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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of this work is to study the genetic basis of tumor pathogenesis in neurofibromatosis type 1 (NF1). This work is testing the two-hit hypothesis in NF1 tumors (benign neurofibromas and MPNSTs), to assay for involvement of the TP53 gene in the various types of tumors, and to determine whether other loci contribute to the formation of NF1 tumors. A large set of human primary tumor samples has been collected, with development of cell culture models from these tumors. One accomplishment is discovery of very strong evidence that the two-hit mechanism operates in at least a large proportion of neurofibromas, and that there is a genetically abnormal Schwann cell clonal component in these tumors, amidst other cell types. The TP53 gene has been found to be implicated only in the malignant tumors. The NF2 gene has allelic loss in some MPNSTs and plexiform tumors, although other tumor suppressor loci studied so far do not show any allelic imbalance. Substantial data from broader surveys of other genes (differential display, cDNA array) are forthcoming. These data are all important in the development of rational therapies to prevent or halt progression of neurofibroma and MPNST growth.

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INTRODUCTION

Neurofibromatosis type 1 (NF1) is a frequent autosomal dominant disease that displays variable expressivity. The features involve localized overgrowths or abnormal growths of neural crest-derived tissues. Thus, NF1 is characterized by abnormal cell proliferation, particularly evident in the formation of benign neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs). Nearly all NF1 patients develop dermal neurofibromas (small tumors in or under the skin), about 40% develop plexiform tumors (larger, arising on deeper nerves), and about 5% develop MPNSTs (these often occur within a previously-existing neurofibroma. Presently there are no known measures for the prevention of NF1 tumor development, and treatment of these tumors using standard therapies has not proven to be particularly effective because this is a lifelong progressive disorder and complete removal of the tumor sacrifices the attached nerve. Understanding of the progression of normal cells to a benign tumor and ultimately to malignancy is lacking in NF1; such knowledge would be invaluable for the development of preventive strategies, diagnostic tools and therapeutic approaches. Our work involves investigating the two-hit hypothesis in neurofibromas, as well as gleaning information about cell type and tumor progression pathways in NF1. Genetic methods are being used to specifically search for abnormalities in NF1, TP53, and other tumor-related genes, as well as screen for genetic and/or regulatory changes that might involve yet-unidentified genes, through several more global genetic analyses. Another aim is to study the functional effect(s) of NF1 and TP53 inactivation in Schwann cells through the use of antisense inhibition technology. The combined use of these complementary approaches will lead to a more comprehensive understanding of the pathogenesis of tumors in NF1.

Technical Objective A. Establish that NF1 Tumors Conform to the "Two-Hit" Hypothesis for the NF1 Gene.

Progress on Task A1 (months 1-36): Continue/complete preparation of samples (DNA and RNA) from the current solid tissue and cultures. Continue subculture of tumoral Schwann cells.

We have prepared RNA and DNA from all of the solid tumors and cultures previously established (prior to Oct. 1999). In this current year we received tumor and blood samples from 18 patients (10 females, 8 males), an extremely fruitful year, along with blood samples from 11 other affected and unaffected relatives. We also obtained fresh tissue from two non-NF schwannomas and an NF2 tumor as controls/comparisons. In total, from 10/99 – 10/00 we procured fresh tissue on 13 dermal neurofibromas (were unable to establish long-term Schwann cell cultures on any, despite several attempts) and 14 plexiform tumors (established 11 early Schwann cell cultures). We also were able to culture Schwann cells from a normal nerve that also came with one sample, which will serve as a rare and extremely useful NF1 heterozygous control. This builds our resources of nucleic acids and cell cultures considerably. Most of the samples received this past year have already had DNA and RNA extracted, including 8 the cultures so far. We are not as actively pursuing tumor samples in this last year (since there will not be as much time to analyze them) but we may receive a few final samples. We are also publishing and sharing these resources with other investigators including two DOD-NF program funded investigators: Dr. Lynn Fieber (Univ. of Miami) and Dr. Karen Stephens (Univ. of Washington). In Jan. 2000 we published our findings of abnormal karyotypes in a majority of the plexiform Schwann cultures but not the dermal tumors, a key publication among several this year supporting that the Schwann cell is genetically abnormal in plexiform neurofibromas (Wallace et al., 2000, see appendix). Also in press is a publication describing the establishment of these cultures and the lack of neurofibromin at the Western blot level, which is another piece of evidence implicating the Schwann cells and indicating that these cultures are homogeneous and valuable for numerous other studies (Muir et al., in press).

Progress on Task A2 (months 1-12): Complete intragenic loss of heterozygosity (LOH) studies on all available tumors; complete studies to outline extent of NF1 deletions.

