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13. ABSTRACT (Maximum 200 Words)

Towards the goal of molecular diagnosis and staging of NF1-related tumors, we proposed to develop and validate a novel PCR-based method, termed QuEST, for rapid and quantitative identification of loci with increases or decreases in genomic copy number in MPNST. The implications of our comprehensive literature search and compilation of genetic changes in NF1-associated MPNST are that this is a feasible and clinically relevant approach. We have developed 6 assays that are sensitive and specific and allow the quantitation of one gene copy versus two gene copies. The method was validated on constitutional DNA using assays that determined the copy number of the NF1 gene. This methodology was used for both deletion) and gain of DNA in tumor cell lines. We demonstrated that tumor cell lines are not the ideal resource for quantitative competitive PCR analyses due to hyperploidy and hypoploidy, which may represent the cellular admixture of the original tumor or selection during growth in culture. We applied our assays to an NF1-associated MPNST cell line from a patient with a constitutional *NF1* microdeletion, but the gene dosage data did not reflect the known deletion, almost surely due to mosaicism for cells of different ploidy in the tumor. So, our idea of developing sensitive quantitative assays for genomic alleles worked splendidly, they were not informative when applied to tumor cell lines.

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Introduction

Lack of a definitive system for the diagnosis and staging of NF1-related tumors is a major obstacle to investigating the molecular basis of tumorigenesis, to our ability to assess prognosis, and consequently, to the rational design and application of stage-specific therapeutic agents (1-3). Locus-specific changes in copy number are a common feature of many types of tumors, including the neurofibromas and malignant peripheral nerve sheath (MPNST) that develop in individuals with NF1 (4-13). Gains or losses at a particular locus are (reviewed in(4)potential biomarkers for the molecular diagnosis and grading of MPNST. Towards the goal of molecular diagnosis and staging of NF1-related tumors, we propose to develop and validate a novel polymerase chain reaction (PCR)-based method, termed QuEST, for rapid and quantitative identification of loci with increases or decreases in genomic copy number in MPNST. The specific aims are [1] to develop a series of reference and target markers for quantitative assessment of gene copy number. This will involve selection, design and optimization of primers and QuEST reactions for reference and target loci. [2] to perform QuEST analysis on series of cell lines and MPNST samples for two target regions where copy number changes are associated with MPNST formation: amplification of 17q22-qter and deletion of 1p. [3] to determine the feasibility of coupling QuEST analysis with laser capture microdissection. This study has the potential of providing direct and precise methods to use in addressing issues important to diagnosis, treatment, and molecular analysis of MPNST.

Body

Research accomplishments associated with each task outlined in the Statement of Work are detailed below.

Approved Statement of Work

Task 1. Design and test a series of primers for analysis of both target loci and reference loci.

Design and synthesize primers for genes in the target regions of chromosomes 1p and 17q22-24 and for reference loci.

Optimize the amplification reaction for each locus using non-tumor genomic DNA

Progress: We need the capability and sensitivity to determine if a locus or gene has no deletion (2 copies), one gene deletion (1 copy), two gene deletion (0 copies), or amplification (likely >10 copies). A deletion of two genes, or a multi-gene amplification, is simple to detect using various methods of quantitative PCR. 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. To determine which quantitative PCR methods were sensitive, we choose to develop an assay at intron 31 of the NF1 gene. To validate the assays we used constitutional genomic (nontumor) DNA from normal individuals (2 NF1 genes present) and DNA from NF1 microdeletion patients (1 NF1 gene present).

Initially, we tried using SYBR green (binds double stranded DNA) fluorescence as a method of detection during real-time PCR in the LightCycler instrument (Roche). There was considerable overlap between the crossing point values (Ct) for samples with one and two NF1 genes indicating that the assay was not as sensitive or specific as required (data not shown).

Secondly, we sought to increase specificity and sensitivity by using a NF1-specific fluorescent labeled PCR primer. We constructed a LUX primer (Invitrogen), which is a hybrid primer comprised of NF1 specific sequences and anonymous sequences that are capable of fold-back annealing. LUX primers are touted as having high specificity because they only fluoresce at high temperatures when the fold backs are melted. We had many problems with LUX primers and decided to abandon that approach (data not shown).

Thirdly, we chose to employ SYBR green for detection in combination with competitive PCR, which we considered a more sensitive method of quantitation one gene versus two genes copies. We adapted and modified a method published by Ruiz-Ponte et al. (5). In this method, a known number of amplicons of a competitor is introduced

directly in the PCR along with the same number of amplicons from the target constitutional DNA of the patient. We constructed the competitor to be an amplicon of the same length, amplified by the same primers, but we changed three of the nucleotides such that the target *NF1* amplicon and the competitor amplicons will have distinguishable melting curves. To increase the probability that the target and competitor reactions will be highly efficient, we designed primers to generate very small products.

Calibration curves of different competitor concentrations determine the optimal concentration that equals that of the target DNA. Figure 1 shows the melting curves of a normal control DNA sample (2 copies of *NF1*), where competitor and intron 31 are co-amplified with equal efficiency and the area under the curves are equal (roughly equivalent to peak height in this example). In this case, we constructed the competitor such that it would be amplified with the intron 31 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 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.

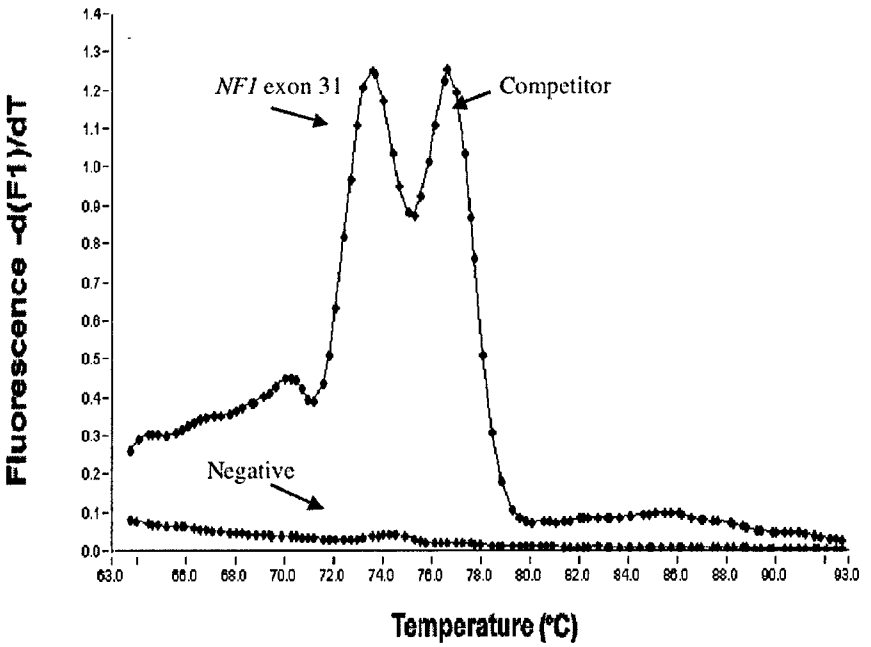

Figure 1. Melting curve analysis after competitive, quantitative PCR at *NF1* intron 31 in genomic DNA of a normal control individual. The peaks representing the melting curve of the amplicon of the competitor and the amplicon of the patient's target DNA are indicated. The negative control without DNA shows evidence of amplificiation.

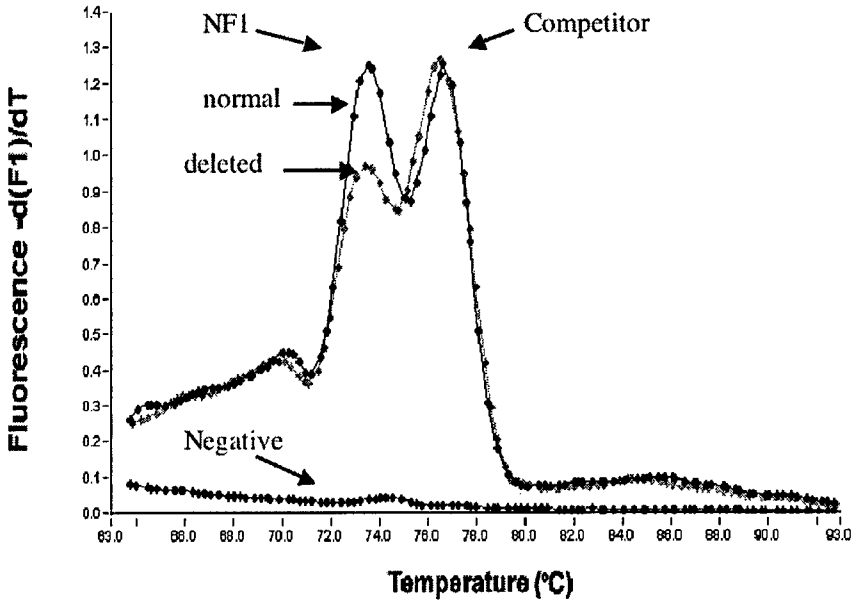

Figure 2. Melting curve analysis after competitive, quantitative PCR at *NF1* intron 31 can differentiate one gene copy versus two gene copies. The results of two reactions are shown, closed circles represent target DNA from a normal control individual and closed squares represent target DNA from an 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. As expected, the negative control without DNA shows no amplification.

