted set of target proteins, generally at one site within a target molecule. Caspases have an absolute requirement for an Asp (D) residue at the P1 position, and a preference for a Ser(S) or Gly(G) in the P1' position. Substrate specificity is determined by the amino acid residues in the P2-P4 positions (Fig. 6) (16).

P4-P3-P2-D--/--P1' (S/G)

Figure 6. Caspases hydrolyze peptides after an Asp(D) residue. Substrate specificity is determined by amino acid residues in the P2-P4 positions. Caspases have a preference for Ser(S) or Gly(G) in P1'.

Neurofibromin contains two potential caspase cleavage sites.

In order to identify novel caspase substrates, we performed a search of non-redundant human protein databases using a scoring matrix derived from the sequences of known caspase substrates and predictions based on *in vitro* peptide analyses. This search generated a list of approximately 300 proteins, which contained potential caspase sites, 160 of which had already been identified as caspase targets, thus validating the search criteria. One of the proteins identified was neurofibromin. Based on the stringent amino acid sequence requirements that caspases have for their substrates, two candidate sites within neurofibromin meet criteria for potential caspase cleavage: DLVD²⁴⁵G, a potential caspase 3 site located in exon 6 at the amino terminus of neurofibromin, and VVLD¹⁹⁹¹S, a potential caspase 6 site located in exon 30 at the carboxy terminus (Fig. 7). Of note, the bipartite nuclear localization sequence in exon 43 is distal to the VVLD¹⁹⁹¹S site.

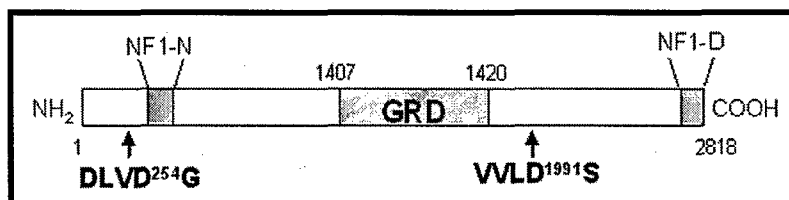


Figure 7. Schematic diagram of neurofibromin showing the relative locations of the caspase cleavage sites, the GRD and the epitopes to which the NF1-N and NF1-D antibodies are directed.

1a. Neurofibromin is cleaved in response to Fas Ligand Binding

To ascertain if the potential caspase cleavage sites found in neurofibromin were functional *in vivo*, we utilized a human B cell line, BJAB, that readily undergoes apoptosis mediated through Fas, caspase 8 and caspase 3 (17,18). Western blot analysis was performed with the NF1-N and NF1-D antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). As can be seen in figure 3, in the absence of stimulation, both antibodies recognize neurofibromin as a band of MW $\approx$ 255kDa, consistent with previous reports. After anti-Fas treatment, the intensity of this band decreased, while lower molecular weight species recognized by the NF1-N antibody ($\approx$ 200kDa) and NF1-D antibodies ($\approx$ 75kDa) were induced. The appearance of these additional bands was prevented by pretreatment with the pan caspase inhibitor, VAD. Initial sequence analysis of neurofibromin revealed two potential caspase sites: a DLVD²⁵⁴/G caspase 3 site in the N-terminus at residue 254, and a VVLD¹⁹⁹¹/S caspase 6 site in the C-terminus at residue 1991 (Fig. 7). The results shown in figure 3 are consistent with cleavage at the VVLD caspase 6 site, as this is predicted to generate a large (>180kDa) fragment identifiable by the NF1-N antibody and a smaller ($\approx$ 75 kDa) fragment identified by the NF1-D antibody (Fig. 8). Neurofibromin may also be cleaved at the N-terminus DLVD site. SDS-PAGE would not detect this cleavage, as the small molecular weight change of the larger fragment would not result in a detectable gel shift and the small 20 kDa N-terminus cleavage product would not be recognized by either the NF1-N or NF1-D antibodies.

