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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, of rapid and quantitative identification of loci with increases of decreases in genomic copy number in MPNST. We have developed new assays that are sensitive and specific and allow the quantification 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 will be used for both loss (deletion) of DNA and gain (amplifications) of DNA in tumor cell lines and primary tumors. Assays are in development for the target areas of chromosome 1p and 17q22-24. The amplification and loss of chromosomal material at these regions was confirmed by high resolution karyotypingin two neuroblastoma-derived cell lines that will be emplyed to test the methodology on tumor tissues. We have made significant progress in assay design for quantification of genomic losses and gains. Implementation of these assays will now test the usefulness of our idea of using QuEST for defining specific losses and gains in tumors.

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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 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 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. We have obtained a one year no-cost extension to complete this project, because our timeline was significantly delayed due to the departure of a postdoctoral fellow who was working on this project. A new postdoctoral fellow (Dr. Wang) is now focusing on the development, optimization, and validation of assays and a new Research Associate (Ms. Vong) is implementing those assays on appropriate samples. With these two new professionals, we anticipate accelerating the timeline to complete the project within the next year. We made significant changes in the methodology of quantitative PCR to increase the sensitivity and precision.

Approved Statement of Work

Task 1. Design and test a series of primers for analysis of both target loci and reference loci.
   a. Design and synthesize primers for genes in the target regions of chromosomes 1p and 17q22-24 and for reference loci.
   b. 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). Two gene deletion and gene amplification are 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 DNA from normal individuals (2 NF1 genes) and DNA from NF1 patients with deletions involving the NF1 gene (1 NF1 gene). 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 show). Secondly, we sought to increase specificity and sensitivity by using a NF1-specific fluorescently-labeled primer. We constructed a LUX primer (Invitrogen), which

4

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 multiple problems with LUX primers and decided to abandon that approach (data not shown). The third method we developed was precise, sensitive, and specific and involves SYBR green for detection, competitive quantitative PCR, and melting curve analysis as detailed below.

We chose to employ SYBR green for detection in combination with competitive PCR, which is the most suitable method of quantification when highly accurate determinations are required. We adapted and modified a method published by Ruiz-Ponte et al. (14). In this method, a known copy number of a competitor is introduced directly in the PCR mixture along with the target DNA of the patient/tumor. The competitor, which is almost identical to the target DNA but distinguishable by product length, is amplified with the same set of primers so that efficiency of amplification for the two amplicons is the same. Calibration curves of different competitor concentrations determines the optimal concentration that equals that of the target DNA. Figure 1 shows the melting curves of a normal control DNA samples (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). 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.

(see figures on next page)

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. The negative control without DNA shows evidence of amplification.
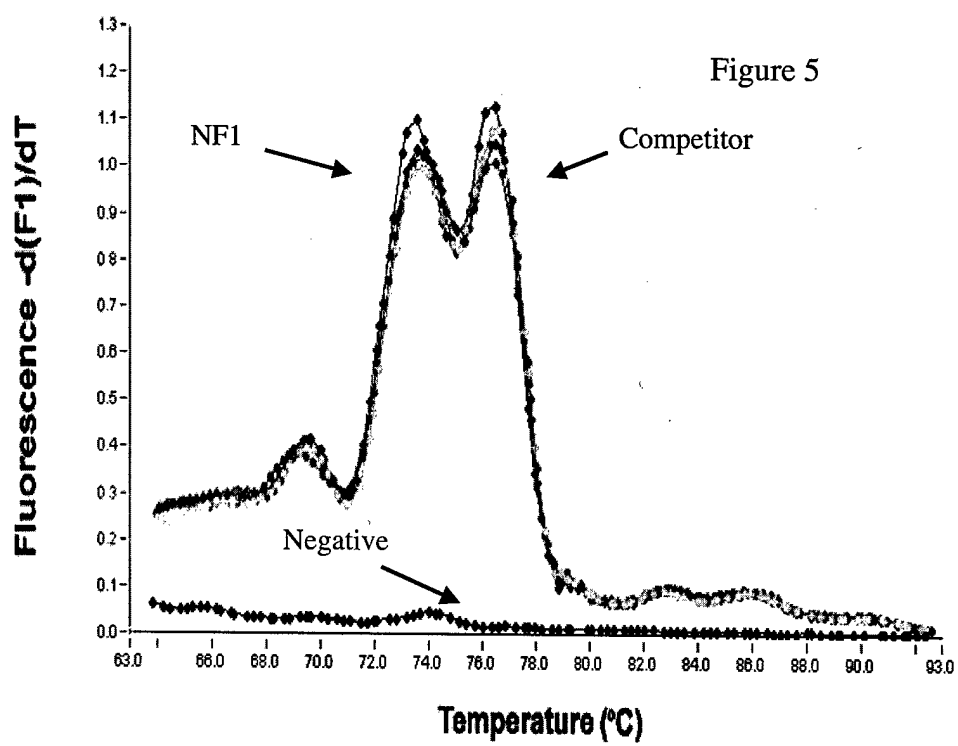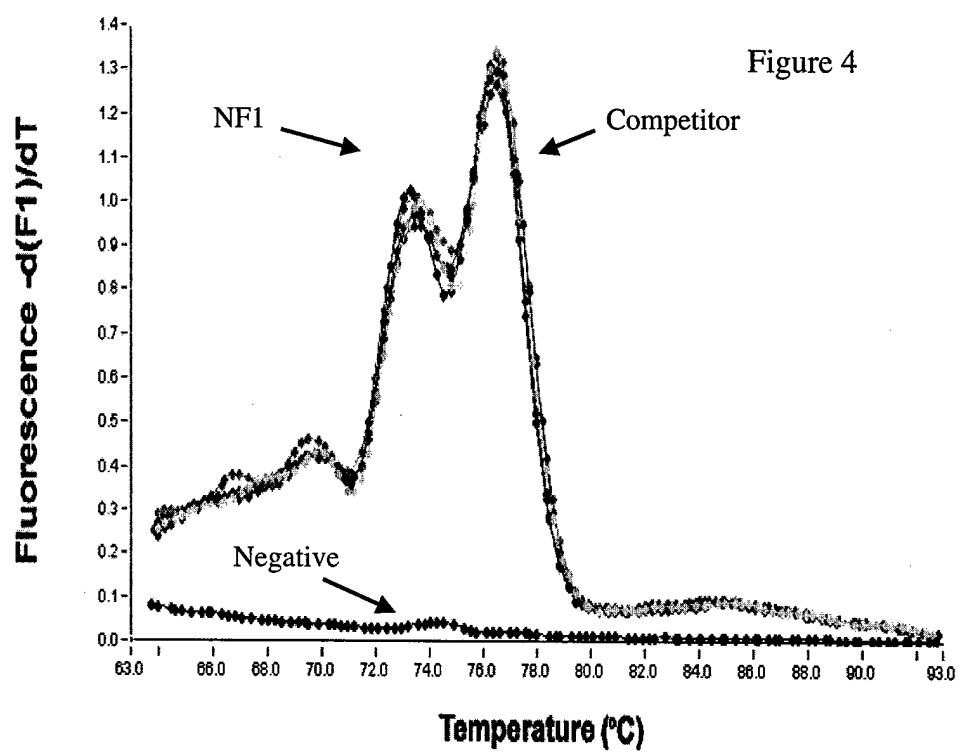
Figure 1