Despite the initial plan to do LOH studies in just months 1-12, LOH analyses have been extended during this past year to include the new tumor samples and culture DNAs. Our previous years' work was just summarized and published this year (Rasmussen et al., 2000, see appendix) in which 5% of dermal and 40% of plexiform neurofibromas (solid tissue only) showed LOH in the NF1 gene region. In several of these cases we also identified the germline mutation and were able to demonstrate both "hits". 60% of the MPNSTs showed LOH, although there is question that the remaining 2 MPNST samples may not represent tumor material due to pre-operative therapies (and thus 60% is conservative). Analysis of markers spanning the rest of chromosome 17 noted that none of the benign tumors had LOH extending above HHH202 or into the p arm, while 2/3 MPNSTs had complete homolog loss. The other MPNST retained the terminal marker on both ends, which seems unique and may indicate a novel mechanism. This also fits with the fact that the MPNSTs lost the TP53 gene, which is consistent with malignancy.

We now have 14 NF1 intragenic markers working in the lab (7 RFLPs, 7 microsatellites). During this past year we have done additional LOH work using many of these markers on new samples. This work is not yet complete, but combined with our previous work we now have found loss of one copy of the NF1 gene in 14/52 dermal neurofibromas, 12/42 plexiform tumors, and 5/7 MPNSTs (solid tissue, except 2 MPNSTs exist only as culture). Thus far 12 dermal tumors have had reasonably pure Schwann cell cultures established, with 5 of these representing tumors showing LOH at the primary tissue level. In comparing primary DNA and culture DNA for these 5 samples, 2 are consistent in the culture also showing LOH, 2 are not yet studied (new samples) and 1 is inconsistent (primary tumor showing more LOH, culture DNA not evident). In the 2 consistent samples the cultures did not show "pure" LOH, although one was clearly more clonal than the primary tumor. Neither of these two cultures are neurofibromin-deficient on Western blot, which suggests that they are enriched for the tumorigenic cells but also contain some normal Schwann cells. Thus, the genetic analysis is helpful in corroboration with Western results and in characterizing the clonality/homogeneity of the samples.

Among the plexiform tumors, relatively pure Schwann cultures exist for 20 samples, with one of these being from a patient with a germline NF1 deletion (and so LOH analysis is not suitable). Of the remaining 19, LOH is found in both the cultures and primary DNA for 3 samples; 5 new samples have not yet been analyzed at all; 1 sample has strong LOH in the primary and the culture has not yet been studied; 7 samples have no LOH in either the primary or culture DNA; and for 3 samples the primary shows no LOH and the cultures have not yet been studied. Like the dermal tumors above, the LOH in the 3 positive cultures was of similar purity to the primary DNA (very pure in one case, not very pure in the other). These are consistent with Western blot results (the purer sample showing no neurofibromin, the other two having some neurofibromin). We have not found cases of increased purity in the cultures compared to primary. Graduate student Susanne Thomson is also applying X-linked clonality analysis to the cultures, which thus far (n = 18 females informative for androgen receptor and/or PGK gene) shows results completely consistent with the LOH (most cultures are not clonal; none of these correspond to the 18 neurofibromin-negative cultures however).

Progress on Task A3 (months 4-12): Complete NF1 protein truncation test (PTT) studies on all tumors for which NF1 LOH was not found to identify somatic mutations; complete NF1 PTT on all blood samples to identify germline mutations; characterize specific mutations (germline and somatic) on cases in which PTT has identified truncated proteins.

As we described last year, our experience shows that only the very best quality RNAs yield unambiguous PTT results, even when the segments are broken down into 1-kb fragments as we have designed. Unfortunately, many of our solid tissue tumor RNAs are not of sufficient quality as we have determined through PTT analysis. We have had 4 PTT-positive tumor samples, and 3 of these reflected the germline mutation and the fourth was rather ambiguous and we have been unable to find any mutations in the exons encoding that segment. However we plan to do another batch of PTTs, on the culture RNAs (which are of better quality) and our newer blood RNAs (no results yet). For any new cultures, we will also attempt to grow some on puromycin for RNA

collection, as we and collaborators in Belgium (Messiaen et al., 1999; appendix) established that this eradicates nonsense-mediated decay in the NF1 gene and therefore increases the likelihood of obtaining a positive PTT result (for either germline or somatic mutation). We may attempt to do short-term culture of blood lymphocytes as well, with puromycin, to optimize the blood RNA for germline analysis. We have had further experience in identifying mutations based on positive PTTs (3 this year), and this has been shown to be challenging when the mutation is actually a splicing error (and the location of the mutation can not be easily estimated). Four positive samples continue to elude characterization, despite having sequenced nearly all the exons in the region; we are now sequencing RNA. We have discovered two novel splicing mutations in this fashion, which we are planning to submit in a manuscript this year. The third PTT mutation was a nonsense mutation.