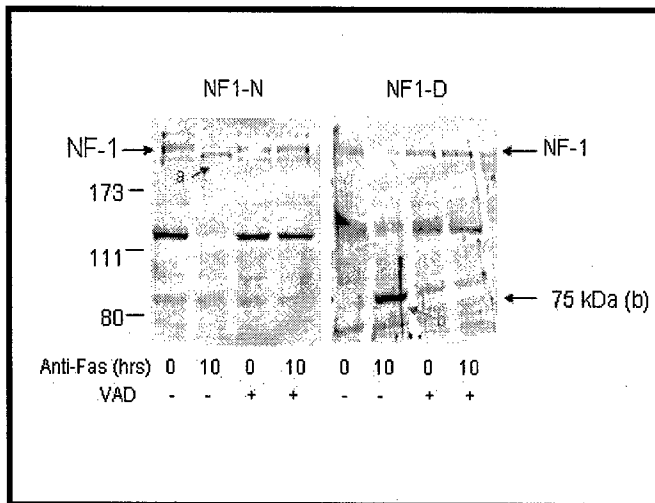


Figure 8. Neurofibromin is cleaved in the presence of caspase activity. BJAB cells were treated with anti-Fas antibody at 1 μ M final concentration for 0 or 10 hrs with (+) or without (-) pretreatment with 20 μ M VAD for 3 hrs. Cleavage is prevented by the pan-caspase inhibitor VAD. Cleavage of neurofibromin generates a large (>180kDa) fragment detected by NF1-N (arrow, a), and a 75kDa fragment detected by NF1-D (arrow, b).

1b. Time course of neurofibromin cleavage

Comparison of the kinetics of cleavage of a novel caspase substrate such as neurofibromin with that of other substrates can provide valuable information about the sequence of events in apoptosis. To investigate the kinetics of neurofibromin cleavage, BJAB cells were stimulated with anti-Fas and harvested at time points between 0 and 10 hrs and lysates were subjected to Western blot analysis using the NF1-N and anti-Caspase 3 antibodies (Cell Signaling Technologies, Beverly, MA). Parallel annexin V assays were performed to determine the degree of apoptosis. Neurofibromin cleavage was detectable within 4 hrs of incubation with anti-Fas, and was essentially complete by 8 hrs (Fig 9A). The kinetics of neurofibromin cleavage lagged behind that of caspase 3, which began to be cleaved within 1 hr (Fig 9B). Neurofibromin cleavage appears to be a mid- to late event in apoptosis, as cleavage is not detectable until the majority of the cells (75%) are annexin V positive, which correlates with the execution phase in apoptosis, characterized by chromatin condensation and DNA fragmentation, but preceding membrane damage. In contrast, caspase 3 cleavage, which is a marker of caspase 3 activation, occurs much earlier, when the majority of the cells are still annexin V negative. The discrepancy between caspase 3 activation and neurofibromin cleavage suggests that caspase 3 is unlikely to be solely responsible for neurofibromin cleavage.

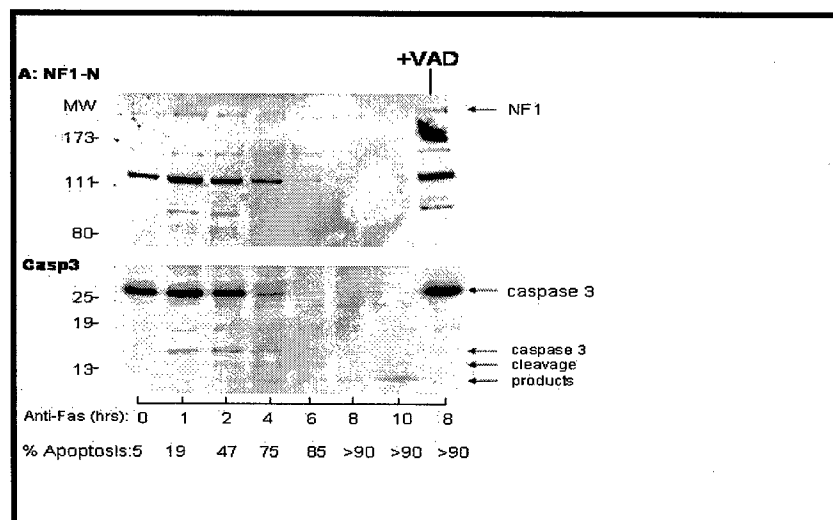


Figure 9. Time course of neurofibromin cleavage in BJAB cells. Cells were pretreated with 20 μ M VAD for 3 hrs, followed by anti-Fas for varying time points. Crude cell lysates were subjected to Western blot analysis and probed with antibodies against neurofibromin (NF1-N, A) or caspase 3 (B). Percent cell apoptosis as determined