Once the optimal concentrations of competitor and genomic target DNAs are determined, it is essential that all subsequent reactions with unknown patient DNA samples contain precisely the same concentrations. Competitor templates are cloned in E. coli for stability and ease of quantitation, aliquoted, and stored frozen. For each patient genomic DNA sample, prior to the competitive quantitative PCR assay, we determine the precise 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 the precise volume of patient DNA to be mixed with the competitortemplate for each assay. An example of the TPA real-time PCR data and standard curve is shown in Figure 3.

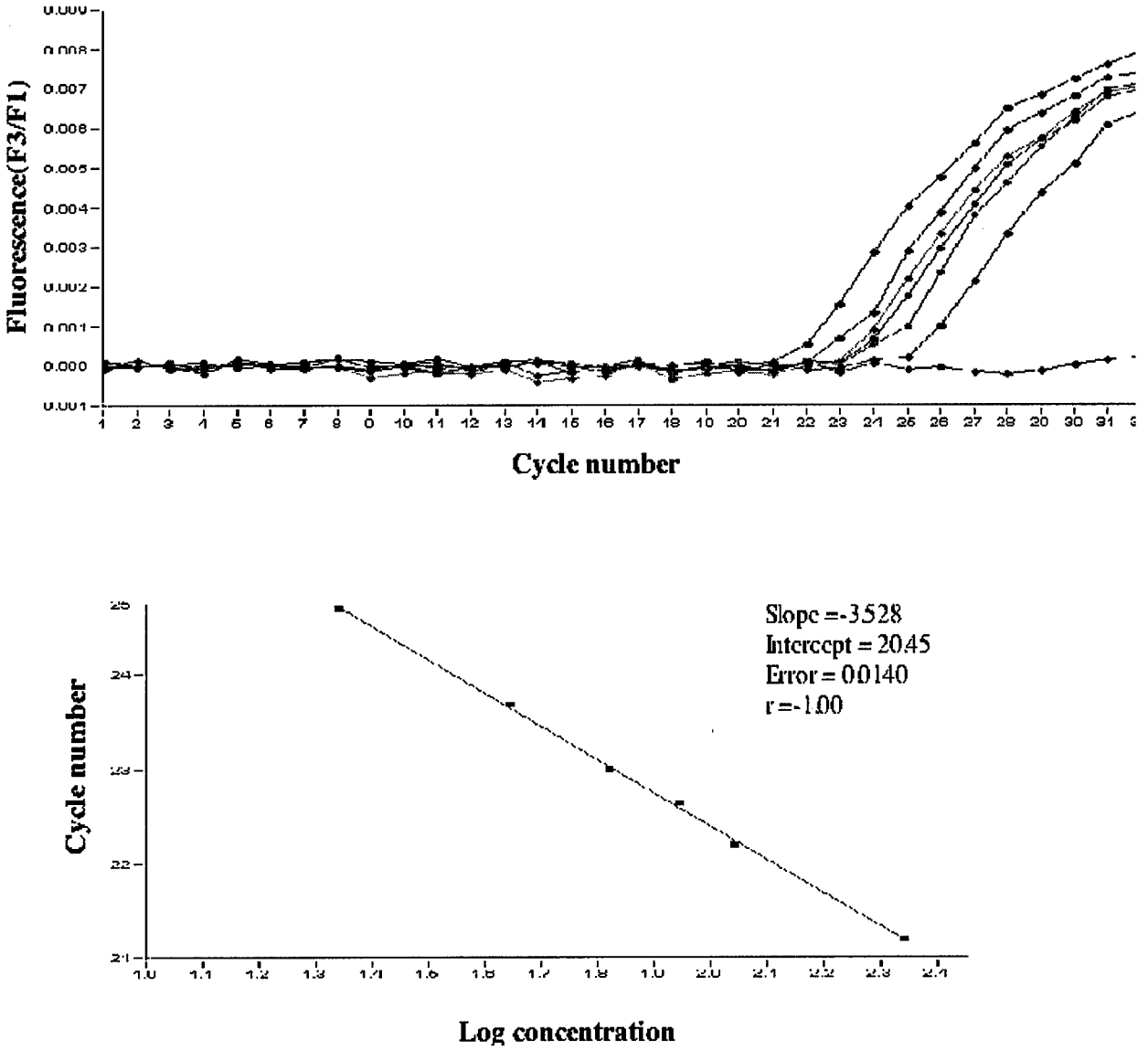

Figure 3. LightCycler real-time PCR at TPA locus showing standard curve. The upper panel shows the results of real-time PCR of the TPA locus of a dilution series of a normal control subject. The reaction consists of unlabeled primers and uses an internal labeled (fluorescence resonance energy transfer, FRET) probe for detection and quantitation of amplicons. 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 of cycle number (Ct) vs log concentration that was calculated from the above data. Note the low error and high correlation coefficient.

The development of additional competitive quantitative PCR assays. We developed additional assays spanning the *NF1* gene region for this project and also for project our project DAMD17-00-1-0542. The advantage of these assays is that they could be validated on genomic DNA of subjects with and without *NF1* regional deletions prior to their application to tumor DNAs, which are genomically more complex. Assay for each locus were screened against an appropriate panel of monochromosomal human-rodent hybrid cell lines to ensure we are not amplifying homologous loci from other chromosomes Location of the loci are shown in Figure 4.

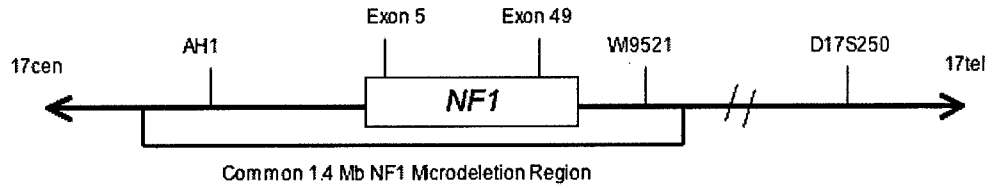

Figure 4. Location of loci in the 17q11.2 *NF1* region for which competitive, quantitative PCR assays were developed. Two loci are intragenic to *NF1* and two are flanking; all four are within the common 1.4 Mb *NF1* microdeletion region previously defined by us (6, 7). D17S250 is at 17q22 region and is never involved in *NF1* microdeletions; it is used as a disomic control locus.

The intra-assay and inter-assay variation of these assays is minimal as shown in Table 1. There is no overlap of values for disomic samples vs. monosomic samples. As expected, the disomic control locus D17S250 gave values of approaching 1.0 for both subjects.

Table 1. Variation of competitive quantitative PCR assays[1]

Intra-assay variation (mean ± 1 S.D.)					
Locus	AH1	*NF1* exon 5	*NF1* exon 49	WI-9521	D17S250
Normal subject	0.97 ± 0.18	1.21 ± 0.13	1.03 ± 0.1	1.10 ± 0.15	1.11 ± 0.16
NF1 microdeletion subject	0.33 ± 0.05	0.52 ± 0.13	0.51 ± 0.11	0.44 ± 0.13	1.15 ± 0.18
Inter-assay variation (mean ± 1 S.D.)					
Normal subject	0.95 ± 0.13	1.23 ± 0.26	0.97 ±0.16	0.98± 0.17	1.07 ±0.15
NF1 microdeletion subject	0.32 ± 0.13	0.53 ± 0.14	0.48 ± 0.06	0.36 ± 0.10	1.12 ± 0.14

[1]Intra-assay variation was determined by assay of 20 replicate samples in one experiment. Inter-assay variation was determined by assay of the same normal subject and deletion subject in 20 independent experiments performed on different days.

We now have considerable expertise in the application of these assays to identify and map the locations of constitutional NF1 regional microdeletions in non-tumor tissues. In our related project (DAMD17-00-1-054) we have screened several hundred NF1 subjects using these assays. However, it was unclear exactly how data derived from application of these and other assays would be interpreted. It is possible that considerable chromosomal rearrangements, along with contaminating normal tissues, might make data interpretation challenging, or possible even impossible.

Task 2. Perform spectral karyotyping (SKY) of the MPNST- and neuroblastoma-derived cell lines. Months 1-12.

Progress: To assess the complexity of the karyotypes of some potential tumor cell lines, we collaborated with Dr. Karen Swisshelm, a internationally-recognized cytogeneticist in the Department of Pathology, University of Washington, who analyzed the karyotypes of two of the

neuroblastoma-derived cell lines. We have performed metaphase G-banded karyotypes at the ~450 band level to determine which bands were deleted and or amplified. The karyotypes were quite complex as documented below in Figure 5. Because this analysis was so informative, we do not plan to perform a SKY analysis as originally proposed.