One issue that together with our experience suggests that the PTT is not an optimal test is the discovery of aberrant cryptic splicing in the NF1 gene due to sample handling. This was originally discovered by us (Wallace et al., 1998 abstract; Messiaen et al., 1999), but was first characterized more fully and published in manuscript form by two European groups this year. We have been working on characterizing these forms, which consist of novel cryptic exons inserted into the NF1 transcript, and/or loss of complete or partial exons. These are related to exposure of blood to room temperature conditions for at least an hour; this is avoided with immediate processing or immediate refrigeration. The papers published describe the 31-bp cryptic exon from intron 4b, but we have found several other forms as well and hope to submit our complete data as a manuscript this year. The main significance of this work is that these aberrant forms can lead to false positive PTT tests. This is a novel finding and it is unclear if it has medical significance in NF1, other than the risk in the PTT test. As an aside, in the splicing analysis we have also found two other aberrant splices (using freshly-processed RNA from 6 human tissues—heart, Schwann cell culture, adult brain, fetal brain, cerebellum, spinal cord, fetal liver, placenta). These forms do not appear to be related to sample handling/environment. One of these appears tissue-specific, and both forms have been amplified and are being sequenced. It is unclear what significance these may have, although they may shed light on neurofibromin function. The expected patterns for inclusion of known alternatively spliced exons 23a, 9br, and 48a were observed.

Related to germline and somatic mutations, we are also instituting several other approaches since PTT has not proven as efficacious as originally hoped. Based on large-scale mutation reports this year and our lab's data, we are specifically analyzing the most frequently-mutated exons (4b, 7, 9, 10a, 10b, 16, 31, 37) in our patients and tumors. This involves a combination of SSCP and direct PCR sequencing. Sequencing of over 150 exon 7 samples has not yet yielded any mutations, although a smaller SSCP analysis (n = 50) has found 1 exon 16 mutation and 3 patients are showing aberrant exon 37 patterns. Also, based on a recent paper (Eisenbarth et al., 2000) the first to publish more than one subtle somatic NF1 tumor mutation) which found nonsense mutations in most of the 7 neurofibromas studied, we are beginning a tumor screen for CpG and CpNpG transition nonsense mutations via looking for altered restriction analysis patterns. 19 such sites have been identified in 14 exons scattered across the gene. This will provide a fast analysis for these potentially most common somatic mutations, and will detect any such germline mutations as well in the tumor tissues. We previously performed a similar analysis for germline mutations (Krkljus et al., 1997) and found the NF1 CpG mutation

rate to be similar to that for other disease genes (20-30%). Thus we will analyze this as a complement to PTT and other approaches. We also have several patients with documented large-gene deletions (Rasmussen et al., 1998), and now have tumor samples from 3 of them (although one of the patients is mosaic for the deletion) and thus the tumors should only reflect the somatically mutated allele which may be easier to characterize. Our collaborator Dr. Karen Stephens (Univ. of Washington) is also studying many of our tumor DNAs to test for the large repeat-mediated gene deletion in tumors. Also, it is possible based on information known about other tumor syndromes (for both benign and malignant tumors) that the mechanism of the second "hit" can be methylation-based silencing. Thus, one student is using the sodium bisulfite sequencing method to assay for methylation in a set of LOH-negative neurofibromas at the promoter region (around transcription start, where the expected "normal" pattern of NF1 gene methylation has already been established in non-Schwann cells). Thus far, 4 tumors have been analyzed with no abnormal results.

Progress on Task A4 (months 6-12): NF1 tumor samples and derivative cultures will be analyzed using immunocytochemical and Western blot analyses to determine if they are indeed devoid of neurofibromin.

This Task continues as new samples are obtained. The finding of neurofibromin-deficiency in 18 Schwann cell cultures derived from neurofibromas (both dermal and plexiform, by Western blot) is in press (Muir et al., in press, appendix). The other cultures (which did not show such strong enrichment for Schwann cells, or seem to have a cell type that is not typical Schwann) do show some neurofibromin upon Western blot, which is harder to interpret due to the culture characteristics. Thus, the 18 cultures above are of a higher priority in various other assays. We have also immunostained over 40 dermal neurofibroma and 30 plexiform NF1 neurofibroma sections with the Santa Cruz N-terminal polyclonal NF1 antibody (corresponding to a peptide encoded within exon 10a), and have found that all have at least patches of neurofibromin-deficient Schwann cells (some completely devoid of neurofibromin staining within the tumor section), and some of these data are included in the above paper. This is consistent with the two hit hypothesis being true in all NF1 neurofibromas, despite the inability at the moment to fully characterize both hits. One note is that the Western blot always shows additional bands below the neurofibromin area, and this has been observed by other labs using this antibody. We have had inconsistent results with Santa Cruz's C-terminal antibody for various purposes, and no other commercial antibodies are available. Thus, as we proposed, we have undertaken creation of a new antibody to see if we can find one that is cleaner on Western blots and possibly other applications. A peptide corresponding to a hydrophilic stretch of 14 amino acids encoded within exon 2 (thus, very N-terminal) was used to for monoclonal antibody production. Thus far, fusions from one mouse have resulted in just two positive hybridomas (which are from adjacent wells and thus may represent the same clone). The hybridomas are producing antibodies very strongly reactive to the original peptide (ELISA) and we are now testing it on tissue sections and Western blots to determine its sensitivity and specificity. This antibody would be the first NF1 monoclonal and could prove very useful for our experiments and future uses by ourselves and other NF researchers.