by the Annexin V assay is shown at the bottom. Caspase 3 is activated within 1 hour of Fas-induced apoptosis, while neurofibromin cleavage is detected between 4 and 6 hours.

Neurofibromin is targeted to the nucleus in suprabasal, differentiating keratinocytes.

The morphology of the epidermis is such that proliferating, differentiating, and dying cells are in close proximity to their origin, rendering the epidermis an ideal model to study common signaling pathways. Because of this, we investigated the expression of neurofibromin in human epidermis and in cultured human epidermal keratinocytes using a battery of antibodies directed towards different epitopes of neurofibromin. Our results relevant to this study show that the post-mitotic, differentiating layers of the epidermis are preferentially recognized by the NF1-D antibody (data not shown).

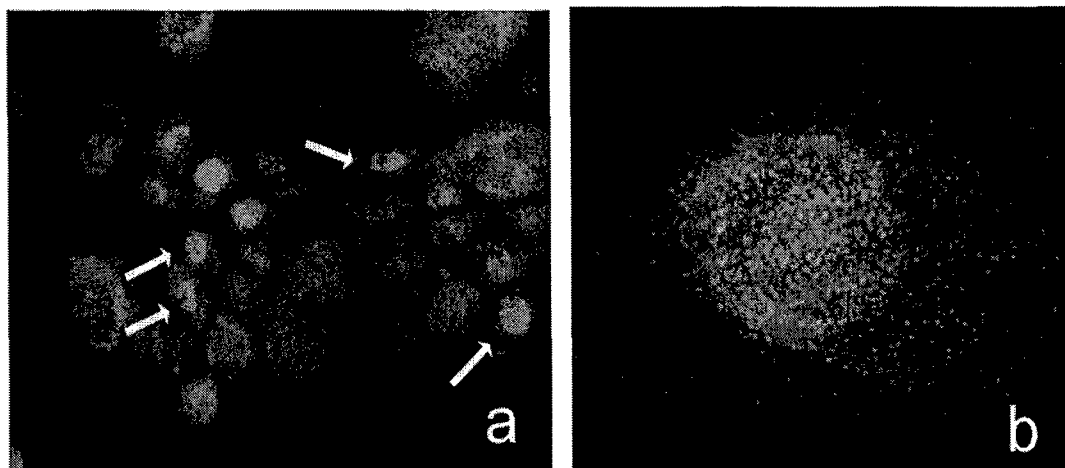


Figure 10. Neurofibromin that contains the NF1-D epitope immunolocalizes to the nucleus in a punctate pattern. A. Cultured human keratinocytes were fixed in methanol/acetone (3:1 v:v) and probed with the NF1-D neurofibromin antibody. FITC (green) secondary was used to visualize staining (original magnification, 20X). B. Deconvolved image of a cultured keratinocyte stained with the NF1-D antibody (FITC, green), keratin-14 (Texas Red, red) and DAPI (blue) to visualize the nucleus (75X original magnification).

Furthermore, cultured keratinocytes induced to differentiate express NF1-D-labeled neurofibromin that is markedly concentrated in the nucleus (fig 10a, arrows). A deconvolved image confirmed nuclear localization in a punctate pattern (Fig 10b). The D epitope includes exons 43-49, and hence the bipartite NLS described by Vandenbroucke et al (see Fig. 2) (19). The punctate nuclear staining suggests that neurofibromin is colocalizing with a particular nuclear body or within a defined nuclear compartment. Different nuclear bodies composed of multi-molecular complexes, such as coiled bodies and promyelocytic leukemia nuclear bodies (PML-NBs), have in recent years been shown to play a role in DNA replication and repair, mRNA and rRNA synthesis and assembly, and chromatin modification. Additionally, we found that the NF1-N antibody did not stain nuclei, indicating that the species of neurofibromin located in the nuclei is not a full-length protein. Whether the neurofibromin species found in the nucleus is the result of a specific caspase cleavage event remains to be determined.