Figure 2
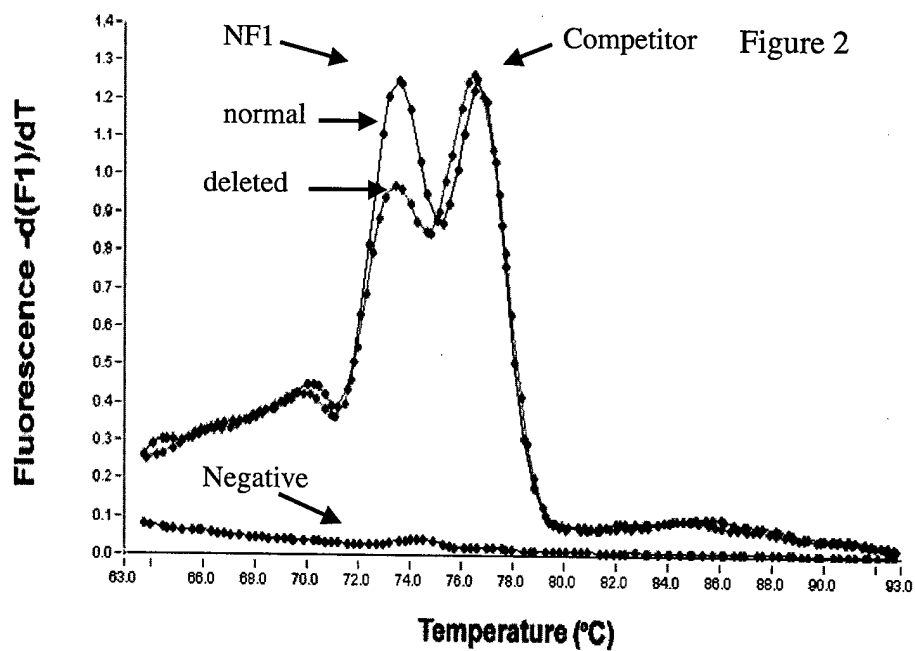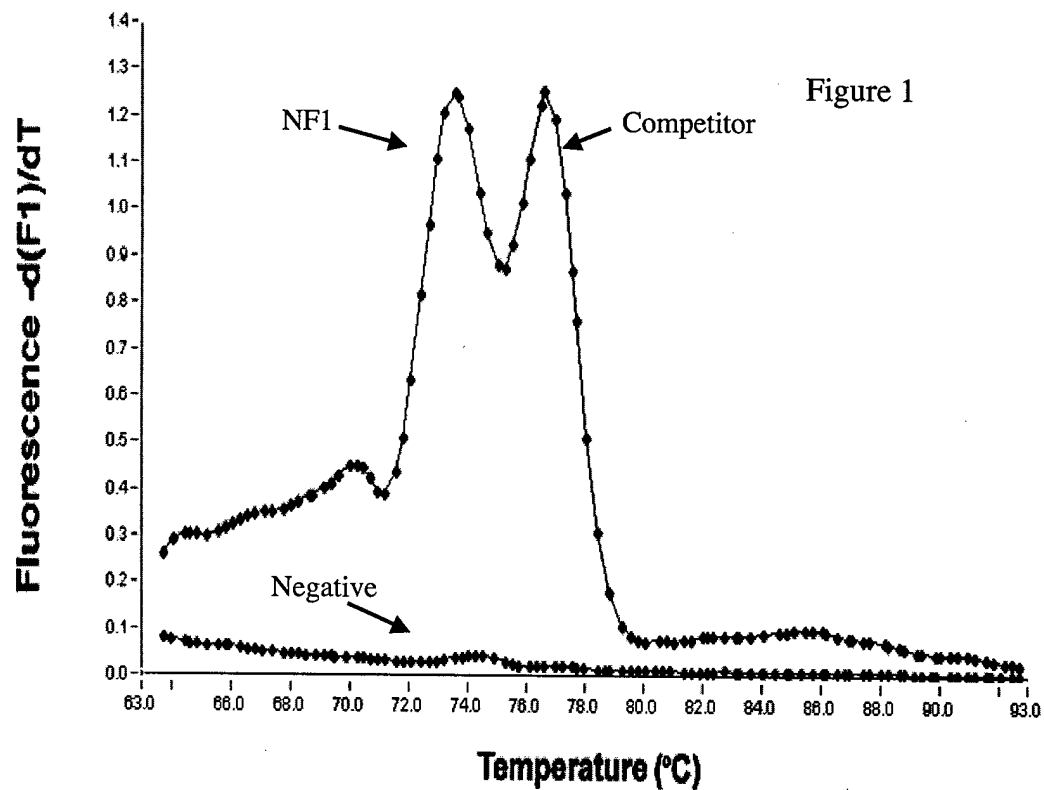
Once the concentration of competitor is determined for a certain concentration of the normal control DNA, it is essential that all subsequent reactions with unknown patient DNA samples contain exactly the same concentration of target DNA. Prior to the competitive quantitative PCR assay, we determine the exact concentration of each patient sample using real-time quantitative PCR at a different locus. We amplify the TPA (tissue plasminogen activator) gene on chromosome 12 in each patient and compare that to a standard curve using the normal control DNA. From this reaction, we can calculate exactly what volume of patient DNA must be added to the competitive quantitative PCR assay. An example of the TPA real-time PCR and standard curve is shown in Figure 3.

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

Figure 4

Figure 5

The competitive quantitative gene dosage assay we have developed for intron 31 of the NF1 gene is very precise and shows minimal variability. Figures 4 and 5 (see legends below and figures on next page) show melting curves for 6 replicates of an NF1 deletion patient and a normal control individual, respectively. The data will be analyzed as shown in the tables below. First, note the precision of the assay; the standard deviations are only 2% and 3% of the means of the assay for normal control individuals and deletion patients, respectively. As expected the ratio of NF1:competitor peak areas is essential 1.0 for samples with two copies of the NF1 gene and is 0.5 for samples with one copy of the NF1 gene. We have screened a large collection of NF1 deletion patients and normal individuals and found that there is no overlap of ratio values between these two populations.

Therefore, we have developed a sensitive and specific assay that differentiates realiably between one versus two copies of a gene. We are now applying this same assay to determine it's ability to quantitate amplified loci.

Replicates of competitive quantitative PCR for intron 33 of NF1
or normal control individual

No.	Area of *NF1* peak	Area of Competitor Peak	Ratio	Mean Ratio	S.D.
1	3.219	2.957	1.088		
2	3.262	3.296	0.989		
3	3.097	3.255	0.951		
4	3.464	3.26	1.062		
5	3.433	3.278	1.047		
6	3.091	2.918	1.059		
				1.033	0.0211

Replicates of competitive quantitative PCR for intron 33 of NF1
for deleted patient

No.	Area of *NF1* peak	Area of Competitor Peak	Ratio	Mean Ratio	S.D.
1	1.937	3.85	0.503		
2	2.103	3.68	0.571		
3	1.952	3.755	0.519		
4	2.052	3.356	0.611		
5	1.851	3.665	0.505		
6	1.934	3.33	0.580		
				0.548	0.0185

Figure 4. Melting curves of competitive quantitative PCR at NF1 intron 31 of 6 replicates of an NF1 deletion patient. Six curves (color replaced by grey tones for this report) are overlaid to demonstrate the precision and lack of variability in replicate samples.

Figure 5. Melting curves of competitive quantitative PCR at NF1 intron 31 of 6 replicates of a normal control individual. Six curves (color replaced by grey tones for this report) are overlaid to demonstrate the precision and lack of variability in replicate samples.