Karyotype of cell line IMR-32:
39-48, XY, dup(1)(?p36.1->p36.3::p36..1->p36.3::p36.1->p36.3)x2, +1, +6[8], -7,-9[3], der(16)t(?15:16)(q15:q24)[8][cp8]/42-50, idem,+12[12],15[3],+mar[4][cp12]

Karyotype of cell line SK-N-AS:
43-48, X,-X,+add(1)(p11),add(3)(p21)?inv(5)(p15.3q11.1),der(6)t(?4:6)(p21;q15), add(8)(q24.1,add(9)(p22),der(11)t(11;?17)(q13;q11.2),-14,add(16)24),-17,i(19)(q10,add(22)(q13)[14],+mar[3]/43-35,idem,-13[3]/45-46,idem,+7[3].

Figure 5. Karyotypes of two neuroblastoma cell lines. The karyotypes were quite complex and demonstrate a significant mixture of cells, some of which are hyperdiploid and some are hypodiploid.

The hyper- and hypo-diploid cells in the tumors diminished our enthusiasm for using these cell lines for quantitative PCR, as it would be difficult to determine appropriate disomic control loci, which in turn would complicate dosage analysis of the target loci. Line IMR-32 was possibly appropriate for analysis of the amplified region of chromosome 1p and line SK-N-AS could possible be used for analysis of the deleted region of 17q22.24. As we were deciding on an appropriate approach to the analysis of these lines, we by happenstance obtained access to MPNST cell lines (see Task 3 below).

Dr. Stephens also performed a comprehensive literature search and compilation of the genetic changes in NF1-associated peripheral nerve sheath tumors (4)(see manuscript in Appendix). This review revealed that there are consistent regions of the genome that undergo gains and losses in MPNST. This review also underscored our choice of the 17q22-q25 region as one of frequent gains in chromosomal loci (74%). While these previous papers concentrated on regions of allele loss and gain, there was little information on mosaicism for hyperdiploid and hypodiploid cells within the tumors.

Task 3. Validate the QuEST method by analysis of target and reference loci in DNA from tumor cell lines. Months 6-12.

a. Perform QuEST analysis of genes in the target regions chromosomes 1p and 17q22-24 in DNA from MPNST- and neuroblastoma-derived cell lines.

Progress: During the last year of this project, Dr. Nancy Ratner (University of Cincinnati recently moved to Children's Hospital Medical Center, Cincinnati) and I were discussing her expression array analyses of 5 MPNST cell lines derived from NF1-associated tumors. Since NF1 affected individuals with *NF1* microdeletions are known to be at increased risk for MPNST, we considered it likely that some of the individuals from whom these lines were derived carried the common germline *NF1* microdeletion (Figure 4). We had recently developed deletion-specific PCR assays to detect these mutations (under our project DAMD17-00-1-0542), which we applied to these DNAs. We found that one NF1-associated MPNST, number 90-8, was positive for the common constitutional 1.4 Mb NF1 microdeletion shown (Figure 4). The most likely explanation for the NF1 subject who developed MPNST 90-8, was that he/she carried a germline *NF1* microdeletion, subsequently acquired a 2nd hit mutation in remaining *NF1* gene in a progenitor Schwann cell, and subsequent mutations and selection led to MPNST. Therefore, we would expect every cell in the tumor, whether Schwann or other, to be hemizygous for *NF1*. We applied 4 of our competitive quantitative PCR assays to these tumors and the results are in Table 2 below.

Table 2. Quantitative locus analysis of human NF1-associated MPNST cell lines

	Dosage values at loci			
MPNST Cell line	***AH1***	***NF1* exon 5**	***WI-9521***	***D17S250***
S26T	0.67	1.40	0.71	0.55
YST1	1.19	1.83	0.48	0.45
90-8*	0.49	1.30	0.77	2.11
S462	0.41	0.65	0.47	1.85
S520	0.26	0.95	0.48	1.57
Constitutional controls				
Normal subject	0.84	1.082	1.18	1.21
NF1-microdeletion subject	0.3	0.34	0.32	1.01

*Cell line positive for common 1.4 Mb *NF1* microdeletion

Focusing on the controls, we see that the majority of values are within 1 S.D. of the mean, and a few are within 2 S.D. of the mean (Table 1 above). So the experiment is working as expected. Now focusing on cell line 90-8, the results are not simply to interpret. We would have expected *AH1*, exon 5 and *WI-9621*, all of which are deleted, to show values approaching monosomy. But *NF1* exon 5 and *WI-9521* are too high. The control locus D17S250 is double the expected value. This might indicate that this region had duplicated or had segmental hyperdiploidy. If that were the case, then we still would have expected the values for the other 3 loci to be similar. For the other tumors, we would have expected that values for the three loci *AH1*, *NF1* and *WI*-9521 would most likely be consistent as they are clustered within 1 Mb, yet they are not. These results were disappointing and indicate to us that qPCR of cellular DNA from tumor cell lines that have grown in culture for many passages is not easily interpretable, presumably due to mosaicism for hyper- and hypo-diploidy at both the target and control loci.

In view of recent literature reports, our results are not unexpected. Quantitative PCR was useful at a specific well-characterized loci such as Her-2/neu in breast cancer (8) or a specific well-defined chromosomal region in clonal tumors with little cellular admixture (unlike MPNST), such as bladder cancer and oral cancer (9, 10). Few studies are reported in complex tumors such as sarcomas.

Task 4. Test the QuEST method by analysis of target and reference loci in DNA from tumors.

Progress: Based on the data above and because we did not have tumor tissue from patient's with constitutional *NF1* microdeletions, this experimental approach seemed inappropriate. Given the complexity of the data and difficulty of interpretation even for a patient with a known constitutional deletion, it is not wise to consider such experiments in tumors from patients with small NF1 intragenic mutations. It is possible, however, that if we did have a tumor from a *NF1* microdeletion patient that it may give more interpretable results. At least the tumor cells would not have undergone selection in culture for innumerable generations. Just last week, one of the NF1 microdeletion patients we were studying under our project DAMD17-00-1-0542 died as a result of metastatic MPNST. We have obtained tumor tissue and if we obtain DNA of sufficient quality for quantitative PCR, we plan to perform these assays again on that tumor in the future.

Task 5. Determine the sensitivity of the QuEST method.

Progress: The sensitivity of the quantitative competitive PCR assays were very high when applied to genomic DNA from constitutional tissues (non-tumor).

Task 6. Couple QuEST with laser capture microdissection of archival MPNST.

Progress: Due to the results of the above Tasks, pursuring this approach was deemed inadvisable and inappropriate.

Key Research Accomplishments

- Performed a comprehensive analysis and summary of genetic changes reported in NF1-associated peripheral nerve sheath tumors. These data were reported in a review article by Dr. Stephens (4) (see Reportable Outcomes).
- Obtained a detailed understanding of the parameters important in quantitative PCR, especially those critical for primer and probe design. These data where extremely helpful in the success of our project DAMD17-00-1-0542, and will be published with those data.
- Developed a specific and sensitive competitive, quantitative PCR methodology that can differentiate one copy from two copies in a genomic DNA of constitutional tissue.
- Developed and employed real-time PCR of the TPA locus as a means of determining the precise concentration of genomic DNA in a patient sample.
- Validated the competitive, quantitative PCR methodology by assay of the NF1 locus using genomic DNA from constitutional tissue of normal control individuals and NF1 deletion patients.
- Documented the reproducibility of the competitive, quantitative PCR method on genomic constitutional tissue DNA.
- Performed and analyzed high-resolution karyotypes of the two neuroblastoma-derived cell lines and determined that their hypo- and hyper-ploidy would significant compromise the planned quantitative DNA assays.
- Identified an MPNST cell line derived from an NF1 patient was carried the common constitutional 1.4 Mb *NF1* microdeletion and demonstrated that quantitative PCR did not give values of haploidy for loci known to occur in the deleted region. This result demonstrated that mosaicism in tumor cells can be significant and hinder the ability to perform qPCR analyses.

Reportable Outcomes

- Manuscript: Stephens K. Genetics of Neurofibromatosis 1-associated peripheral nerve sheath tumors. Cancer Investigation 21:901-918, 2003.
- Book Chapter. Stephens K. Neurofibromatosis. In *Molecular Pathology in Clinical Practice*. D.G.B. Leonard, Ed. New York: Springer –Verlag, in press.
- Abstract: Wang B, Bong S, Le R, Stephens K. Identification and Mapping of NF1 contiguous gene deletions using real-time competitive PCR. National Neurofibromatosis Foundation International Consortium for the Molecular Biology of NF1 and NF2, Aspen, CO, June, 2004.
- Invited Speaker: Stephens K. Neurocutaneous Syndromes in the Developmental Age, "Molecular Genetics of NF1 and NF12", Fondazione Mariani, Lucca, Italy, March 3-5, 2004.
- Invited Speaker: Division of Medical Genetics, University of Washington, "Mechanisms and phenotypic consequences of recurrent, uniform *NF1* contiguous gene deletions", February 22, 2002.
- Invited Speaker: Division of Laboratory Genetics, Dept of Laboratory Medicine and Pathology, Mayo Clinic, "Neurofibromatosis: Phenotype and Mechanism of *NF1* Microdeletion, June 19, 2002.
- Invited Speaker: National Neurofibromatosis Foundation International Consortium on Gene Cloning and Gene Function of NF1 and NF2. "Megabase Deletions", Aspen, CO, June, 2003.
- Karyotypes of two neuroblastoma-derived cell lines.