Based on these data, we hypothesize that neurofibromin is cleaved by caspase 6 and that the cleaved isoform translocates to the nucleus where it controls cellular processes either directly by regulation of transcription or indirectly by regulating signaling proteins.

2. We also had the opportunity to collaborate with Drs. Beverly Dale (University of Washington) and David Kelsell (Barts and the London School of Medicine and Dentistry, London) to identify the gene mutated in Harlequin Ichthyosis, a generally neonatal fatal form of ichthyosis. Harlequin Ichthyosis is a very rare disease and we were able to provide the valuable DNA samples and cell lines that had been previously banked in my laboratory for this study.

Key Research Accomplishments

- Developed a database of NF1 subjects, clinical manifestations, and results of molecular assays to screen for microdeletions
- Designed and validated a real-time competitive PCR assay at 5 loci for rapid screening of NF1 subjects for NF1 deletion. This assay can detect novel deletions in addition to the recurrent 1.4 Mb microdeletion.
- Screened 207 unselected NF1 subjects and 34 DNAs from known NF1 deletion patients with deletion-specific assays for the 1.4 Mb deletion and competitive PCR assays for novel deletions. We identified 56 subjects with the recurrent 1.4 Mb deletion and 16 subjects with novel deletions.
- We have identified an apparent new function for neurofibromin as a target of caspase. Based on these data, we hypothesize that neurofibromin is cleaved by caspase 6 and that the cleaved isoform translocates to the nucleus where it controls cellular processes either directly by regulation of transcription or indirectly by regulating signaling proteins. We have submitted a new grant proposal to pursue this important research to understand the role of caspases in NF1-related tumorigenesis.

Reportable Outcomes

- Developed immortalized lymphoblastoid cell lines from 38 newly enrolled NF1 subjects
- Developed a database of NF1 subjects
- Manuscript: Stephens K. Neurofibromatosis. In *Molecular Pathology in Clinical Practice*. D.G.B. Leonard, Ed. New York: Springer-Verlag, in press.
- Manuscript: Stephens K. Neurofibromatosis 1. In *Genomic Disorders: The Genomic Basis of Disease*, Eds. JR Lupski, PT Stankiewicz. New Jersey: Humana Press, in press.
- Manuscript: Kelsell DP, Norgett EE, Unsworth H, Teh MT, Cullup T, Mein CA, Dopping-Hepenstal PJ, Dale BA, Tadini G, Fleckman P, Stephens KG, Sybert VP, Mallory SB, North BV, Witt DR, Sprecher E, E M Taylor A, Ilchysyn A, Kennedy CT, Goodyear H, Moss C, Paige D, Harper JJ, Young BD, Leigh IM, Eady RA, O'toole EA. Mutations in ABCA12 Underlie the Severe Congenital Skin Disease Harlequin Ichthyosis. *Am J Hum Genet* Mar 8;76, 2005.
- Manuscript: Lombillo VA and Sybert VP. Mosaicism in Cutaneous pigmentation. *Curr Opin in Pediatr*, submitted and now in revision.
- Abstract: De Raedt, Heyns I, Brems H, Stephens K, Marynen P, Legius E. On the origin of NF1 microdeletions. The CTF International Consortium for the Molecular and Cell Biology of NF1, NF2 and Schwannomatosis, Aspen, June, 2005
- Abstract: Shinohara MM, Kuechle MK, Graves J, Stephens K. Neurofibromin is a caspase target. The CTF International Consortium for the Molecular and Cell Biology of NF1, NF2 and Schwannomatosis, Aspen, June, 2005.
- Abstract: Shinohara MM, Kuechle MK, Graves J, Stephens K. Caspase mediated proteolysis of neurofibromin. Society of Investigative Dermatology, St. Louis, May, 2005.
- Training of Dr. Paula Zook, a dermatology fellow, in the field of neurofibromatosis, 8/04 – present.