An additional extension of this work, which had been proposed as a possible approach, is being tested now—laser capture microdissection using a new Arcturus

instrument here at U.F. We are troubleshooting the system to attempt to select just Schwann cells from tumor sections, and see if we can also use staining of adjacent sections to help ensure selection of neurofibromin-negative Schwann cells. Pilot studies have shown mixed results on obtaining DNA from 500 cells or less, although we are also testing whole-genome amplification as a means to increase the amount of genomic DNA available. We are disappointed that the optics are not as good as we'd hoped, such that selection of cells from stained sections can be very difficult depending on the intensity, color, and cellular location of the stain. Thus, we may not be able to be as selective as hoped. Successful DNA extractions will be used for limited LOH analysis (to compare to data from solid tissue and culture) and for clonality and methylation analyses.

Progress on Task A5 (months 6-12): Complete immunocytochemistry analysis for neurofibromin protein of tumors/cell cultures; complete Western blot studies for neurofibromin on tumors/cell cultures.

See Task A4.

Progress on Task A6 (months 1-36): Complete antisense inactivation of NF1 to model NF1 inactivation in NF1-relevant cells; perform tumorigenicity related assays in antisense inhibited cells.

Oligonucleotide antisense inhibition has not been further pursued because the antisense construct will provide stable cultures that can be manipulated in the laboratory to obtain much greater information about tumorigenicity. Part of the reasoning behind this is the success of Dr. Muir at engrafting the human neurofibroma Schwann cell cultures in scid mice sciatic nerves (Muir et al., in press). Thus, the ability to take the cultures to the in vivo model supports the approach of having cultures that lack only NF1, as compared to the heterogeneous human samples (many of which clearly have other genetic abnormalities). In the past year we created a new antisense construct of 450 bp of rat NF1 cDNA (corresponding to exons 4a-6) in an inducible Invitrogen expression vector, and transfected this construct into rat Schwann cells as a test of the system. Both protein and RNA level analysis (Western and semi-quantitative RT-PCR) showed that the construct did not express as expected, consistent with no detectable reduction in neurofibromin. Even though the control genes on that vector and co-transfected vectors apparently had induction, the insert's transcript did not respond. Although the company does not completely understand why there was insufficient expression, it is remotely possible that the lack of an ATG and Kozak start signal (as is typically done in these vectors) might have had a role. This did not seem significant in the design since we were not interested in any protein expression, just transcripts. Thus, at the advice of the company, we are now constructing two new inserts, both of which contain the natural NF1 start sequences (one is into exon 3, the other into exon 7). This also fits better with general antisense folklore that having an antisense construct over the translation start site has a better likelihood of success; however there are no clear rules for predicting whether an antisense construct or oligo will function as predicted. The cloning has just been completed, and one of each insert (using both orientations, one as control) will be grown and used in the same experiments. The utility of having cell lines lacking just NF1 will be very useful to test whether lack of NF1 alone does or does not promote tumorigenic properties, as well as for future in vivo experiments (the inducing agent is non-toxic and can be fed to the mice), and thus we are not giving up easily. Since

antisense approaches have not been reported for genes as large as NF1, it raises the possibility that there are underlying mechanisms that may effectively prohibit successful antisense inhibition for this gene and tolerate some degree of double stranded RNA (perhaps supported by the fact that some tissues express both NF1 and embedded genes such as EVI2A, EVI2B, and OMGP simultaneously). Nonetheless we will continue our efforts in the hope of success. We may produce several lines with differing amounts of antisense activity, and this may be important as well, since having a certain level of neurofibromin activity may rescue a cell from a tumorigenic fate. This latter notion would have important implications clinically.

Technical Objective B. Evaluate the Involvement of the TP53 Gene in NF1 Tumors.

Progress on Task B1 (months 1-6): Complete TP53 LOH studies on all available tumors.

Completed (Rasmussen et al., 2000). It is clear that alterations in TP53 do not play a role in benign neurofibromas.

Progress on Task B2 (months 12-24): Complete TP53 sequencing on all tumors.

Completed (Rasmussen et al., 2000). It is clear that MPNSTs typically do have involvement of TP53, usually in the form of LOH, but occasionally in the form of a point mutation (not in any of our samples, however).

Progress on Task B3 (months 12-36): Complete inactivation of TP53 by antisense technology in Schwann cells; perform transformation related assays in TP53 antisense inhibited cells.

As described last year, this Task is not being pursued further since TP53 is only involved in MPNSTs. Furthermore, the recent creation of the NF1/TP53 double knockout cross (laboratories of Tyler Jacks and Luis Parada) provides an animal MPNST system that fills the need that an antisense approach would have created.

Progress on Task B4 (months 18-24): Perform immunocytochemistry studies for p53 on all tumors.

Completed. None of our seven MPNST sections were positive for p53 staining, as expected based on our LOH data (and failure to find ORF point mutations in the sequencing). As described last year, there is no need to examine any further benign tumors for p53 staining.