Figure 3

Slope =-3.528
Intercept = 20.45
Error = 0.0140
r =-1.00
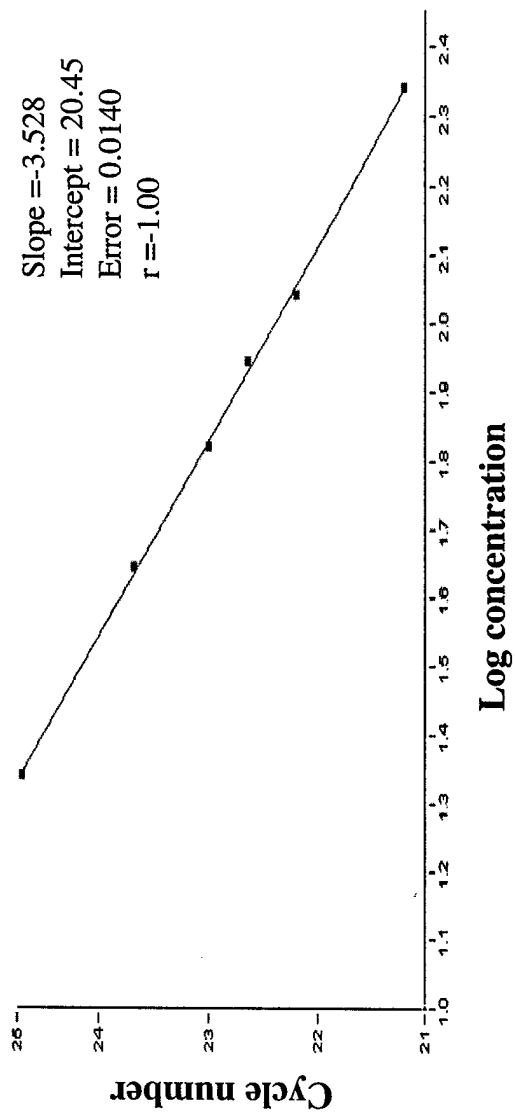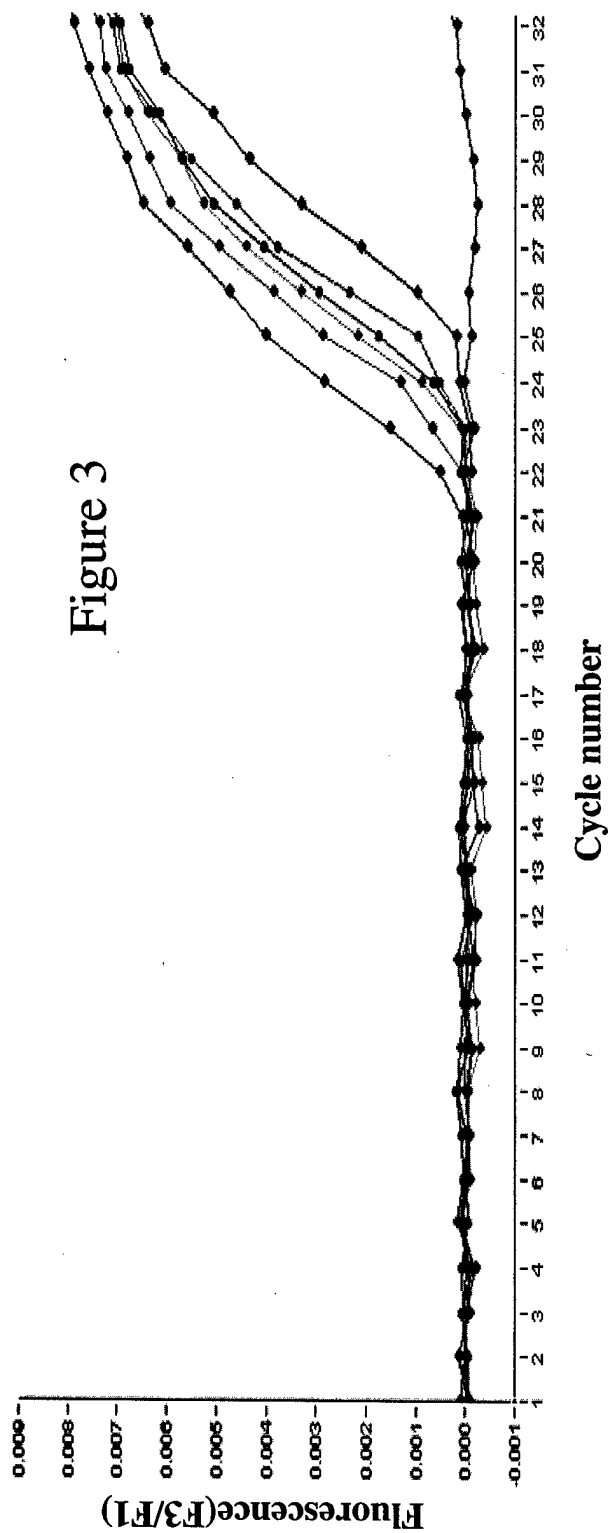
Task 2. Perform spectral karyotyping (SKY) of the MPNST- and neuroblastoma-derived cell lines. Months 1-12.

Progress: In collaboration with Dr. Karen Swisshelm, Department of Pathology, University of Washington, we have analyzed the karyotype of two of the neuroblastoma-derived cell lines. We have performed a routine metaphase G-banded karyotype, which was very informative in determining exactly which bands were deleted and or amplified. One line will be ideal for analysis of the amplified region of chromosome 1p and the other line for the deleted region of 17q22.24. Other regions of amplification and deletion were observed and may be targeted later in this project. Because this analysis was so informative, we do not plan to perform a SKY analysis unless our PCR results are inconsistent with the karyotype.

Dr. Stephens performed a comprehensive literature search and compilation of the genetic changes in NF1-associated peripheral nerve sheath tumors (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%).

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. We anticipate analysis of 4 lines.
      b. Evaluate the results of QuEST compared to the SKY analysis.

Progress: This analysis is in progress. Now that the competitive quantitative PCR methodology has been validated, we are developing and optimizing similar assays for loci that span the 1p and 17q22-24 regions. Development and ptimization of each assays includes:

- Choosing primer pairs for loci that are single-copy in the genome.
- Screening against the appropriate monochromosomal human-rodent hybrid cell line to ensure we are not amplifying related loci from other chromosomes
- Optimizing PCR reaction
- Developing competitor target DNA
- Titrating competitor target DNA to determine the concentration equivalent to the normal diploid DNA control target
- Performing real-time PCR at TPA locus for each tumor DNA sample (in reality, we will have 5 such loci as controls in case one of them is altered in the tumor sample). Determine the concentration to add to the competitive quantitative PCR assay.
- Performing competitive, quantitative PCR assay
- Analyzing data

As stated in the original Statement of Work, tasks 4-6 will be performed in the later period of the research project.

Task 4. Test the QuEST method by analysis of target and reference loci in DNA from tumors. This awaits completion of the assays in the tumor cell lines. Anonyous MPNST DNA from NF1 patients are being collected from collaborators for these future assays.

Task 5. Determine the sensitivity of the QuEST method.
    This await completion of Task 4.

Task 6. Couple QuEST with laser capture microdissection of archival MPNST.
    After Dr. Wang has the assays developed and they are being implemented by Ms. Vong, he will begin performing laser capture microdissection of archival MPNST at the University of Washington Medical Center and assessing the quality of the DNA using the TPA assay. When this protocol is optimized, we will apply the assays for deletion of 17q22-q25 region in MPNST.

Key Research Accomplishments

- 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 that is in press (see Reportable Outcomes).
- Developed a specific and sensitive competitive, quantitative PCR methodology that can differentiate one copy from two copies in a genome.
- Employed real-time PCR of the TPA locus as a means of precise DNA quantification
- Validated the competitive, quantitative PCR methodology by assay of the NF1 locus in normal control individuals and NF1 deletion patients.
- Documented the reproducibility of the competitive, quantitative PCR method.
- Performed and analyzed high-resolution karyotype of the two neuroblastoma-derived cell lines that we will be using to validate the competitive, quantitative PCR method for analysis of DNA gains and losses in tumor tissue.

Reportable Outcomes

- Manuscript: Stephens K. Genetics of Neurofibromatosis 1-associated peripheral nerve sheath tumors. Cancer Invest, in press. (See proofs of article in appendix).
- Karyotypes of two neuroblastoma-derived cell lines.
- Development of competitive, quantitative PCR assays for differentiating one versus two copies of NF1 gene intron 31.

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 new 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 will be used for both loss (deletion) of DNA and gain (amplifications) of DNA in tumor cell lines and primary tumors. Assays are in development for the target areas of chromosome 1p and 17q22-24. The amplification and loss of chromosomal material at these regions was confirmed by high resolution karyotyping in two neuroblastoma-derived cell lines that will be employed to test the methodology on tumor tissues. We have made significant progress in assay design for quantification of genomic losses and gains. Implementation of these assays will now test the usefulness of our idea of using QuEST for defining specific losses and gains in tumors.