- New grant application: Based on our results that neurofibromin is a caspase target, we submitted a grant application to the Neurofibromatosis Program of the U.S. Army Medical Research and Materiel Command entitled, "Caspase mediate cleavage of neurofibromin: Generation of functionally distinct peptide fragments". Principal Investigator: Melanie Kuechle; Co-investigator: Karen Stephens. Submitted Feb, 2005.
- Seminar: Medical Genetics Seminar, University of Washington, Neurofibromatosis 1: Different paralogous recombination sites for meiotic versus mitotic NF1 microdeletion. Jan 21, 2005.
- Invited international 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 courses at the University of Washington:
 - PATH 516 Molecular Basis of Human Genetic Disease
 - Lecture 1: "NF1, Genomic Disorders and their Mechanisms." April 6, 2005
 - Lecture 2: "Fragile X Mental Retardation Syndrome", April 11, 2005.
 - Lecture 3: "Genetic Testing", April 25, 2005.
 - GENOME 531 Genetics of Human Disease
 - Genetics of Human Disease, "Neurofibromatosis", May, 2004
 - Introduction to Medical Genetics
 - Lecture: Molecular Diagnostics, Feb 25 9-10 am, 2005
 - Lecture: Fragile X Mental Retardation Syndrome, Feb 25 10-11 am, 2005.

Conclusions

We have developed rapid and sensitive assays for the detection and mapping of NF1 microdeletions. Using these assays, we screened 242 patients and identified 56 carriers of the recurrent 1.4 Mb NF1 microdeletion and 16 carriers of novel deletions. Clinical evaluation of these patients, along with age/sex matched NF1 patients that are not NF1 deletion carriers, is in progress to delineate the clinical manifestations most prevalent in deletion patients. We have collected tumors from NF1 microdeletion carriers, which are now being examined for mutations and genomic instability. We began a new collaboration and generated preliminary data that identified an apparent new function of neurofibromin as a caspase target. Since this work is outside the scope of this grant, we have submitted a new application for investigating the role of caspase in NF1 disease and tumorigenesis. We have written 2 reviews on NF1 and given local and international seminars on NF1. Therefore, our progress on genotype/phenotype correlations in carriers of NF1 microdeletions, along with our new work identifying neurofibromin as a caspase target, will provide clinically applicable information for patient diagnosis and management and basic knowledge about neurofibromin function in disease pathogenesis.

REFERENCES

1. Dorschner MO, Sybert, VP, Weaver, M, Pletcher, BA and Stephens, K (2000) NF1 microdeletion breakpoints are clustered at flanking repetitive sequences. *Hum Mol Genet*, 9, 35-46.
2. Forbes SH, Dorschner, MO, Le, R and Stephens, K (2004) Genomic context of paralogous recombination hotspots mediating recurrent NF1 region microdeletion. *Genes Chromosomes Cancer*, 41, 12-25.
3. Lopez-Correa C, Dorschner, M, Brems, H, Lazaro, C, Clementi, M, Upadhyaya, M, Dooijes, D, Moog, U, Kehrer-Sawatzki, H, Rutkowski, JL et al. (2001) Recombination hotspot in NF1 microdeletion patients. *Hum Mol Genet*, 10, 1387-1392.
4. Dorschner MO, Brems, H, Le, R, De Raedt, T, Wallace, MR, Curry, CJ, Aylsworth, AS, Haan, EA, Zackai, EH, Lazaro, C et al. (in revision) Tightly clustered breakpoints permit detection of the recurrent 1.4 Mb NF1 microdeletion by deletion-specific amplification.
5. 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. *Clin Chem*, 46, 1574-1582.