- Development of competitive, quantitative PCR assays for differentiating one versus two copies of *NF1* and virtually any other locus (manuscript in preparation).

Conclusions

Towards the goal of molecular diagnosis and staging of NF1-related tumors, we proposed to develop and validate a novel PCR-based method, termed QuEST, for rapid and quantitative identification of loci with increases or decreases in genomic copy number in MPNST. We have performed a comprehensive literature search and compilation of the genetic changes in NF1-associated peripheral nerve sheath tumors. The implications of this analysis are that consistent regions of the genome frequently undergo gains and losses in NF1-associated MPNST. This phenomenon underscores the validity of our approach and increases the probability that it may be of clinical and/or research use. We have developed 6 assays that are sensitive and specific and allow the quantitation of one gene copy versus two gene copies. The method was validated on constitutional DNA using assays that determined the copy number of the *NF1* gene. This methodology was used for both loss (deletion) of DNA and gain (amplifications) of DNA in tumor cell lines. We demonstrated that tumor cell lines are not the ideal resource for quantitative competitive PCR analyses due to hyperploidy and hypoploidy, which may represent the cellular admixture of the original tumor or selection during growth in culture. We applied our assays to an NF1-associated MPNST cell line from a patient with a constitutional *NF1* microdeletion, but the gene dosage data did not reflect the known deletion, almost surely due to mosaicism for cells of different ploidy in the tumor. So, our idea of developing sensitive quantitative assays for genomic alleles worked splendidly, they were not informative when applied to tumor cell lines.

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Appendix

The following items are attached.

1. Manuscript: Stephens K. Genetics of Neurofibromatosis 1-associated peripheral nerve sheath tumors. Cancer Investigation 21:901-918, 2003. (a preprint is provided for your review).
2. Book Chapter. Stephens K. Neurofibromatosis. In *Molecular Pathology in Clinical Practice.* D.G.B. Leonard. Ed. New York: Springer –Verlag, in press.
3. Abstract: Wang B, Vong S, Le R, Stephens K. Identification and Mapping of NF1 contiguous gene deletions using real-time competitive PCR. National Neurofibromatosis Foundation International Consortium for the Molecular Biology of NF1 and NF2, Aspen, CO, June, 2004.

Genetics of neurofibromatosis 1-associated peripheral nerve sheath tumors

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INTRODUCTION

Much of our current understanding of tumorigenesis is founded upon genetic studies of relatively rare individuals with inherited disorders that predispose to certain cancers, for example, retinoblastoma (1) and colorectal tumors (2, 3). Such genetic studies are consistent with a model whereby normal tissues become highly malignant due to successive mutation of multiple genes that dysregulate cellular proliferation and homeostasis. Important classes of mutated genes include oncogenes (positive growth regulators), tumor suppressors (negative growth regulators), and those encoding cell cycle regulators, anti-apototic signals, and components of the DNA replication and repair machinery. Mutation during tumorigenesis can occur at the nucleotide level as inactivation of a single gene or at the chromosomal level as losses of large segments or entire chromosomes, as a fusion of two different chromosomal segments, or as a high level amplification of a segment (4). Screening normal and tumor tissues of patients for common genetic alterations has been a productive strategy for identifying genes that contribute to tumor formation. This article will focus on genetic changes commonly associated with peripheral nerve sheath tumors in individuals with the autosomal dominant tumor-prone disorder neurofibromatosis 1 (NF1).

Virtually all individuals affected with NF1 develop multiple peripheral nerve sheath tumors. The most common are neurofibromas, which are benign tumors that can occur anywhere along the length of epidermal, dermal, deep peripheral (including dorsal nerve roots in the paraspinal area), or cranial nerves. They do not occur in the brain or the spinal cord proper (5). Although benign, neurofibromas can cause considerable morbidity by, for example, infiltrating and functionally impairing normal tissues, causing limb hypertrophy, or masking an emerging malignancy. Some types of neurofibromas can transform into malignant peripheral nerve sheath tumors (MPNST), previously termed neurofibrosarcoma or malignant schwannoma. An estimated 20-50% of MPNSTs are associated with NF1 disease (6) and they are a significant cause of the decreased life expectancy in the NF1 patient population (7, 8). Recent population-based longitudinal studies detected an annual incidence of 1.6/1000 and a lifetime risk of 8-13% for NF1-associated MPNST (9), which is much higher than detected in previous cross-sectional studies (10) and over three orders of magnitude greater than that of the general population (~0.001%) (11). Furthermore, NF1-associated MPNST were diagnosed at an earlier age compared to sporadic tumors (26 vs. 62 years, $p<0.001$) and associated with a poorer prognosis compared to sporadic MPNST (5 yr survival of 21 vs. 42%, $p=0.09$) (9).

This article will review the genetic abnormalities that have been identified at the level of the gene, chromosome, and genome in NF1-associated neurofibromas and MPNST. Identifying commonly-associated genetic alterations and the mechanisms by which they arise, may potentially lead to markers for tumor staging, to new research approaches to pathogenesis, and to the identification of gene targets, which in conjunction with NF1, will be useful for mouse models.

THE MOLECULAR BASIS OF NF1

NF1 is a common autosomal dominant disorder that affects about 1/3500 individuals worldwide. About 30-50% of cases are sporadic, caused by *de novo* mutation in the *NF1* gene of an individual without a family history of the disorder. In addition to predisposing to tumorigenesis, NF1 is associated with characteristic changes in pigmentation and can be associated with a wide range of other manifestations such as learning disabilities and bony abnormalities (reviewed by (12-16). All cases are caused by mutation of the *NF1* gene at chromosome 17 band q11.2, which contains 60 exons that encode the 2818 amino acid protein called neurofibromin (17-21). Neurofibromin is widely expressed, predominantly in the central nervous system and sensory neurons and Schwann cells of the peripheral nervous system (22, 23). One functional domain of neurofibromin has been defined, the GAP-related domain (GRD), so called because of its structural and functional homology to mammalian p120-GAP (GTPase activating protein) and yeast genes known to regulate the Ras pathway (24, 25). Ras-GAP proteins function as negative regulators of Ras by catalyzing the conversion of active GTP-bound Ras to the inactive GDP-bound Ras form (26). In NF1-associated peripheral nerve sheath tumors, it is hypothesized that neurofibromin deficiency leads to increased activated Ras, resulting in aberrant mitogenic signaling and the consequent growth of a tumor. The identification of an NF1 patient with a missense mutation in the GRD that specifically abolished the RAS-GAP activity of neurofibromin demonstrated the importance of neurofibromin GAP function in NF1 pathogenesis (27). In at least some tissues, there is evidence that it is the Ras-GAP activity that accounts for the tumor suppressor function of neurofibromin. The most complete evidence comes from genetic and biochemical analyses of NF1-associated malignant myeloid disorders (28, 29). The tumor suppressor function of *NF1* has been reviewed recently (30-32) along with its potential as a therapeutic target (33).

NF1 disease is caused by haploinsufficiency for neurofibromin. The vast majority of *NF1* mutations are not in the GRD, but are distributed throughout the gene. Over 80% of mutations inactivate or predict inactivation of

neurofibromin; splicing defects and sequence alterations that create a premature translation termination codon are the most common (34, 35). About 10% of *NF1* mutations are missense (35, 38), which are typically clustered in the GRD or an upstream cysteine/serine-rich domain that may play a role in ATP binding (38). Definitive evidence that neurofibromin haploinsufficiency underlies NF1 came with the identification of *NF1* whole gene deletions in an estimated 5-10% of affected individuals (36, 37). Mutational analyses of patients with specific features, such as plexiform neurofibroma (39), spinal neurofibroma (40), or malignant myeloid disorders (41) failed to detect any correlation between genotype and phenotype. A notable exception is the subset of patients heterozygous for a microdeletion spanning the entire *NF1* gene, who consistently show an early age at onset of cutaneous neurofibromas (see below).