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3   MOLECULAR BIOLOGY AND GENETICS OF CANCER

Genetics of Neurofibromatosis 1-Associated Peripheral Nerve Sheath Tumors

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INTRODUCTION

Much of our current understanding of tumorigenesis is founded on genetic studies of relatively rare individuals with inherited disorders that predispose to certain cancers, e.g., 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, antiapototic 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 focuses 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, e.g., 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 ($\sim$0.001%).[11] Furthermore, NF1-associated MPNST were diagnosed at an earlier age than sporadic tumors (26 vs. 62 years, $p < 0.001$) and associated with a poorer prognosis than sporadic MPNST (5-year survival of 21 vs. 42%, $p = 0.09$).[9]

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63 This article reviews the genetic abnormalities that
64 have been identified at the level of the gene, chro-
65 mosome, and genome in NF1-associated neurofibromas
66 and MPNST. Identifying commonly associated genetic
67 alterations and the mechanisms by which they arise
68 may potentially lead to markers for tumor staging, to
69 new research approaches to pathogenesis, and to the
70 identification of gene targets, which in conjunction
71 with NF1, will be useful for mouse models.

72 **THE MOLECULAR BASIS OF NF1**

73 The NF1 is a common autosomal dominant dis-
74 order that affects about 1 in 3500 individuals world-
75 wide. About 30%–50% of cases are sporadic, caused
76 by de novo mutation in the *NF1* gene of an individual
77 without a family history of the disorder. In addition
78 to predisposing to tumorigenesis, NF1 is associated
79 with characteristic changes in pigmentation and can be
80 associated with a wide range of other manifestations
81 such as learning disabilities and bony abnormalities
82 (reviewed by Refs. [12–16]). All cases are caused by
83 mutation of the *NF1* gene at chromosome 17 band
84 q11.2, which contains 60 exons that encode the 2818
85 amino acid protein called neurofibromin.[17–21] Neuro-
86 fibromin is widely expressed, predominantly in the
87 central nervous system and sensory neurons and
88 Schwann cells of the peripheral nervous system.[22,23]
89 One functional domain of neurofibromin has been
90 defined, the GAP-related domain (GRD), so called
91 because of its structural and functional homology to
92 mammalian p120-GAP (*G*TPase *a*ctivating *p*rotein) and
93 yeast genes known to regulate the Ras pathway.[24,25]
94 The Ras-GAP proteins function as negative regulators
95 of Ras by catalyzing the conversion of active GTP-
96 bound Ras to the inactive GDP-bound Ras form.[26] In
97 NF1-associated peripheral nerve sheath tumors, it is
98 hypothesized that neurofibromin deficiency leads to
99 increased activated Ras, resulting in aberrant mitogenic
100 signaling and the consequent growth of a tumor. The
101 identification of an NF1 patient with a missense muta-
102 tion in the GRD that specifically abolished the Ras-
103 GAP activity of neurofibromin demonstrated the
104 importance of neurofibromin GAP function in NF1
105 pathogenesis.[27] In at least some tissues, there is evi-
106 dence that it is the Ras-GAP activity that accounts for
107 the tumor suppressor function of neurofibromin. The
108 most complete evidence comes from genetic and bio-
109 chemical analyses of NF1-associated malignant mye-
110 loid disorders.[28,29] The tumor suppressor function of
111 *NF1* has been reviewed recently[30–32] along with its
112 potential as a therapeutic target.[33]

113 The NF1 disease is caused by haploinsufficiency for
114 neurofibromin. The vast majority of *NF1* mutations are
115 not in the GRD but are distributed throughout the gene.
116 Over 80% of mutations inactivate or predict inactivation
117 of neurofibromin; splicing defects and sequence altera-
118 tions that create a premature translation termination co-
119 don are the most common.[34,35] About 10% of *NF1*
120 mutations are missense,[35,36] which are typically clus-
121 tered in the GRD or an upstream cysteine/serine-rich
122 domain that may play a role in ATP binding.[36] Defi-
123 nitive evidence that neurofibromin haploinsufficiency
124 underlies NF1 came with the identification of *NF1* whole
125 gene deletions in an estimated 5%–10% of affected
126 individuals.[37,38] Mutational analyses of patients with
127 specific features, such as plexiform neurofibroma,[39]
128 spinal neurofibroma,[40] or malignant myeloid disor-
129 ders,[41] failed to detect any correlation between geno-
130 type and phenotype. A notable exception is the subset of
131 patients heterozygous for a microdeletion spanning the
132 entire *NF1* gene, who consistently show an early age at
133 onset of cutaneous neurofibromas (see below).

134 Virtually all NF1 patients develop neurofibromas.
135 Neurofibromas are comprised of an admixture of large-
136 ly Schwann cells and fibroblasts, along with mast cells,
137 endothelial cells, and pericytes.[42,43] Although classi-
138 fication schemes vary, Friedman and Riccardi[13] define
139 four types of neurofibromas. Discrete cutaneous neuro-
140 fibromas of the epidermis or dermis are the most com-
141 mon, typically appearing near or at puberty and in-
142 creasing in number to over 100 by the fourth decade of
143 life.[44] They are a localized tumor of small nerves in
144 the skin that feels fleshy and soft; they are more
145 prevalent on the trunk but also occur frequently on the
146 face and extremities. Discrete subcutaneous neurofi-
147 bromas have a spherical or ovoid shape, feel firm or
148 rubbery, and may be painful or tender. Deep nodular
149 neurofibromas, also called nodular plexiform neurofi-
150 bromas, involve major or minor nerves in tissues be-
151 neath the dermis. On gross pathology, they appear to
152 grow inside the peripheral nerve, causing a fusiform
153 enlargement, and may extend the entire length of a
154 nerve.[43] Diffuse plexiform neurofibromas have a cel-
155 lular composition similar to that of cutaneous neuro-
156 fibromas, but in contrast, they have a tendency to be-
157 come locally invasive.[5,43] Histologically, this tumor is
158 a tangled network involving multiple nerve fascicles or
159 branches of major nerves with poorly defined margins
160 that makes complete surgical resection virtually im-
161 possible. Plexiform neurofibromas can be superficial
162 with extensive involvement of underlying tissues, or
163 they may involve deep tissues, particularly in the cra-
164 niofacial region, paraspinal structures, retroperitoneum,
165 and gastrointestinal tract.[13] They can infiltrate soft

166 tissues resulting in localized hypertrophy and signifi-
167 cant functional impairment. In contrast to other types
168 of neurofibromas, diffuse plexiform tumors are con-
169 sidered congenital because they typically become evi-
170 dent in infancy or childhood and rarely, if ever, in late
171 adulthood.[45,46] In a population-based study, 32% of
172 individuals with NF1 had a plexiform neurofibroma(s)
173 on physical examination.[47] Korf[5] recently reviewed
174 plexiform neurofibromas.
175   Plexiform neurofibromas, both diffuse and nodular,
176 are at a greater risk of transforming to an MPNST
177 than other types of neurofibromas (reviewed in
178 Refs. [5,43,48]). Pathological examination of MPNST
179 from individuals, both with and without NF1, most often
180 shows an association with a neurofibroma.[11,49–51]
181 These data suggest that an early step in MPNST devel-
182 opment may be preneoplastic process in the nerve sheath.
183 Multiple pathological and molecular criteria are used to
184 evaluate a neurofibroma for malignant transforma-
185 tion.[48] Several lines of evidence are consistent with
186 cutaneous and plexiform neurofibromas and MPNSTs
187 being clonal tumors that arise from an ancestral Schwann
188 cell (see below).