Technical Objective C. Examine the Involvement of Genes other than NF1 and TP53 in NF1 Tumorigenesis.

Progress on Task C1 (months 1-12): Complete cytogenetic analyses on neurofibroma and neurofibrosarcoma cell cultures.

Completed (Wallace et al., 2000) except that we did not pursue our 3 MPNST cultures since literature suggests that there will be extreme heterogeneity in the karyotypes of these tumors which will be of little strategic use. The plexiform data

suggest that there may be chromosomal instability, and one culture showed telomere associations. These observations suggest that many plexiforms become quite heterogeneous after a first step toward tumorigenesis of loss of the other NF1 allele in a founder Schwann cell. This may lead to future investigations into instability-related genes and/or telomerase.

Progress on Task C2 (months 11-12): Perform comparative genomic hybridization on 5 cutaneous, 5 plexiform tumors and 5 neurofibrosarcomas.

As mentioned at last year's progress report, our original collaborator has withdrawn; we have been unable to set up a new collaboration with an appropriately experienced cytogenetics lab. In the meantime there have been a few other reports of CGH applications to NF1 and these have been uniformly disappointing in their results (little rearrangement, and not consistent areas). It is possible that our own UF cytogenetics lab may hire a new director in the next few months who would be willing/able to set up CGH and so it is possible this will still be pursued. But this approach is now clearly a low priority. Another broad approach pilot study is underway that is actually more powerful, asking questions more directly about gene involvement rather than genomic region involvement: tumor culture RNAs are being hybridized to the Clontech Atlas Cancer cDNA array membranes, compared to normal Schwann cell RNA, to identify aberrantly expressed transcripts (known to be involved in cancer-related systems such cell cycle, proliferation, apoptosis, DNA repair, etc.). This array has over 1100 genes. The method is proving technically exacting but some data are being generated. We hope to analyze RNA from up to 12 tumors (mostly plexiforms) to identify the most commonly-involved genes. These data will be compared with that from differential display as well. It is also possible that we may be able to test some of the tumor RNAs on the Affymetrix Human Cancer Chips, since U.F. just got the Affymetrix system and might provide a few of these chips for pilot studies. The Affymetrix chips have the added advantage of better internal controls for quantitation, which makes analysis a bit cleaner.

Progress on Task C3 (months 13-24): Perform LOH studies for other tumor suppressor loci on all NF1 tumors.

LOH at other loci has been pursued this year as well. TP53 LOH was finished the previous year, as described above (Rasmussen et al., 2000). A microsatellite marker in NF2 has now shown LOH in 4 MPNSTs and 2/29 plexiforms (all numbers reflect results in informative situations). No dermal tumors have yet been examined, but we predict that there will be few, if any, positive samples. This is very interesting and suggests that loss of the NF2 protein may contribute to Schwann cell malignancy, and may be a marker for plexiform tumors at highest risk for malignancy. Of the two positive plexiforms, one is an aggressively growing cervical tumor, and the other was a relatively quiescent neurofibroma in a thigh (whose cells grow very well in culture however). Analysis of further samples may shed light on this possible significance. We have had significant technical problems with the best-informative RB1 marker, despite several primer designs, and so we are looking into just testing RFLPs. The p16 (CDKN2A) gene analysis (one tetranucleotide) has found no LOH in 21 informative plexiform tumors and

MPNSTs studied thus far. TP73 LOH analysis has detected no LOH in 12 informative tumors (RFLP, but we are now testing a new RFLP that has better informativeness). Thus, this work is still underway, in part to accommodate analysis of new tumor and culture samples. We had originally planned to do LOH of 8 genes, but we will now curtail this analysis after we get the RB1 data since the cDNA arrays contain these genes and would provide a more efficient analysis rather than the laborious LOH genotyping.

Progress on Task C4 (months 25-26): Perform second CGH series on tumors of interest (probably all MPNSTs, unless alterations identified in plexiforms in first series of CGH).
See Task C2.

Progress on Task C5 (months 18-24): Submit samples for differential display (DD) analysis to Core Lab.

Very nearly completed except for final confirmation of RNA differences by RNA dot-blot, underway in the core lab. The analysis included 2 normal human Schwann cell cultures, 3 MPNSTs, 4 dermal neurofibromas, and 5 plexiform neurofibromas (all representing Schwann cell cultures). 18 primer sets were used for the analysis to increase chances of detecting changes. A total of 23 bands were chosen that had consistently different intensities than normal or other tumor types as a class, from the autoradiograms. 22 have been cloned and sequenced. Results are listed below—some sequences are known genes and some are ESTs of unknown function. These are not considered conclusive until the RNA dot blot experiments show independently that these RNA level differences are reproducible. If/when we find reproducible differences, we will consider how the alteration in each gene's level might affect the cell's biology and tumorigenic properties. Additional tumor samples will be screened with RNA-based analysis to test for frequency of involvement of those genes. Possibly, DNA-based analysis will be undertaken to see if reduced genes might be due to LOH.