Virtually all NF1 patients develop neurofibromas. Neurofibromas are comprised of an admixture of largely Schwann cells and fibroblasts, along with mast cells, endothelial cells, and pericytes (42, 43). Although classification schemes vary, Friedman and Riccardi (13) define 4 types of neurofibromas. Discrete cutaneous neurofibromas of the epidermis or dermis are the most common, typically appearing near or at puberty and increasing in number to over 100 by the fourth decade of life (44). They are a localized tumor of small nerves in the skin that feels fleshy and soft; they are more prevalent on the trunk but also occur frequently on the face and extremities. Discrete subcutaneous neurofibromas have a spherical or ovoid shape, feel firm or rubbery and may be painful or tender. Deep nodular neurofibromas, also called nodular plexiform neurofibromas, involve major or minor nerves in tissues beneath the dermis. Upon gross pathology, they appear to grow inside the peripheral nerve causing a fusiform enlargement and may extend the entire length of a nerve (43). Diffuse plexiform neurofibromas have a cellular composition similar to that of cutaneous neurofibromas but in contrast, they have a tendency to become locally invasive (5, 43). Histologically, this tumor is a tangled network involving multiple nerve fascicles or branches of major nerves with poorly defined margins that makes complete surgical resection virtually impossible. Plexiform neurofibromas can be superficial with extensive involvement of underlying tissues or they may involve deep tissues, particularly in the craniofacial region, paraspinal structures, retroperitoneum, and gastrointestinal tract (13). They can infiltrate soft tissues resulting in localized hypertrophy and significant functional impairment. In contrast to other types of neurofibromas, diffuse plexiform tumors are considered congenital because they typically become evident in infancy or childhood and rarely, if ever, in late adulthood (45, 46). In a population-based study, 32% of individuals with NF1 had a plexiform neurofibroma(s) on physical examination (47). Korf (5) has recently reviewed plexiform neurofibromas.

Plexiform neurofibromas, both diffuse and nodular, are at a greater risk of transforming to an MPNST compared to other types of neurofibromas (reviewed in 5, 43, 48). Pathological examination of MPNST from individuals, both with and without NF1, most often shows an association with a neurofibroma (11, 49-51). These data suggest that an early step in MPNST development may be pre-neoplastic process in the nerve sheath. Multiple pathological and molecular criteria are used to evaluate a neurofibroma for malignant transformation (48). Several lines of evidence are consistent with cutaneous and plexiform neurofibromas and MPNSTs being clonal tumors that arise from an ancestral Schwann cell (see below).

GENETICS OF NEUROFIBROMAGENESIS

Homozygous inactivation of *NF1*.

Homozygous inactivation of a tumor suppressor gene(s) is a fundamental mechanism of tumorigenesis. It occurs by either sequential somatic inactivation of both alleles or by a somatic mutation in the single normal homolog in individuals who inherit a germline mutation in one allele. Somatic inactivation is frequently associated with loss of heterozygosity (LOH) at the tumor suppressor locus and at multiple flanking loci (reviewed in 52, 53). Evidence for such "2nd hit" somatic *NF1* mutations in neurofibromas has been sought in support of the hypothesis that neurofibromin functions as a tumor suppressor in Schwann cells. Initial reports of LOH at *NF1* (54) have been confirmed and extended by analyses of both primary tumor tissue and neurofibroma-derived Schwann cells. At least 25% of neurofibromas undergo LOH at *NF1* (55, 56). The cellular admixture in neurofibromas can mask allelic loss (55, 56), which is the likely explanation for reports that detected few, if any, tumors with LOH (57-62). Compelling evidence that the somatic inactivation of the *NF1* gene itself is important was provided by the identification of a 4 bp deletion in exon 4b in a cutaneous neurofibroma of a patient with a germline *NF1* microdeletion (63). Subsequent analyses of cDNA from neurofibromin-derived Schwann cells showed that 19% of neurofibromas carried somatic intragenic mutations, which were typically mRNA splicing defects (34). Mutation frequency may be underrepresented due to the difficulty of recovering high quality tumor RNA and the under-representation of mutant transcripts observed in some tumors. The latter could be attributed to mutations that induce nonsense-mediated decay or other mechanisms that affect mRNA

content (reviewed in 64) or to reduced expression for other reasons (34), and/or a low proportion of mutant to normal Schwann cells in some tumors (65, 66). Homozygous inactivation of *NF1* also occurs in plexiform neurofibromas, where an estimated 40% (n=10) showed LOH (67); a result confirmed by subsequent reports (57, 58, 68, 69).

A predominant mechanism of somatic *NF1* inactivation in neurofibromas that underwent LOH was mitotic recombination (56). A 17q proximal single mitotic recombination event near the centromere of the q arm between the normal chromosome 17 and the homolog carrying the germline *NF1* mutation can generate a cell in which both *NF1* genes carry the germline mutation and all loci distal to the recombination site are identical. Less common were double recombination events that result in chromosome 17 interstitial loci showing LOH. *NF1* mRNA in some neurofibromas is edited such that an arginine codon is changed to a nonsense codon (70). Although only one-third of neurofibromas examined showed a low level (<2.5%) of edited *NF1* transcripts (71), such modulation of neurofibromin expression may be important if, for example, editing occurred at high frequency in transcripts from a specific minor cellular component of the tumor. In other neurofibromas, somatic mutations appear to destabilize *NF1* mRNA (66). Transcriptional silencing via hypermethylation of promoter regions, a prominent mechanism of inactivating other tumor suppressor genes (72), has not been detected in neurofibroma tissue (73, 74). As expected from the mutational and LOH analyses, some neurofibromas had no detectable *NF1* transcripts or neurofibromin (75, 76). A quantitative Ras activity assay demonstrated that activated Ras-GTP levels were about four-fold higher in neurofibromas compared to levels in non-NF1-associated schwannomas (75, 77).

Neurofibromas are most likely clonal tumors derived from a Schwann cell progenitor. The detection of LOH in a tumor operationally defines it as being clonal in origin and these data are consistent with direct marker analyses in neurofibromas (57, 61). LOH or other somatic mechanisms that inactivate *NF1* in a Schwann cell progenitor may be an early or initiating genetic event in neurofibromagenesis. Klewe and colleagues showed LOH in primary neurofibroma tissue and in tumor-derived Schwann cells, but not fibroblasts (78). Other investigators confirmed this observation (66, 68). Two genetically distinct Schwann cell populations, $NF1^{+/-}$ and $NF1^{-/-}$, were successfully cultured from 10 mutation-characterized neurofibromas, while tumor-derived fibroblasts carried only the germline $NF1^{+/-}$ genotype (65). Sherman et al., (79) used an elegant single cell Ras-GTP assay to show elevated levels in neurofibroma-derived Schwann cells but not fibroblasts. Consistent with two Schwann cell populations, only a fraction (12-62%) of neurofibroma-derived Schwann cells had elevated Ras-GTP levels. The basis of the Schwann cell heterogeneity is not known, but the authors speculate that the cells with high Ras-GTP (presumably neurofibromin-deficient) may recruit Schwann cells with lower Ras-GTP levels (presumably the constitutional neurofibromin-haploinsufficient cells) via the synthesis of growth factors. Whether this admixture of Schwann cells is important in tumorigenesis remains to be determined, however, its observation in primary neurofibroma tissues makes it more likely. Fuorescence *in situ* hybridization and immunohistochemistry demonstrated that S-100 protein (Schwann cell marker) immunopositive cells in sections of 4 out of 7 primary plexiform neurofibromas were monosomic for *NF1* (80). Since other cells types were disomic at *NF1*, these results strongly implicate the Schwann cell as the target of the *NF1* 2nd hit mutation and verify that the results from neurofibroma-derived Schwann cells were not biased by cell culture. These data also support Schwann cell genetic heterogeneity, as the fraction of S-100 protein positive cells showing *NF1* deletion ranged from 50-93%. Further evidence for the importance of Schwann cells in neurofibromagenesis comes from the tumorigenic properties exhibited by neurofibroma-derived cells (81-84). In the most comprehensive studies, Muir and colleagues (83, 84) showed that neurofibromin-deficient Schwann cells derived from either dermal or plexiform neurofibromas, had high invasive potential and produce neurofibroma-like tumors when engrafted into peripheral nerves of *scid* mice.

Germline alterations that may modify neurofibromagenesis

The considerable variable expressivity of NF1 disease among family members with presumably the same *NF1* mutation and results of a statistical trait analysis led to the contention that variation in an individual's genetic background modified the NF1 phenotype (46, 85). To date, no such germline modifying genes have been identified. However, the study of patients carrying *NF1* microdeletions has provided compelling evidence for a gene that modifies neurofibromagenesis. We first recognized that *NF1* microdeletion patients typically showed an early age at onset of localized cutaneous neurofibromas or, in cases in which age at onset could not be documented, the had significantly greater numbers of cutaneous neurofibromas relative to their age (37, 86). It is common to observe multiple cutaneous neurofibromas during physical examination of children under age 10 that are heterozygous for an *NF1* microdeletion (37, 87, 88), while only about 10% in the general NF1 population have a tumor(s) by that age (47). In our most severe case, a 5 year old with an *NF1* microdeletion had 51-100

tumors (37). *NF1* microdeletions can be inherited from an affected parent and in these cases, the microdeletion co-segregated with the early age at onset of neurofibromas (88, 89). Other investigators have confirmed the early onset and heavy burden of cutaneous neurofibromas in *NF1* microdeletion patients and over 50 such patients have been identified (90-98).