189   **GENETICS OF**
190   **NEUROFIBROMAGENESIS**

191   **Homozygous Inactivation of NF1**

192   Homozygous inactivation of a tumor suppressor
193 gene(s) is a fundamental mechanism of tumorigenesis. It
194 occurs by either sequential somatic inactivation of both
195 alleles or by a somatic mutation in the single normal
196 homolog in individuals who inherit a germline mutation
197 in one allele. Somatic inactivation is frequently
198 associated with loss of heterozygosity (LOH) at the
199 tumor suppressor locus and at multiple flanking loci
200 (reviewed in Refs. [52,53]). Evidence for such "2nd
201 hit" somatic NF1 mutations in neurofibromas has been
202 sought in support of the hypothesis that neurofibromin
203 functions as a tumor suppressor in Schwann cells. Initial
204 reports of LOH at NF1[54] have been confirmed and
205 extended by analyses of both primary tumor tissue and
206 neurofibroma-derived Schwann cells. At least 25% of
207 neurofibromas undergo LOH at NF1.[55,56] The cellular
208 admixture in neurofibromas can mask allelic loss,[55,56]
209 which is the likely explanation for reports that detected
210 few, if any, tumors with LOH.[57–62] Compelling
211 evidence that the somatic inactivation of the NF1 gene
212 itself is important was provided by the identification of a
213 4 bp deletion in exon 4b in a cutaneous neurofibroma of
214 a patient with a germline NF1 microdeletion.[63]

215 Subsequent analyses of cDNA from neurofibromin-
216 derived Schwann cells showed that 19% of neurofibro-
217 mas carried somatic intragenic mutations, which were
218 typically mRNA splicing defects.[34] Mutation frequen-
219 cy may be underrepresented due to the difficulty of
220 recovering high-quality tumor RNA and the underrep-
221 resentation of mutant transcripts observed in some
222 tumors. The latter could be attributed to mutations that
223 induce nonsense-mediated decay or other mechanisms
224 that affect mRNA content (reviewed in Ref. [64]) or to
225 reduced expression for other reasons,[34] and/or a low
226 proportion of mutant to normal Schwann cells in some
227 tumors.[65,66] Homozygous inactivation of NF1 also
228 occurs in plexiform neurofibromas, where an estimated
229 40% (n = 10) showed LOH,[67] a result confirmed by
230 subsequent reports.[57,58,68,69]
231   A predominant mechanism of somatic NF1 inac-
232 tivation in neurofibromas that underwent LOH was
233 mitotic recombination.[56] A 17q proximal single mito-
234 tic recombination event near the centromere of the q
235 arm between the normal chromosome 17 and the ho-
236 molog carrying the germline NF1 mutation can gene-
237 rate a cell in which both NF1 genes carry the germline
238 mutation and all loci distal to the recombination site
239 are identical. Less common were double recombination
240 events that result in chromosome 17 interstitial loci
241 showing LOH. The NF1 mRNA in some neurofibromas
242 is edited such that an arginine codon is changed to a
243 nonsense codon.[70] Although only one-third of neuro-
244 fibromas examined showed a low level (<2.5%) of
245 edited NF1 transcripts,[71] such modulation of neurofi-
246 bromin expression may be important if, e.g., editing
247 occurred at high frequency in transcripts from a spe-
248 cific minor cellular component of the tumor. In other
249 neurofibromas, somatic mutations appear to destabilize
250 NF1 mRNA.[66] Transcriptional silencing via hyper-
251 methylation of promoter regions, a prominent mecha-
252 nism of inactivating other tumor suppressor genes,[72]
253 has not been detected in neurofibroma tissue.[73,74] As
254 expected from the mutational and LOH analyses, some
255 neurofibromas had no detectable NF1 transcripts or
256 neurofibromin.[75,76] A quantitative Ras activity assay
257 demonstrated that activated Ras-GTP levels were about
258 fourfold higher in neurofibromas than levels in non-
259 NF1-associated schwannomas.[75,77]
260   Neurofibromas are most likely clonal tumors de-
261 rived from a Schwann cell progenitor. The detection of
262 LOH in a tumor operationally defines it as being clonal in
263 origin, and these data are consistent with direct marker
264 analyses in neurofibromas.[57,61] The LOH or other
265 somatic mechanisms that inactivate NF1 in a Schwann
266 cell progenitor may be an early or initiating genetic event
267 in neurofibromagenesis. Klewe and colleagues showed

268 LOH in primary neurofibroma tissue and in tumor-
269 derived Schwann cells but not fibroblasts.[78] Other in-
270 vestigators confirmed this observation.[66,68] Two genet-
271 ically distinct Schwann cell populations, $NF1^{+/-}$ and
272 $NF1^{-/-}$, were successfully cultured from 10 mutation-
273 characterized neurofibromas, whereas tumor-derived
274 fibroblasts carried only the germline $NF1^{+/-}$ geno-
275 type.[65] Sherman et al.[79] used an elegant single cell
276 Ras-GTP assay to show elevated levels in neurofibroma-
277 derived Schwann cells but not fibroblasts. Consistent
278 with two Schwann cell populations, only a fraction
279 (12%–62%) of neurofibroma-derived Schwann cells had
280 elevated Ras-GTP levels. The basis of the Schwann cell
281 heterogeneity is not known, but the authors speculate that
282 the cells with high Ras-GTP (presumably neurofibromin-
283 deficient) may recruit Schwann cells with lower Ras-
284 GTP levels (presumably the constitutional neurofibro-
285 min-haploinsufficient cells) via the synthesis of growth
286 factors. Whether this admixture of Schwann cells is
287 important in tumorigenesis remains to be determined;
288 however, its observation in primary neurofibroma tissues
289 makes it more likely. Fluorescence in situ hybridization
290 and immunohistochemistry demonstrated that S-100
291 protein (Schwann cell marker) immunopositive cells in
292 sections of four of seven primary plexiform neurofibro-
293 mas were monosomic for $NF1$.[80] Because other
294 cells types were disomic at $NF1$, these results strongly
295 implicate the Schwann cell as the target of the $NF1$ 2nd
296 hit mutation and verify that the results from neurofibro-
297 ma-derived Schwann cells were not biased by cell
298 culture. These data also support Schwann cell genetic
299 heterogeneity, because the fraction of S-100 protein
300 positive cells showing $NF1$ deletion ranged from 50%–
301 93%. Further evidence for the importance of Schwann
302 cells in neurofibromagenesis comes from the tumor-
303 igenic properties exhibited by neurofibroma-derived
304 cells.[81–84] In the most comprehensive studies, Muir
305 and colleagues[83,84] showed that neurofibromin-defi-
306 cient Schwann cells, derived from either dermal or
307 plexiform neurofibromas, had high invasive potential
308 and produce neurofibroma-like tumors when engrafted
309 into peripheral nerves of scid mice.

310 **Germline Alterations That May**
311 **Modify Neurofibromagenesis**

312 The considerable variable expressivity of NF1
313 disease among family members with presumably the
314 same $NF1$ mutation and results of a statistical trait
315 analysis led to the contention that variation in an
316 individual's genetic background modified the NF1
317 phenotype.[46,85] To date, no such germline modifying

318 genes have been identified. However, the study of
319 patients carrying $NF1$ microdeletions has provided
320 compelling evidence for a gene that modifies neurofi-
321 bromagenesis. We first recognized that $NF1$ micro-
322 deletion patients typically showed an early age at onset
323 of localized cutaneous neurofibromas or, in cases in
324 which age at onset could not be documented, they had
325 significantly greater numbers of cutaneous neurofibro-
326 mas relative to their age.[38,86] It is common to observe
327 multiple cutaneous neurofibromas during physical
328 examination of children under age 10 that are
329 heterozygous for an $NF1$ microdeletion,[38,87,88] where-
330 as only about 10% in the general NF1 population have
331 a tumor(s) by that age.[47] In our most severe case, a 5
332 year old with an $NF1$ microdeletion had 51–100
333 tumors.[38] The $NF1$ microdeletions can be inherited
334 from an affected parent, and in these cases, the micro-
335 deletion cosegregated with the early age at onset of
336 neurofibromas.[88,89] Other investigators have con-
337 firmed the early onset and heavy burden of cutaneous
338 neurofibromas in $NF1$ microdeletion patients, and over
339 50 such patients have been identified.[90–98]

340 Most $NF1$ microdeletions span 1.5 MB of DNA,[87]
341 which harbors the entire 350 kb $NF1$ gene and at least
342 11 additional genes, most of unknown function.[87,99]
343 The microdeletions are preferentially maternal in ori-
344 gin[87,91,93,100,101] and arise by homologous recombina-
345 tion between 60 kb misaligned paralogs (termed
346 NF1REP) that flank the $NF1$ gene.[87] Paralogs are
347 nonallelic DNA sequences with a high degree of identity
348 that arose via duplication, e.g., the two functional α
349 globin genes. Over 25 human disorders are known to be
350 caused by gene or chromosomal rearrangements medi-
351 ated by homologous recombination between paralogs, a
352 process correctly referred to as nonallelic homologous
353 recombination in the review by Stankiewicz and
354 Lupski.[102] Here, I propose that this process be called
355 by the less awkward term of paralogous recombination
356 and that disorders arising by this mechanism be called
357 paralogous recombination disorders, rather than the cur-
358 rently used genomic disorders. Although the NF1REPs
359 share >95% sequence identity over 60 kb of sequence,
360 about 80% of the $NF1$ deletion alleles are virtually
361 identical because their breakpoints map to one of either
362 two recombination hotspots of several kb in size located
363 within the NF1REP[103] (M. Dorschner et al., in pre-
364 paration). Although the molecular basis for these
365 hotspots is not yet known, their existence means that
366 the majority of microdeletion patients will be deleted
367 for the same set of genes. The early age at onset of
368 cutaneous neurofibromas observed in several pa-
369 tients with larger deletions and/or different breakpoints