← unpublished data

Band 1: 6x reduced in two dermals, down even more in most plexiforms.
Sequence homology with EST R42462 (novel).
Band 2: 6x-15x reduced in dermals and plexiforms, 3x down in MPNSTs.
Sequence homology: one clone with EST W72647, one with EST AI804845.
Band 3: ≥4x reduced in dermals and most plexiforms and MPNSTs.
Sequence homology with caldesmon and ESTs (F07712).
Band 4: 20x reduced in two dermals and most plexiforms and MPNSTs.
Sequence homology (with a gap in the sequence) to okadaic acid-inducible and cAMP regulated phosphoprotein 19 (ARPP-19), L-myc, ESTs.
Band 5: (intensity varied between normals), 2-3x down in nearly all tumors.
Sequence homology: one clone has no matches, other clone matches best with BAC sequence on chrom. 6 but not so well with ESTs.
Band 6: slightly increased in two dermals and all plexiforms and MPNSTs.
Sequence homology: no matches.
Band 7: reduced or absent in two dermals and nearly all other tumors.
Sequence homology: no matches.
Band 8: reduced in all tumors (most 4x-10x)
Sequence homology ESTs (N95437).
Band 9: increased in two dermals and all other tumors (3-5x)

unpublished data

Sequence homology: perfect match to BACs on chrom. 6
Band 10: increased in two dermals and all other tumors (2-3x)
Sequence homology: one clone EST KIAA0108, other clone ESTs (AA845347).
Band 11: 8x increased in two dermals and all others except sNF96.2
Sequence homology: EST AI672860, some homology to PEX gene.
Band 12: increased in sNF96.2 (15x), normal in all other tumors.
Sequence homology: adenosine A2b receptor
Band 13: varied in normal, reduced a lot in most tumors except 2 dermals.
Sequence homology to MIHD (inhibitor of apoptosis protein 1)
Band 14: absent in normal, increased in tumors 0.5-3x (not type consistent)
Sequence homology: EST AA406425
Band 15: decreased 1X – 40X in tumors, low in all MPNSTs, others vary.
Sequence homology to calumenin.
Band 16: decreased 4-10X in most tumors, especially plexiforms and MPNSTs
Sequence homology to ESTs (AW339630).
Band 17: most tumors absent compared to clear band in normals.
Sequence homology: BAC hit chrom. 22, and ESTs.
Band 18: most tumors decreased 2x or more.
Sequence homology: alpha3 type IX collagen.
Band 19: most tumors decreased 10x or more.
Sequence homology to cytochrome b gene (mitochondrial).
Band 20: absent in most tumors.
Sequence homology to chrom. 18 BAC and HAMY2 gene.
Band 21: most tumors decreased 2x or more
Sequence homology: not yet cloned
Band 22: tumors show 2x or more increase, variable by type
Sequence homology to nickel-induced gene CAP43
Band 23: 2 dermals show 2x increase, most others decreased by 3x or more.
Sequence homology to HLA class II antigen DPW2 beta chain.

Progress on Task C6 (months 25-36): Characterize and analyze specific genes suggested by CGH and DD experiments.

To be done in the last year of the grant. This may also include genes implicated by the cDNA array analysis, should that analysis be completed early enough in the year.

KEY RESEARCH ACCOMPLISHMENTS

- An additional 27 NF1 tumors (dermal and plexiform neurofibromas) were obtained from participating subjects.
- A total of 50 NF1 tumor cultures have been established; 18 of these are clearly tumorigenic Schwann cells (neurofibromin negative), and 10 new ones are still being characterized.
- NF1 LOH analysis has been performed on a total of 115 NF1 tumors and cultures; 75 of these have been analyzed most fully.
- NF1 PTT and mutation analysis have detected 3 new germline mutations, no subtle somatic mutations. Two of the new mutations are novel splicing mutations.
- Neurofibromin staining has been done on all tumors where fixed sections are available, all show at least some neurofibromin-negative Schwann cells. Western blot analysis for neurofibromin has been performed on all cultures that have shown good enrichment for Schwann cells.
- A new NF1 antibody (monoclonal, to the N-terminus) has been made, and is being tested for specificity.
- Two new NF1 rat antisense constructs spanning the translation start site have been made and will be ready to test shortly.
- NF2 LOH analysis was done on over 40 plexiform and MPNSTs, and 2/29 plexiforms were positive, and 4/7 MPNSTs were positive.
- TP73 LOH analysis of a limited sample of tumors has shown no LOH.
- P16 LOH analysis of 21 plexiforms and MPNSTs showed no LOH.
- An erbB2 mutation was found in a non-NF1 plexiform tumor, mimicking the known rodent ENU mutagen model of sporadic neurofibromas.
- Cytogenetic analysis has been performed on 13 neurofibroma cultures.
- Differential display has been performed on 11 tumor cultures, 22 positive bands have been sequenced and differences are being tested for confirmation.

REPORTABLE OUTCOMES

1. From the PTT-based mutation studies, we published our findings for exon 10b (Messiaen et al., 1999). Additional mutation data are being gathered for another publication this year, which will encompass several observations such as splicing errors.