Most *NF1* microdeletions span 1.5 MB of DNA (87), that harbors the entire 350 kb *NF1* gene and at least 11 additional genes, most of unknown function (87, 99). The microdeletions are preferentially maternal in origin (87, 91, 93, 100, 101) and arise by homologous recombination between 60 kb misaligned paralogs (termed NF1REP) that flank the *NF1* gene (87). Paralogs are nonallelic DNA sequences with a high degree of identity that arose via duplication, for example, the two functional α globin genes. Over 25 human disorders are known to be caused by gene or chromosomal rearrangements mediated by homologous recombination between paralogs, a process correctly referred to as nonallelic homologous recombination in the review by Stankiewicz and Lupski (102). Here, I propose that this process be called by the less awkward term of paralogous recombination and that disorders arising by this mechanism by called paralogous recombination disorders, rather than the currently used genomic disorders. Although the NF1REPs share >95% sequence identity over 60 kb of sequence, about 80% of the *NF1* deletion alleles are virtually identical because their breakpoints map to one of either two recombination hotspots of several kb in size located within the NF1REP (103)(M. Dorschner et al., in preparation). Although the molecular basis for these hotspots is not yet known, their existence means that the majority of microdeletion patients will be deleted for the same set of genes. The early age at onset of cutaneous neurofibromas observed in several patients with larger deletions and/or different breakpoints indicate that generation of a deletion-specific fusion gene product is unlikely (87).

We hypothesized that the co-deletion of *NF1* and an unknown linked gene potentiates cutaneous neurofibromagenesis (37, 87). What might be the function of the putative neurofibromagenesis-potentiating locus (*NPL*)? Here I propose two models for the early age at onset of cutaneous neurofibromas in microdeletion patients. Haploinsufficiency for *NPL* could increase the frequency of somatic 2nd hit mutations in the *NF1* gene. This could result from a genomic instability in microdeletion patients, which is intriguing in view of reports detecting cytogenetic abnormalities and microsatellite instability in some neurofibromas (see below). It would be interesting to determine if somatic *NF1* mutations in cutaneous neurofibromas of microdeletion patients occur by a predominant mechanism. Only a single tumor from each of 2 deletion patients have been analyzed, one had a 4 bp intragenic deletion (63) and the other a splice site (65). A second model for early onset of cutaneous neurofibromas proposes that *NPL* haploinsufficiency increases the probability that a neurofibromin-deficient progenitor cell proliferates and manifests as a neurofibroma. Multiple mechanisms could be proposed. For example, *NPL* could encode (or regulate) a cytokine, cell cycle regulator, tumor suppressor, or oncogene, which exerts a positive proliferative advantage on the progenitor cell. Because of the cellular heterogeneity of neurofibromas, this model does not necessarily require that the abnormal expression of *NPL* or its putative downstream targets occur in the neurofibromin-deficient Schwann clone.

Preliminary, but intriguing evidence suggests that genetic background, other than *NF1* microdeletion, may influence the somatic inactivation of *NF1*. In patients with multiple neurofibromas, it was determined that each of the tumors showed the same type of somatic mutation event (e.g., LOH of the entire q arm or interstitial LOH)(56). Depending upon the extent of LOH and the particular genes involved, this could explain differences in the age at onset and/or numbers of neurofibromas that develop in an individual.

Somatic alterations that may modify neurofibromagesis.

NF1-associated neurofibromas have been analyzed for genetic abnormalities at the chromosomal level by comparative genome hybridization (CGH) and cytogenetic analyses. CGH is a powerful technique to detect and map chromosomal regions with copy number imbalances in tumor specimens (reviewed in 104). Only 2 out of 8 neurofibromas (type not specified) examined showed chromosomal imbalances; one tumor showed 3 gains, the other only a single gain (105, 106). This observation is consistent with cytogenetic studies. Although Schwann cell cultures from dermal neurofibromas had normal karyotypes, cells derived from plexiform neurofibromas had abnormalities, which in some tumors consisted of unrelated non-clonal abnormalities (107). One plexiform had structural abnormalities predominantly involving telomeres, which are typically associated with genomic instability in other syndromes/tumors (107). Wallace et al. proposed that chromosomal abnormalities might be important in the development of plexiform neurofibromas. Chromosomal abnormalities in plexiform neurofibromas may account, at least in part, for their increased risk of malignant transformation. Whether other cellular components of neurofibromas show cytogenetic abnormalities is unclear. Some neurofibroma fibroblast-like derived cultures were reported to show an increased frequency of chromosomal aberrations (108), while others were typically negative (109).

Conflicting data have been reported regarding the presence of microsatellite instability in NF1-associated neurofibromas. Some human tumors, most notably those of patients with hereditary nonpolyposis colon cancer HNPCC, show microsatellite instability, which is detected by random changes in the length of microsatellite (simple nucleotide repeats) loci. Length mutations at multiple microsatellite loci in a tumor reflect a genome-wide instability, which in the case of HNPCC is due to a defect in any of several mismatch repair genes (reviewed in 2). Ottini et al. (110) reported that 50% (n=16) of neurofibromas showed altered allele lengths compared to matched normal tissue and instability at chromosome 9 loci has also been reported (111). Birindelli et al. (112) observed instability in a primary MPNST and a metastasis in one of 25 cases. However, no evidence of instability was detected in two subsequent studies of 80 neurofibromas, of which 5% appear to be of the plexiform type (55, 73). This disparity may be due to technical differences, the number and type of microsatellite loci examined, and/or the stage of the neurofibromas. LOH at *NF1* was not a factor, as all three studies analyzed neurofibromas that were both positive and negative. Of the 8 neurofibromas with microsatellite instability, 7 were unstable at only one of the 5 loci tested (110). Due to the important implications that microsatellite instability would have for neurofibromagenesis, additional loci should be examined in these tumors including those analyzed by the other investigators. In addition, it would be interesting to know if the neurofibromas that showed microsatellite instability were of the larger, central plexiform type, in which case they may have been transforming to malignancy. The proportion of loci that display instability is known to increase with tumor progression in HNPCC (113).

GENETICS OF MPNST DEVELOPMENT

Homozygous inactivation at *NF1*.

The homozygous inactivation of *NF1* in NF1-associated MPNST, first reported by Skuse et al. (60), has been confirmed in about 50% of tumors (n=22) by LOH (67, 109, 112, 114, 115). Mutations in both *NF1* alleles have been identified in a single MPNST (114). Although the mechanism of LOH is not known, it does not generally involve cytogenetically-detectable losses at 17q1 (116). Furthermore, NF1-associated MPNST derived cell lines showed decreased or absent neurofibromin and high levels of active Ras-GTP (117, 118) and a quantitative Ras activity assay demonstrated that activated Ras-GTP levels in tumors were about 15-fold higher compared to levels in non-NF1-associated schwannomas (77). Because homozygous inactivation of *NF1* occurs in benign neurofibromas, it is considered an early or initiating event that is necessary and sufficient for neurofibromagenesis but not MPNST development. Malignant transformation is presumably driven by predisposing genetic factors in the germline and/or by additional somatic mutations and positive growth selection in a malignant precursor cell. The role of *NF1* in the development of sporadic MPNST is not clear; only about 10% of these tumors show LOH at *NF1*(112).

Germline alterations that may modify MPNST development.

Little is known about germline genetic modifiers that predispose to MPNST. Early speculation that patients with *NF1* microdeletion may be at increased risk for malignancy (36) is now supported by indirect evidence. Mutational analysis of germline DNA from 7 patients that developed MPNST, determined that 3 (42%) were heterozygous for an *NF1* microdeletion (119). In another study, 2 of 17 (11%) unrelated *NF1* microdeletion patients developed MPNST and in addition, affected first-degree relatives of two microdeletion patients had other malignancies (87). Although additional cases are needed, these data suggest that the lifetime risk of MPNST in microdeletion patients may exceed the already high 10% in the general NF1 population (9). If so, the underlying mechanism may be essentially the same as that proposed above for early onset neurofibromagenesis. Deletion of the putative *NPL* gene could result in genomic instability or exert positive growth selection for the malignant clone. If microdeletions do predispose to both cutaneous neurofibromas and MPNST, it seems reasonable to speculate that in at least this subset of patients, either the discrete cutaneous type of neurofibroma is at increased risk of malignant transformation or the frequency of nodular or diffuse plexiform neurofibromas is high.