370 indicates that generation of a deletion-specific fusion
371 gene product is unlikely.[87]
372   We hypothesized that the codeletion of *NF1* and
373 an unknown linked gene potentiates cutaneous neurofi-
374 bromagenesis.[38,87] What might be the function of the
375 putative neurofibromagenesis-potentiating locus (*NPL*)?
376 Here I propose two models for the early age at onset
377 of cutaneous neurofibromas in microdeletion patients.
378 Haploinsufficiency for *NPL* could increase the fre-
379 quency of somatic 2nd hit mutations in the *NF1* gene.
380 This could result from a genomic instability in
381 microdeletion patients, which is intriguing in view of
382 reports detecting cytogenetic abnormalities and micro-
383 satellite instability in some neurofibromas (see below).
384 It would be interesting to determine if somatic *NF1*
385 mutations in cutaneous neurofibromas of microdeletion
386 patients occur by a predominant mechanism. Only a
387 single tumor from each of two deletion patients have
388 been analyzed; one had a 4 bp intragenic deletion[63]
389 and the other a splice site.[65] A second model for early
390 onset of cutaneous neurofibromas proposes that *NPL*
391 haploinsufficiency increases the probability that a
392 neurofibromin-deficient progenitor cell proliferates
393 and manifests as a neurofibroma. Multiple mechanisms
394 could be proposed. For example, *NPL* could encode
395 (or regulate) a cytokine, cell cycle regulator, tumor
396 suppressor, or oncogene, which exerts a positive pro-
397 liferative advantage on the progenitor cell. Because of
398 the cellular heterogeneity of neurofibromas, this model
399 does not necessarily require that the abnormal expres-
400 sion of *NPL* or its putative downstream targets occur in
401 the neurofibromin-deficient Schwann clone.
402   Preliminary, but intriguing evidence suggests that
403 genetic background, other than *NF1* microdeletion,
404 may influence the somatic inactivation of *NF1*. In
405 patients with multiple neurofibromas, it was deter-
406 mined that each of the tumors showed the same type of
407 somatic mutation event (e.g., LOH of the entire q arm
408 or interstitial LOH).[56] Depending on the extent of
409 LOH and the particular genes involved, this could
410 explain differences in the age at onset and/or numbers
411 of neurofibromas that develop in an individual.

412   **Somatic Alterations That May**
413   **Modify Neurofibromagesis**

414   The NF1-associated neurofibromas have been
415 analyzed for genetic abnormalities at the chromosomal
416 level by comparative genome hybridization (CGH) and
417 cytogenetic analyses. Comparative genome hybridiza-
418 tion is a powerful technique to detect and map chromo-
419 somal regions with copy number imbalances in tumor

specimens (reviewed in Ref. [104]). Only two of eight 420
neurofibromas (type not specified) examined showed 421
chromosomal imbalances; one tumor showed three 422
gains; the other only a single gain.[105,106] This obser- 423
vation is consistent with cytogenetic studies. Although 424
Schwann cell cultures from dermal neurofibromas had 425
normal karyotypes, cells derived from plexiform neuro- 426
fibromas had abnormalities, which in some tumors 427
consisted of unrelated non-clonal abnormalities.[107] 428
One plexiform had structural abnormalities predomi- 429
nantly involving telomeres, which are typically associ- 430
ated with genomic instability in other syndromes/ 431
tumors.[107] Wallace et al. proposed that chromosomal 432
abnormalities might be important in the development 433
of plexiform neurofibromas. Chromosomal abnormali- 434
ties in plexiform neurofibromas may account, at least 435
in part, for their increased risk of malignant transfor- 436
mation. Whether other cellular components of neurofi- 437
bromas show cytogenetic abnormalities is unclear. 438
Some neurofibroma fibroblast-like derived cultures 439
were reported to show an increased frequency of chro- 440
mosomal aberrations,[108] whereas others were typically 441
negative.[109] 442
    Conflicting data have been reported regarding the 443
presence of microsatellite instability in NF1-associated 444
neurofibromas. Some human tumors, most notably 445
those of patients with hereditary nonpolyposis colon 446
cancer HNPCC, show microsatellite instability, which 447
is detected by random changes in the length of 448
microsatellite (simple nucleotide repeats) loci. Length 449
mutations at multiple microsatellite loci in a tumor 450
reflect a genome-wide instability, which in the case of 451
HNPCC is due to a defect in any of several mismatch 452
repair genes (reviewed in Ref. [2]). Ottini et al.[110] 453
reported that 50% (n = 16) of neurofibromas showed 454
altered allele lengths compared with matched normal 455
tissue, and instability at chromosome 9 loci has also 456
been reported.[111] Birindelli et al.[112] observed 457
instability in a primary MPNST and a metastasis in 458
one of 25 cases. However, no evidence of instability 459
was detected in two subsequent studies of 80 neuro- 460
fibromas, of which 5% appear to be of the plexiform 461
type.[55,73] This disparity may be due to technical dif- 462
ferences, the number and type of microsatellite loci 463
examined, and/or the stage of the neurofibromas. The 464
LOH at *NF1* was not a factor, because all three studies 465
analyzed neurofibromas that were both positive and 466
negative. Of the eight neurofibromas with microsatel- 467
lite instability, seven were unstable at only one of the 468
five loci tested.[110] Due to the important implications 469
that microsatellite instability would have for neurofi- 470
bromagenesis, additional loci should be examined in 471

472 these tumors, including those analyzed by the other
473 investigators. In addition, it would be interesting to
474 know if the neurofibromas that showed microsatellite
475 instability were of the larger, central plexiform type, in
476 which case they may have been transforming to malig-
477 nancy. The proportion of loci that display instability
478 is known to increase with tumor progression in
479 HNPCC.[113]

GENETICS OF MPNST
DEVELOPMENT

Homozygous Inactivation at *NF1*

483 The homozygous inactivation of *NF1* in NF1-
484 associated MPNST, first reported by Skuse et al.,[60]
485 has been confirmed in about 50% of tumors (n = 22) by
486 LOH.[67,109,112,114,115] Mutations in both *NF1* alleles
487 have been identified in a single MPNST.[114] Although
488 the mechanism of LOH is not known, it does not
489 generally involve cytogenetically detectable losses at
490 17q1.[116] Furthermore, NF1-associated MPNST-derived
491 cell lines showed decreased or absent neurofibromin and
492 high levels of active Ras-GTP,[117,118] and a quantitative
493 Ras activity assay demonstrated that activated Ras-GTP
494 levels in tumors were about 15-fold higher than levels in
495 non-NF1-associated schwannomas.[77] Because homo-
496 zygous inactivation of *NF1* occurs in benign neurofi-
497 bromas, it is considered an early or initiating event that is
498 necessary and sufficient for neurofibromagenesis but not
499 MPNST development. Malignant transformation is
500 presumably driven by predisposing genetic factors in
501 the germline and/or by additional somatic mutations and
502 positive growth selection in a malignant precursor cell.
503 The role of *NF1* in the development of sporadic MPNST
504 is not clear; only about 10% of these tumors show LOH
505 at *NF1*.[112]

Germline Alterations That May
Modify MPNST Development

508 Little is known about germline genetic modifiers
509 that predispose to MPNST. Early speculation that
510 patients with *NF1* microdeletion may be at increased
511 risk for malignancy[38] is now supported by indirect
512 evidence. Mutational analysis of germline DNA from
513 seven patients who developed MPNST determined that
514 three (42%) were heterozygous for an *NF1* microdele-
515 tion.[119] In another study, 2 of 17 (11%) unrelated *NF1*
516 microdeletion patients developed MPNST, and affected
517 first-degree relatives of two microdeletion patients had