2. NF1 tumor LOH data (NF1, TP53, and other chrom. 17 markers) have been published (Rasmussen et al., 2000).

3. We published the tumor Schwann cell culture technique and cytogenetics findings (Wallace et al., 2000). This plus more complete data were also displayed on a poster at the Aspen NNFF International Research Conference in June 2000, which has stimulated interest from potential collaborators. This more complete data has been accepted for publication in the American Journal of Pathology, and includes the finding of neurofibromin-negativity in cultures, and the ability of these cultures to survive and grow as sciatic nerve xenoplants in scid (immunodeficient) mice (Muir et al., in press). This latter finding was the basis for a DOD application with Dr. Muir as PI, to develop this mouse model, which was funded and began this summer.

4. The aberrant NF1 splicing data (including sequence on new isoforms) are nearly complete and should be submitted before the end of 2000.

5. The erbB2 mutation finding is reportable as a case report, which will be written up by graduate student Lauren Fishbein this year.

6. A license to sell the monoclonal neurofibromin antibody to a company has been applied for, pending quality control results.

7. Graduate student Lauren Fishbein has joined this project (MD/PhD student), and she recently gained her Ph.D. candidacy. She joins Ph.D. candidate Susanne Thomson on this project. Lauren will be developing a new project based on cell biology analysis of the tumor cultures; this is the basis for a new DOD Army NF Program Investigator Initiated grant that we submitted in Sept. 2000 (Steroid Hormones in NF1 Tumorigenesis).

8. Differential Display data, possibly with cDNA array data, may be analyzed sufficiently to be submitted as a manuscript prior to the end of this grant. These data (along with many of the other findings above) will be preliminary data for future grant applications to continue study of NF1 tumors.

CONCLUSIONS

The data substantially support the two-hit hypothesis in NF1 tumors, specifically in a genetically-abnormal clonal Schwann cell population in neurofibromas. This suggests that loss of neurofibromin is associated with tumorigenesis, although whether this is sufficient is still not answered (until the antisense experiments work). It is unclear whether having even just minimal functional neurofibromin is enough to offset tumor formation, and we may be able to use this project's resources to determine this. Splicing errors, for example, are often leaky and thus one would expect that tumors containing one or two NF1 splicing errors would still produce a trace of normal neurofibromin. This does not seem sufficient to stop tumorigenesis, however. Thus, replacement of neurofibromin itself may not be a good therapeutic target because it may have to be present at greater than minor levels to be effective, and replacement of this gene and/or protein is not feasible due to the size and complexity and the number/location of target cells. It is hoped that mutation data such as we are generating will ultimately shed some light on possible functional domains of the molecule, though, so that there might be compensation for one or more functions without replacing the whole molecule. The cytogenetic complexity of the plexiform tumors suggests that these tumors are more abnormal than dermal neurofibromas, and it is a combined effect of a number of gene aberrations that support the unusual growth. Since the plexiform tumors are more medically significant than dermal and affect more patients than MPNSTs, our focus on this tumor type in most of our experiments is well justified. The approaches in our work are aimed at finding common biochemical abnormalities in the different tumor types, or defining the range of pathway heterogeneity present in the population. Both issues are crucial to develop new therapeutic targets, and so our data, particularly data about up- and down-regulated genes, will be very helpful to others who have expertise in biochemistry and pharmaceutics.

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APPENDIX

Rasmussen et al., 2000 reprint
Wallace et al., 2000 reprint
Messiaen et al., 1999 reprint
Human subjects renewal letter.

GENES, CHROMOSOMES & CANCER 28:425–431 (2000)

Chromosome 17 Loss-of-Heterozygosity Studies in Benign and Malignant Tumors in Neurofibromatosis Type 1

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Neurofibromatosis type 1 (NF1) is a common autosomal dominant condition characterized by benign tumor (neurofibroma) growth and increased risk of malignancy. Dermal neurofibromas, arising from superficial nerves, are primarily of cosmetic significance, whereas plexiform neurofibromas, typically larger and associated with deeply placed nerves, extend into contiguous tissues and may cause serious functional impairment. Malignant peripheral nerve sheath tumors (MPNSTs) seem to arise from plexiform neurofibromas. The *NF1* gene, on chromosome segment 17q11.2, encodes a protein that has tumor suppressor function. Loss of heterozygosity (LOH) for *NF1* has been reported in some neurofibromas and NF1 malignancies, but plexiform tumors have been poorly represented. Also, the studies did not always employ the same markers, preventing simple comparison of the frequency and extent of LOH among different tumor types. Our chromosome 17 LOH analysis in a cohort of three tumor types was positive for *NF1* allele loss in 2/15 (13%) dermal neurofibromas, 4/10 (40%) plexiform neurofibromas, and 3/5 (60%) MPNSTs. Although the region of loss varied, the p arm (including *TP53*) was lost only in malignant tumors. The losses in the plexiform tumors all included sequences distal to *NF1*. No subtle *TP53* mutations were found in any tumors. This study also reports the identification of both *NF1* "hits" in plexiform tumors, further supporting the tumor suppressor role of the *NF1* gene in this tumor type. *Genes Chromosomes Cancer 28:425–431, 2000.*