Evidence from two families suggests the intriguing possibility that MLH1-deficiency predisposes to NF1 and early onset extracolonic tumors (120, 121). Germline heterozygous inactivating mutations in *MLH1* cause inefficient DNA mismatch repair, with the consequent increase in mutation frequency and susceptibility to hereditary nonpolyposis colorectal cancer (reviewed in 122). Two rare and independent cases of consanguineous marriages between *MLH1* heterozygous first cousins each produced two children with NF1 disease and hematological malignancies (120, 121). The parents had no signs of NF1 and there was no family history of the disease. MLH1 mutations were identified confirming homozygosity in 3 of the 4 deceased children, who presented with mutiple café au lait spots (4/4), dermal neurofibromas (2/4), tibial pseudoarthrosis (1/4), non-

Hodkin's lymphoma (2/4), myeloid leukemia (2/4), and medulloblastoma (1/4). The authors suggest that these patients had a *de novo* post-zygotic *NF1* mutation and that the *NF1* gene may be preferentially susceptible to mismatch repair deficiency. Unfortunately, the *NF1* gene was not analyzed for mutations, due in part to lack of patient tissue (120), which could have confirmed mutation of *NF1* and differentiated between post-zygotic mutation and germline mosaicism in a parent.

Somatic alterations in NF1-associated MPNST

All NF1-associated MPNST showed significant chromosomal imbalances by CGH (105, 106, 123, 124). Analysis of 27 total tumors from 19 NF1 patients showed an average of >7 imbalances per MPNST (Table 1). One tumor had only a single imbalance, a gain of chromosome 8 (123). The studies lead by Mechtersheimer and Schmidt (105, 106), which include 74% of tumors examined, detected chromosomal gains more frequently than losses (Table 1). Each of the 20 tumors in these studies had ≥4 chromosomal gains. While the results of these two studies were comparable, they differed from a third study that found chromosomal losses more prevalent than gains (123). The reason for the disparity is unclear, however it was also evident in the analyses of sporadic MPNST in each study (Table 1). Table 2 summarizes the chromosomal segments that most frequently showed gains in NF1-associated MPNST. The most common segments were on 17q22-q24, 17q25, 7p14, 7p21, 8q22, 8q23-q24, and 7q31. Chromosomal loss of these segments was rarely, if ever, observed (105, 106, 123). A combined analysis of sporadic and NF1-associated MPNST revealed a significantly decreased survival rate of patients with MPNST with gains at both 7p15-21 and 17q22-qter (124).

In addition to chromosomal gains, CGH analyses also revealed large-scale chromosomal amplifications (105, 106, 124). One-third of MPNST of both NF1 and non-NF1 patients had at least one amplified chromosomal segment (Table 3). Although the number of tumors is small, there are differences in amplification patterns. In NF1-associated MPNST 7p and 17q22-qter, the same regions that commonly showed chromosomal gains (Table 1), were amplified frequently. The frequency of 7p amplifications may be overestimated since these 4 MPNST (considered as different primary tumors) were from a single patient (106, 124). In contrast, amplifications of 5p, 8q, and 12q were the most prevalent in sporadic MPNST. There was a significant difference in the frequency of tumors with more than one chromosomal segment amplified. Only 10% of NF1-associated MPNST had more than one amplified segment, while the frequency was 72% in sporadic tumors (Table 3). Differences in the location and number of chromosomal segments amplified were unlikely to be due to tumor grade, as the majority of MPNST, 14/20 (70%) of NF1-associated and 22/34 (64%) of sporadic, were grade 3 (poorly differentiated). Although amplifications were more frequent in NF1-associated compared to sporadic MPNST (50% vs. 32%), the number of NF1-associated tumors may be overestimated because it includes multiple tumors from the same patient. Adjustment for one tumor/patient gives a frequency of 25% (5/20) for NF1-associated MPNST. Similarly, there was no correlation between chromosomal amplification and progression to metastasis. Analysis of tissue from a primary tumor, recurrence, and metastasis from a single patient showed a single amplification (8q12-qter) in the recurrent tumor (124).

Comparison of the chromosomal losses detected in the 3 CGH studies revealed the following 5 most frequent losses in 27 NF1-associated MPNST: 13q21-22 (12/27), 11q23-25 (10/27), 1p22-31 (9/27), 3q11-21 (8/27) and 17p12-pter (7/27) (105, 106, 123). Interestingly, the loss of 17p12-pter, was detected in 50% (7/14) tumors in one study (106) and in none of the tumors of the other studies. The relatively low detection rate of chromosomal losses may be due to the decreased sensitivity of CGH in polyploid MPNST (125).

CGH analysis of multiple, presumably synchronous or metachronus, primary MPNST at different sites in three NF1 patients revealed a remarkable similarity in chromosomal gains and losses (106, 124). For example, five grade 3 (poorly differentiated) tumors of one patient each showed imbalances of +7p, −13q21, and +17q22-qter. These data showed that in the specific genetic background of each patient, a relatively limited, and defined, number of rearrangements were shared among the tumors. Similarly, nearly identical aberrations were found in different MPNST from the same patient (125). Although limited, these data suggest that each individual's constitutional genotype sets a certain "baseline" upon which a minimal and limited number of genetic alterations are necessary and sufficient for MPNST development.

Consistent with CGH analysis, the karyotype of NF1-associated MPNST-derived cells are complex with chromosomal numbers ranging from 34-270 indicative of hypodiploidy, hypotriploidy, hypotetraploidy and hypertriploidy and hypertetraploidy (109, 116, 125 and references therein). While breakpoints were frequent, a common specific breakpoint was not detected. A comparison of CGH and karyotying in 6 MPNST revealed significant overlap in the most frequent gains and in addition, detected losses at 19q (3/6 tumors), a region not analyzed in the CGH studies (106, 125). Plaat et al. (116) performed a computer-assisted cytogenetic analysis of 46 MPNST reported in the literature and 7 new cases of both NF1-associated and sporadic tumors (116 and

references therein). These studies confirmed the CGH observation of high frequency gains at chromosomes 7p and 7q and losses at 1p and 17p. However, their reported cytogenetic differences between NF1-associated and sporadic MPNST (116) were not confirmed in later studies (125, 126). In one study, near triploid or near tetraploid clones were associated with grade 3 tumors and a poor prognosis (126). The detection of a t(X;18) translocation in MPNST (127) was not confirmed in either sporadic (128) or NF1-associated neurofibroma or MPNST (129).

Inactivating mutations in several tumor suppressor genes have been identified in NF1-associated MPNST. Both LOH and intragenic missense mutations of *TP53* (50, 62, 112, 130) have been detected. Like many sarcomas, NF1-associated MPNST often showed overexpression of p53 (the protein product of the *TP53* gene) as assayed by immunoreactive positivity in the nucleus (50, 112, 131, 132). In keeping with findings in other tumors, this most likely is mutant p53 protein, which accumulates due to its increased stability. Mutant protein is thought to promote cancer by either complexing with and sequestering functional p53 or by complexing with p63 and p73, and blocking their normal transcription factor activities (reviewed in 133). Immunohistochemical detection of p53 was more common in NF1-associated versus sporadic MPNST and it was associated with poor prognosis in NF1 children (132). About 50% of NF1-associated MPNST showed homozygous deletion for exon 2 of the *INK4A* gene, and over 90% were immunonegative for its protein product p16 (112, 134, 135). Homozygous deletion of exon 2 results in deficiency for both p16 and $p14^{ARF}$, two proteins encoded by alternative splicing of *INK4A* (also known as *CDKN2A*). Both of these proteins are tumor suppressors that modulate activities of the RB and p53 pathways, which are critical for cell cycle control and tumor surveillance (reviewed in 136). MXI1 mutations in regions that encode known functional domains have been detected in the 2 NF1-associated MPNST analyzed (137). Mxi1 is an agonist of the oncoprotein Myc and is thought to limit cell proliferation and help maintain the differentiated state (reviewed in138). *Mxi1*-deficient mice develop tumors and *Mxi1* deficiency decreases the latency of tumors that arise in Ink4a-deficient mice (139). If additional studies show that somatic inactivation of MXI1 is common, it would suggest a link between the pathways of Ink4a, Myc, and Ras in NF1-associated MPNST (140, 141).

Several differences observed in NF1-associated MPNST are likely involved in the malignant transformation of a preexisting neurofibroma. *TP53* or *INK4A* mutations/altered expression were not detected in neurofibromas (50, 51, 54, 62, 67, 69, 112, 132, 134, 135). Furthermore, p53-positive nuclei, typically associated with MPNST, were observed in a few cells at the transitional zone between an existing plexiform neurofibroma and an arising MPNST (51). One plexiform neurofibroma proximal to an MPNST did show p16 immunonegativity (112). Microdissection of a preexisting neurofibroma from its MPNST focal malignant process revealed 5 chromosomal imbalances in the neurofibroma, all of which were novel compared to the 10 imbalances in the MPNST component (106). These data are consistent with a restructuring of the genome during transformation. An additional distinguishing feature of MPNST is the high labeling index of the nuclear proliferating antigen Ki67, which was correlated with reduced survival in a study that combined NF1-associated and sporadic MPNST (51, 132, 142).