518 other malignancies.[87] Although additional cases are
519 needed, these data suggest that the lifetime risk of
520 MPNST in microdeletion patients may exceed the
521 already high 10% in the general NF1 population.[9] If
522 so, the underlying mechanism may be essentially the
523 same as that proposed above for early onset neurofi-
524 bromagenesis. Deletion of the putative *NPL* gene could
525 result in genomic instability or exert positive growth
526 selection for the malignant clone. If microdeletions
527 do predispose to both cutaneous neurofibromas and
528 MPNST, it seems reasonable to speculate that in at least
529 this subset of patients, either the discrete cutaneous
530 type of neurofibroma is at increased risk of malignant
531 transformation or the frequency of nodular or diffuse
532 plexiform neurofibromas is high.
533 Evidence from two families suggests the intriguing
534 possibility that MLH1 deficiency predisposes to NF1
535 and early onset extracolonic tumors.[120,121] Germline
536 heterozygous inactivating mutations in *MLH1* cause
537 inefficient DNA mismatch repair, with the consequent
538 increase in mutation frequency and susceptibility to
539 hereditary nonpolyposis colorectal cancer (reviewed in
540 Ref. [122]). Two rare and independent cases of con-
541 sanguineous marriages between *MLH1* heterozygous
542 first cousins each produced two children with NF1
543 disease and hematological malignancies.[120,121] The
544 parents had no signs of NF1 and there was no family
545 history of the disease. The MLH1 mutations were
546 identified, confirming homozygosity in three of the
547 four deceased children, who presented with mutiple
548 café au lait spots (4/4), dermal neurofibromas (2/4),
549 tibial pseudoarthrosis (1/4), non-Hodgkin's lymphoma
550 (2/4), myeloid leukemia (2/4), and medulloblastoma
551 (1/4). The authors suggest that these patients had a de
552 novo postzygotic *NF1* mutation and that the *NF1* gene
553 may be preferentially susceptible to mismatch repair
554 deficiency. Unfortunately, the *NF1* gene was not
555 analyzed for mutations, due in part to lack of patient
556 tissue,[120] which could have confirmed mutation of
557 *NF1* and differentiated between postzygotic mutation
558 and germline mosaicism in a parent.

Somatic Alterations in
NF1-Associated MPNST

561 All NF1-associated MPNST showed significant
562 chromosomal imbalances by CGH.[105,106,123,124] Anal-
563 ysis of 27 total tumors from 19 NF1 patients showed an
564 average of >7 imbalances per MPNST (Table 1). One T1
565 tumor had only a single imbalance, a gain of chro-
566 mosome 8.[123] The studies led by Mechtersheimer and
567 Schmidt,[105,106] which include 74% of tumors exam-
568 ined, detected chromosomal gains more frequently than

T1.1 **Table 1.** Chromosomal gains and losses in MPNST detected by CGH.

	NF1-associated MPNST			Sporadic MPNST		
	Loethe[a]	Mechtersheimer[b]	Schmidt[c]	Loethe[a]	Mechtersheimer[b]	Schmidt[c]
No. tumors/No. 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

a: Ref. [123].
b: Ref. [105].
c: Ref. [106].

losses (Table 1). Each of the 20 tumors in these studies had ≥4 chromosomal gains. Although 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

T2.1 **Table 2.** Frequency of chromosomal gains in NF1-associated MPNST.

	% MPNST			
	Loethe[a]	Mechtersheimer[b]	Schmidt[c]	Total
No. MPNST/No. patients	7/7	6/6	14/6	27/19
Chromosome				
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

a: Refs. [105,123].
b: Ref. [105].
c: Ref. [106].

593 gains (Table 1) were amplified frequently. The fre-
594 quency of 7p amplifications may be overestimated
595 because these four MPNST (considered as different
596 primary tumors) were from a single patient.[106,124] In
597 contrast, amplifications of 5p, 8q, and 12q were the most
598 prevalent in sporadic MPNST. There was a significant
599 difference in the frequency of tumors with more than
600 one chromosomal segment amplified. Only 10% of
601 NF1-associated MPNST had more than one amplified
602 segment, whereas the frequency was 72% in sporadic
603 tumors (Table 3). Differences in the location and

604 number of chromosomal segments amplified were
605 unlikely to be due to tumor grade, because the majority
606 of MPNST, 14 of 20 (70%) of NF1-associated and 22 of
607 34 (64%) of sporadic, were grade 3 (poorly differentiat-
608 ed). Although amplifications were more frequent in NF1-
609 associated than sporadic MPNST (50% vs. 32%), the
610 number of NF1-associated tumors may be overestimated
611 because it includes multiple tumors from the same
612 patient. Adjustment for one tumor/patient gives a
613 frequency of 25% (5 of 20) for NF1-associated MPNST.
614 Similarly, there was no correlation between chromosomal

T3.1 *Table 3.* Large-scale chromosomal amplifications in MPNST.[a]

	Chromosome segment amplified	No. NF1-associated MPNST[b] (N = 20)	No. sporadic MPNST[c] (N = 34)
T3.3	4q12-q13		1
T3.4	5p11-p15		1
T3.5	5p14		1
T3.6	5p15		2
T3.7	5p13-pter		2
T3.8	7p14-pter	2[d]	1
T3.9	7p13-pter	1[d]	
T3.10	7p12-pter	1[d]	
T3.11	7p11-p12		1
T3.12	8q12-qter		2
T3.13	8q13		1
T3.14	8q21-q22		1
T3.15	8q22-q23		1
T3.16	8q24		1
T3.17	8q23-qter	1	
T3.18	9p21-p23	1[e]	
T3.19	9q31-q33		1
T3.20	12p12	1[e]	
T3.21	12p13		1
T3.22	12q13-q14		1
T3.23	12q14-q21		3
T3.24	12q24		3
T3.25	13q13-q33		1
T3.26	13q32-q33		1
T3.27	17p11-p12		1
T3.28	17q24-qter	2	
T3.29	17q22-q24	1	
T3.30	20q12-qter		1
T3.31	Xp11		1
T3.32	Xp21-p22		1
T3.33	*Summary*		
T3.34	% tumors with an amplification	50 (10/20)	32 (11/34)
T3.35	% tumors with >1 amplified segment	0.1 (1/10)	72 (8/11)
T3.36	% patients with ≥ 1 tumor with an amplification	30 (4/12)	32 (11/34)

a: Centromeric regions, chromosomes 19 and Y, and 1p32-p36 were not scored for technical reasons. (From Ref. [105].)
b: Refs. [105,106,124].
c: Refs. [105,124].
d: Four of five primary MPNST from one patient. (From Ref. [106].)
e: Two of four primary MPNST from one patient. (From Ref. [106].)