6. Yang, J. Y., and Widmann, C. (2002) *J Biol Chem* 277, 14641-6.
7. Wen, L. P., Madani, K., Martin, G. A., and Rosen, G. D. (1998) *Cell Death Differ* 5, 729-34.
8. Kim, H. S., Lee, J. W., Soung, Y. H., Park, W. S., Kim, S. Y., Lee, J. H., Park, J. Y., Cho, Y. G., Kim, C. J., Jeong, S. W., Nam, S. W., Kim, S. H., Lee, J. Y., Yoo, N. J., and Lee, S. H. (2003) *Gastroenterology* 125, 708-15.
9. Harada, K., Toyooka, S., Shivapurkar, N., Maitra, A., Reddy, J. L., Matta, H., Miyajima, K., Timmons, C. F., Tomlinson, G. E., Mastrangelo, D., Hay, R. J., Chaudhary, P. M., and Gazdar, A. F. (2002) *Cancer Res* 62, 5897-901.
10. Park, W. S., Lee, J. H., Shin, M. S., Park, J. Y., Kim, H. S., Kim, Y. S., Lee, S. N., Xiao, W., Park, C. H., Lee, S. H., Yoo, N. J., and Lee, J. Y. (2002) *Oncogene* 21, 2919-25.
11. Shivapurkar, N., Reddy, J., Matta, H., Sathyanarayana, U. G., Huang, C. X., Toyooka, S., Minna, J. D., Chaudhary, P. M., and Gazdar, A. F. (2002) *Oncogene* 21, 8510-4.
12. Soung, Y. H., Lee, J. W., Kim, S. Y., Park, W. S., Nam, S. W., Lee, J. Y., Yoo, N. J., and Lee, S. H. (2004) *Hum Genet* 115, 112-5.
13. Soengas, M. S., Capodieci, P., Polsky, D., Mora, J., Esteller, M., Opitz-Araya, X., McCombie, R., Herman, J. G., Gerald, W. L., Lazebnik, Y. A., Cordon-Cardo, C., and Lowe, S. W. (2001) *Nature* 409, 207-11.
14. Philchenkov, A. (2004) *J Cell Mol Med* 8, 432-44.
15. Philchenkov, A., Zavelevich, M., Krocak, T. J., and Los, M. (2004) *Exp Oncol* 26, 82-97.
- 16.. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T., and Nicholson, D. W. (1997) *J Biol Chem* 272, 17907-11.
17. Yamada, H., Tada-Oikawa, S., Uchida, A., and Kawanishi, S. (1999) *Biochem Biophys Res Commun* 265, 130-3.
18. Thomas, L. R., Johnson, R. L., Reed, J. C., and Thorburn, A. (2004) *J Biol Chem* 279, 52479-86.
19. Vandenbroucke, I., Van Oostveldt, P., Coene, E., De Paepe, A., and Messiaen, L. (2004) *FEBS Lett* 560, 98-102.

Appendices

PDF files of the following manuscripts/documents are attached and can be opened by double clicking on the icon.

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

Manuscript: Stephens K. Neurofibromatosis 1. In Genomic Disorders: The Genomic Basis of Disease, Eds.. JR Lupski, PT Stankiewicz. New Jersey: Humana Press, in press.

Manuscript: Kelsell DP, Norgett EE, Unsworth H, Teh MT, Cullup T, Mein CA, Dopping-Hepenstal PJ, Dale BA, Tadini G, Fleckman P, Stephens KG, Sybert VP, Mallory SB, North BV, Witt DR, Sprecher E, E M Taylor A, Ilchyshyn A, Kennedy CT, Goodyear H, Moss C, Paige D, Harper JI, Young BD, Leigh IM, Eady RA, O'toole EA. Mutations in ABCA12 Underlie the Severe Congenital Skin Disease Harlequin Ichthyosis. Am J Hum Genet Mar 8;76, 2005.

Abstract: De Raedt, Heyns I, Brems H, Stephens K, Marynen P, Legius E. On the origin of NF1 microdeletions. The CTF International Consortium for the Molecular and Cell Biology of NF1, NF2 and Schwannomatosis, Aspen, June, 2005

Abstract: Shinohara MM, Kuechle MK, Graves J, Stephens K. Neurofibromin is a caspase target. The CTF International Consortium for the Molecular and Cell Biology of NF1, NF2 and Schwannomatosis, Aspen, June, 2005.

Abstract: Shinohara MM, Kuechle MK, Graves J, Stephens K. Caspase mediated proteolysis of neurofibromin. Society of Investigative Dermatology, St. Louis, May, 2005.