OTHER NF1-ASSOCIATED NEOPLASMS AND MOUSE MODELS

In addition to peripheral nerve sheath tumors, individuals affected with NF1 are at increased risk for an array of other tumors. Epidemiologically-associated neoplasms include medulloblastoma, pheochromocytoma, astrocytoma, adenocarcinoma of the ampulla of Vater (143 and references therein)(144). Primarily children affected with NF1 are at increased risk for optic pathway gliomas and brainstem gliomas, rhabdomyosarcomas, and malignant myeloid leukemias (41, 145-149). NF1 patients are also at increased risk for a second malignancy, some of which may be treatment-related (146, 150, 151). In different studies, 8-21% of NF1 patients with a first malignancy developed a second cancer, compared to a frequency of 4% in the general population (143). Malignancy in NF1 has been reviewed recently (152).

Although heterozygous mutant mice ($Nf1^{+/-}$) develop tumors, they are not the characteristic peripheral nerve sheath tumors characteristic of the human disease (153-155). *Nf1*-deficient mice ($Nf1^{-/-}$) die in utero from cardiac defects. Mice chimeric for ($Nf1^{-/-}$) and ($Nf1^{+/+}$) cells were able to develop many microscopic plexiform neurofibromas, but dermal tumors did not develop (156). In addition, mice doubly heterozygous for mutations in *Nf1* and *p53* developed MPNST that showed LOH at both tumor suppressor loci (156, 157). Recently, mice were constructed such that only their Schwann cells were *Nf1* –deficient (158). Different tumor phenotypes were observed depending upon whether the *Nf1*–deficient Schwann cells were in an animal with an $Nf1^{+/-}$ or an $Nf1^{+/+}$ constitutional genotype. In the $Nf1^{+/-}$ genetic background, plexiform neurofibromas composed of *Nf1*–deficient

Schwann cells, and the fibroblasts and mast cells that normally occur in human neurofibromas, developed on peripheral nerves. In the $Nf1^{+/+}$ genetic background, *Nf1*–deficient Schwann cells did not participate in neurofibromagenesis but did form relatively small hyperplastic lesions of the cranial nerves containing minimal mast cells. This mouse model demonstrates that neurofibromin deficiency in Schwann cells is sufficient for generating nascent tumor lesions, but frank plexiform neurofibroma development requires neurofibromin haploinsufficiency in cells of the surrounding tissues.

With the development of mouse models, an understanding the genetics, pathology, and natural history of human benign and malignant peripheral nerve sheath tumors takes on new importance. It is only by accurate modeling of the human disease that progress can be made toward therapies that can slow or halt neurofibromagenesis and reduce the high risk of malignancy associated with NF1 disease.

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Table 1. Chromosomal gains and losses in MPNST detected by CGH.

	NF1-associated MPNST			Sporadic MPNST		
	Loethe[1]	Mechter-sheimer[2]	Schmidt[3]	Loethe[1]	Mechter-sheimer[2]	Schmidt[3]
# tumors / # patients	7 / 7	6 / 6	14 / 6	3 / 3	13 / 13	22 / 20
Imbalances	52	77	188	14	176	200
per tumor	7.4	11.7	13.4	4.6	13.5	9.1
range	1-17	6-30	7-29	1-7	0-34	0-25
Chromosome gains	14	48	139	4	125	179
per tumor	2	8.0	10.0	1.3	9.6	8.1
range	0-3	4-18	5-20	0-2	0-23	0-21
Chromosome losses	38	29	49	10	51	33
per tumor	5.4	4.8	3.5	3.3	3.9	1.5
range	0-14	1-12	0-11	1-4	0-11	0-9

[1]Reference123.
[2]Reference 105.
[3]Reference 106.

Table 2. Frequency of chromosomal gains in NF1-associated MPNST

	Loethe[1]	Mechter-sheimer[2]	Schmidt[3]	Total
# MPNST / # patients	7 / 7	6 / 6	14 / 6	27 / 19
Chromosome	% MPNST			
1q33	0	50	50	37
5p15	28	50	35	37
7p14	28	83	71	63
7p21	28	50	64	52
7q31	28	33	64	48
8q22	28	33	64	48
8q23-q24	14	33	85	55
15q24-q25	0	17	71	41
17q22-q24	42	83	85	74
17q25	71	83	78	78

[1]Reference 105, 123.
[2]Reference 105.
[3]Reference 106.

Table 3. Large scale chromosomal amplifications in MPNST[1]

Chromosome segment amplified	No. NF1-associated MPNST[2] (N=20)	No. Sporadic MPNST[3] (N=34)
4q12-q13		1
5p11-p15		1
5p14		1
5p15		2
5p13-pter		2
7p14-pter	2[4]	1
7p13-pter	1[4]	
7p12-pter	1[4]	
7p11-p12		1
8q12-qter		2
8q13		1
8q21-q22		1
8q22-q23		1
8q24		1
8q23-qter	1	
9p21-p23	1[5]	
9q31-q33		1
12p12	1[5]	
12p13		1
12q13-q14		1
12q14-q21		3
12q24		3
13q13-q33		1
13q32-q33		1
17p11-p12		1
17q24-qter	2	
17q22-q24	1	
20q12-qter		1
Xp11		1
Xp21-p22		1

Summary

% tumors with an amplification	50 (10/20)	32 (11/34)
% tumors with >1 amplified segment	0.1 (1/10)	72 (8/11)
% patients with ≥1 tumor with an amplification	30 (4/12)	32 (11/34)

[1]Centromeric regions, chromosomes 19 and Y, and 1p32-p36 were not scored for technical reasons (105).
[2]References 105, 106, 124.
[3]References 105, 124.
[4]Four out of 5 primary MPNST from one patient (106).
[5]Two of 4 primary MPNST from one patient (106).

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Chapter 20.

Neurofibromatosis

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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.

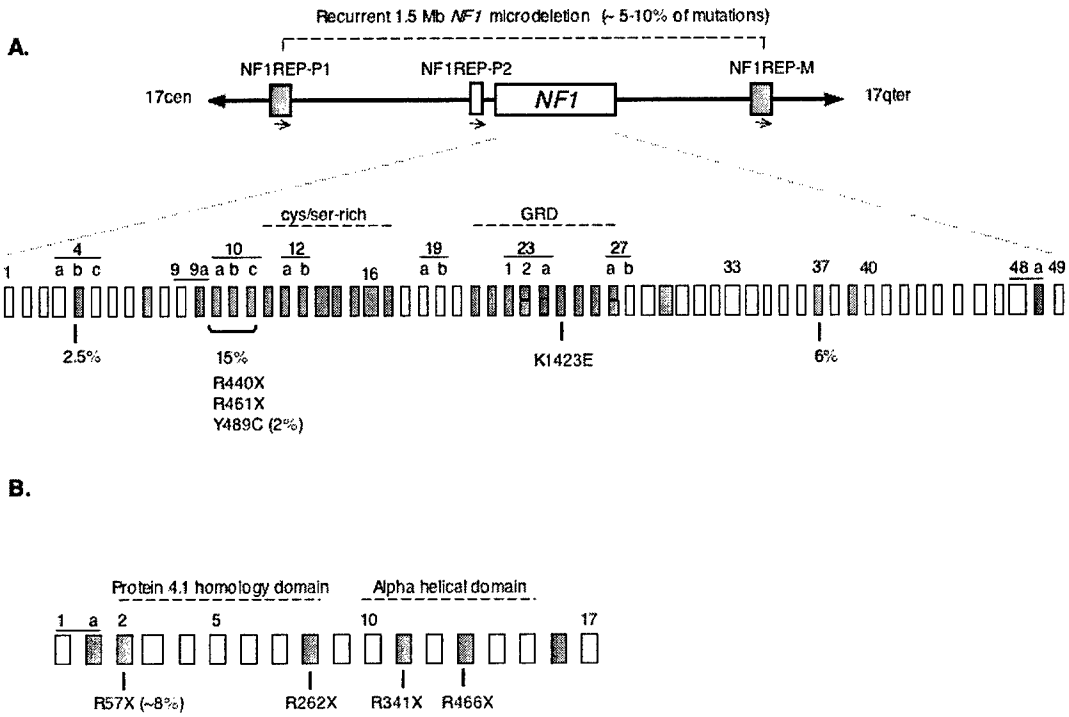
A.
Recurrent 1.5 Mb NF1 microdeletion (~ 5-10% of mutations)
NF1REP-P1
NF1REP-P2
NF1REP-M
17cen
NF1
17qter
cys/ser-rich
GRD
1
4
a b c
9 9a
10
a b c
12
a b
16
19
a b
23
1 2 a
27
a b
33
37
40
48 a 49
2.5%
15%
R440X
R461X
Y489C (2%)
K1423E
6%
B.
Protein 4.1 homology domain
Alpha helical domain
1 a
2
5
10
17
R57X (~8%)
R262X
R341X
R466X

Figure 1.

National Neurofibromatosis Foundation International Consortium for the Molecular Biology of NF1 and NF2, Aspen, CO, June, 2004.

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.