615 amplification and progression to metastasis. Analysis of
616 tissue from a primary tumor, recurrence, and metastasis
617 from a single patient showed a single amplification
618 (8q12-qter) in the recurrent tumor.[124]
619 Comparison of the chromosomal losses detected in
620 the three CGH studies revealed the following five most
621 frequent losses in 27 NF1-associated MPNST: 13q21-
622 22 (12/27), 11q23-25 (10/27), 1p22-31 (9/27), 3q11-21
623 (8/27), and 17p12-pter (7/27).[105,106,123] It is of interest
624 that the loss of 17p12-pter was detected in 50% (7 of
625 14) tumors in one study[106] and in none of the tumors
626 of the other studies. The relatively low detection rate of
627 chromosomal losses may be due to the decreased
628 sensitivity of CGH in polyploid MPNST.[125]
629 The CGH analysis of multiple, presumably syn-
630 chronous or metachronus, primary MPNST at different
631 sites in three NF1 patients revealed a remarkable
632 similarity in chromosomal gains and losses.[106,124] For
633 example, five grade 3 (poorly differentiated) tumors of
634 one patient each showed imbalances of +7p, −13q21,
635 and +17q22-qter. These data showed that in the speci-
636 fic genetic background of each patient, a relatively
637 limited, and defined, number of rearrangements were
638 shared among the tumors. Similarly, nearly identical
639 aberrations were found in different MPNST from the
640 same patient.[125] Although limited, these data suggest
641 that each individual's constitutional genotype sets a
642 certain "baseline" on which a minimal and limited
643 number of genetic alterations are necessary and suf-
644 ficient for MPNST development.
645 Consistent with CGH analysis, the karyotype of
646 NF1-associated MPNST-derived cells are complex with
647 chromosomal numbers ranging from 34 to 270 indica-
648 tive of hypodiploidy, hypotriploidy, hypotetraploidy, hy-
649 pertriploidy, and hypertetraploidy (Refs. [109,116,125]
650 and references therein). Although breakpoints were fre-
651 quent, a common specific breakpoint was not detected.
652 A comparison of CGH and karyotying in six MPNST
653 revealed significant overlap in the most frequent gains
654 and detected losses at 19q (3 of 6 tumors), a region not
655 analyzed in the CGH studies.[106,125] Plaat et al.[116]
656 performed a computer-assisted cytogenetic analysis of
657 46 MPNST reported in the literature and 7 new cases of
658 both NF1-associated and sporadic tumors (Ref. [116]
659 and references therein). These studies confirmed the
660 CGH observation of high-frequency gains at chromo-
661 somes 7p and 7q and losses at 1p and 17p. However,
662 their reported cytogenetic differences between NF1-
663 associated and sporadic MPNST[116] were not con-
664 firmed in later studies.[125,126] In one study, near triploid
665 or near tetraploid clones were associated with grade 3
666 tumors and a poor prognosis.[126] The detection of a
667 t(X;18) translocation in MPNST[127] was not confirmed

668 in either sporadic[128] or NF1-associated neurofibroma
669 or MPNST.[129]
670 Inactivating mutations in several tumor suppressor
671 genes have been identified in NF1-associated MPNST.
672 Both LOH and intragenic missense mutations of
673 TP53[50,62,112,130] have been detected. Like many sarco-
674 mas, NF1-associated MPNST often showed overexpres-
675 sion of p53 (the protein product of the TP53 gene) as
676 assayed by immunoreactive positivity in the nucle-
677 us.[50,112,131,132] In keeping with findings in other tumors,
678 this most likely is mutant p53 protein, which accumulates
679 due to its increased stability. Mutant protein is thought to
680 promote cancer by either complexing with and seques-
681 tering functional p53 or by complexing with p63 and p73
682 and blocking their normal transcription factor activities
683 (reviewed in Ref. [133]). Immunohistochemical detec-
684 tion of p53 was more common in NF1-associated vs.
685 sporadic MPNST, and it was associated with poor
686 prognosis in NF1 children.[132] About 50% of NF1-
687 associated MPNST showed homozygous deletion for
688 exon 2 of the INK4A gene, and over 90% were
689 immunonegative for its protein product p16.[112,134,135]
690 Homozygous deletion of exon 2 results in deficiency for
691 both p16 and p14^{ARF}, two proteins encoded by
692 alternative splicing of INK4A (also known as CDKN2A).
693 Both of these proteins are tumor suppressors that
694 modulate activities of the RB and p53 pathways, which
695 are critical for cell cycle control and tumor surveillance
696 (reviewed in Ref. [136]). MXI1 mutations in regions that
697 encode known functional domains have been detected in
698 the two NF1-associated MPNST analyzed.[137] Mxi1 is
699 an agonist of the oncoprotein Myc and is thought to limit
700 cell proliferation and help maintain the differentiated
701 state (reviewed in Ref. [138]). Mxi1-deficient mice
702 develop tumors, and Mxi1 deficiency decreases the
703 latency of tumors that arise in Ink4a-deficient mice.[139]
704 If additional studies show that somatic inactivation of
705 MXI1 is common, it would suggest a link between the
706 pathways of Ink4a, Myc, and Ras in NF1-associated
707 MPNST.[140,141]
708 Several differences observed in NF1-associated
709 MPNST are likely involved in the malignant transfor-
710 mation of a preexisting neurofibroma. TP53 or INK4A
711 mutations/altered expression were not detected in
712 neurofibromas.[50,51,54,62,67,69,112,132,134,135] Further-
713 more, p53-positive nuclei, typically associated with
714 MPNST, were observed in a few cells at the transitional
715 zone between an existing plexiform neurofibroma and
716 an arising MPNST.[51] One plexiform neurofibroma
717 proximal to an MPNST did show p16 immunonegativ-
718 ity.[112] Microdissection of a preexisting neurofibroma
719 from its MPNST focal malignant process revealed 5
720 chromosomal imbalances in the neurofibroma, all of

721 which were novel compared to the 10 imbalances in the
722 MPNST component.[106] These data are consistent with
723 a restructuring of the genome during transformation.
724 An additional distinguishing feature of MPNST is the
725 high labeling index of the nuclear proliferating antigen
726 Ki67, which was correlated with reduced survival in a
727 study that combined NF1-associated and sporadic
728 MPNST.[51,132,142]

729 ## OTHER NF1-ASSOCIATED
730 ## NEOPLASMS AND
731 ## MOUSE MODELS

732 In addition to peripheral nerve sheath tumors,
733 individuals affected with NF1 are at increased risk for
734 an array of other tumors. Epidemiologically associated
735 neoplasms include medulloblastoma, pheochromocyto-
736 ma, astrocytoma, and adenocarcinoma of the ampulla
737 of Vater (Ref. [143] and references therein).[144]
738 Primarily children affected with NF1 are at increased
739 risk for optic pathway gliomas and brainstem gliomas,
740 rhabdomyosarcomas, and malignant myeloid leuke-
741 mias.[41,145−149] The NF1 patients are also at increased
742 risk for a second malignancy, some of which may be
743 treatment-related.[146,150,151] In different studies, 8%−
744 21% of NF1 patients with a first malignancy developed
745 a second cancer, compared to a frequency of 4% in the
746 general population.[143] Malignancy in NF1 has been
747 reviewed recently.[152]

748 Although heterozygous mutant mice $(Nf1^{+/-})$
749 develop tumors, they are not the characteristic peri-
750 pheral nerve sheath tumors characteristic of the human
751 disease.[153−155] The $Nf1$-deficient mice $(Nf1^{-/-})$
752 die in utero from cardiac defects. Mice chimeric for
753 $(Nf1^{-/-})$ and $(Nf1^{+/+})$ cells were able to develop many
754 microscopic plexiform neurofibromas, but dermal
755 tumors did not develop.[156] In addition, mice doubly
756 heterozygous for mutations in $Nf1$ and $p53$ developed
757 MPNST that showed LOH at both tumor suppressor
758 loci.[156,157] Recently, mice were constructed such that
759 only their Schwann cells were $Nf1$ deficient.[158]
760 Different tumor phenotypes were observed, depending
761 on whether the $Nf1$-deficient Schwann cells were in an
762 animal with an $Nf1^{+/-}$ or an $Nf1^{+/+}$ constitutional
763 genotype. In the $Nf1^{+/-}$ genetic background, plexiform
764 neurofibromas composed of $Nf1$-deficient Schwann
765 cells, and the fibroblasts and mast cells that normally
766 occur in human neurofibromas, developed on periphe-
767 ral nerves. In the $Nf1^{+/+}$ genetic background, $Nf1-$
768 deficient Schwann cells did not participate in neurofi-
769 bromagenesis but did form relatively small hyperplastic
770 lesions of the cranial nerves containing minimal mast

771 cells. This mouse model demonstrates that neurofibro-
772 min deficiency in Schwann cells is sufficient for
773 generating nascent tumor lesions, but frank plexiform
774 neurofibroma development requires neurofibromin
775 haploinsufficiency in cells of the surrounding tissues.

776 With the development of mouse models, under-
777 standing the genetics, pathology, and natural history of
778 human benign and malignant peripheral nerve sheath
779 tumors takes on new importance. It is only by accurate
780 modeling of the human disease that progress can be
781 made toward therapies that can slow or halt neurofi-
782 bromagenesis and reduce the high risk of malignancy
783 associated with NF1 disease.

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