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common autosomal dominant condition, occurring in approximately 1/3,000 individuals. It is characterized primarily by neurofibromas, axillary freckling, café-au-lait spots, and Lisch nodules (Huson, 1994). Affected individuals also have an increased risk of malignancy. Dermal neurofibromas are benign lesions, primarily of cosmetic significance. These occur in most adult NF1 patients, typically developing during adolescence and adulthood, with most individuals developing dozens to thousands in their lifespan. These tumors have virtually no risk of malignant transformation. In contrast, plexiform neurofibromas usually involve deep nerves, have a complex architecture, and frequently extend into contiguous structures. They are often congenital and can cause severe functional impairment. Plexiform tumors occur in a minority of NF1 patients but are not uncommon [32% by macroscopic examination (Huson et al., 1988); 40% by chest/abdominal/pelvic CT imaging (Tonsgard et al., 1998)]. These tumors are believed to degenerate to more highly transformed malignant peripheral nerve sheath tumors (MPNSTs) in an estimated 6% of cases (Gutmann and Collins, 1995).

NF1 is caused by mutations in the *NF1* gene, a 60-exon tumor suppressor locus at 17q11.2 that encodes neurofibromin (Gutmann and Collins, 1995). Loss of heterozygosity (LOH) for chromosome 17 markers (including the *NF1* locus) has been seen in many NF1 malignancies (Skuse et al.,

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1989; Menon et al., 1990; Glover et al., 1991; Xu et al., 1992; Legius et al., 1993; Lothe et al., 1993, 1995; Shannon et al., 1994; Gutmann et al., 1995; Martinsson et al., 1997; Side et al., 1997). Other studies support a tumor suppressor function, such as neurofibromin's GTPase activating protein (ras-GAP) function, and late-onset tumor predisposition in heterozygous knockout mice (Gutmann and Collins, 1995). Colman et al. (1995) showed LOH involving *NF1* in 8/22 dermal neurofibromas, and Serra et al. (1997) found LOH in 15/60 dermal neurofibromas, with a combined percentage of 28% of these tumors showing *NF1* LOH. The region of *NF1* loss varied in these studies, even among tumors from one patient, supporting the hypothesis that *NF1* loss is an independent, somatic event. Sawada et al. (1996) found a somatic 4 bp *NF1* deletion in a dermal tumor in a patient with a constitutional *NF1* deletion, also supportive of the two-hit hypothesis. The only studies that have examined plexiform tumors used four intragenic *NF1* markers, but no flanking markers, and found LOH in a total of 7/14 independent tumors (Daschner et al., 1997; Kluwe et al., 1999). A study of *NF1* mRNA editing also showed a small degree of conversion to a nonsense codon in dermal and plexiform neurofibromas (with a higher level of editing in malignant NF1 tumors), supporting a minor role for epigenetic *NF1* inactivation in all three types of tumors (Cappione et al., 1997).

Plexiform neurofibromas are the least studied of NF1 tumors, yet understanding the pathogenesis of these tumors is particularly important, because these tumors often cannot be completely removed surgically and seem to have a risk for malignant transformation. For a better study of these, along with a cohort of the other NF1 tumor types for comparison, we analyzed 15 dermal neurofibromas, 10 plexiform neurofibromas, and five MPNSTs for LOH at *NF1* and other chromosome 17 loci, to gather further evidence for the two-hit hypothesis and to evaluate the types and frequencies of somatic chromosome 17 events in these tumors. The status of the *TP53* gene, encoding the important p53 cell cycle control molecule [mutated in half of human cancers (Soussi, 1996)], was also examined.

MATERIALS AND METHODS

Genotyping for LOH

Blood and tumor samples were collected under an approved Institutional Review Board protocol. Patients met diagnostic criteria for NF1 (Gutmann et al., 1997). For tumors showing LOH, the following data are available: 319T was a tumor of unknown location from a patient who was 47 years old; 328T5 was from the back of a 24-year-old; 386T1 was excised from the arm of a 12-year-old; 452T came from the arm of a 19-year-old; 454-v came from the buttock of a 21-year-old; the PD tumor was a congenital orbital plexiform on a patient who had this operation at age 34 years; 396T was a neck MPNST from a 43-year-old; 459T was an MPNST from the leg of a 28-year-old; 441T was an MPNST from the cheek of an 18-year-old. DNA was isolated from blood and tissue samples as described in our previous LOH study (Colman et al., 1995).

To assay for *NF1* LOH, blood samples from patients from whom tumors were available were initially genotyped for the intragenic markers (denoted NF-) listed in Figures 1 and 2 [methods for these polymorphisms are referenced in a table in Colman et al. (1995) and Rasmussen et al. (1998), except for Evi-20 that was done as described in Lazaro et al. (1993)]. The *TP53* intron 1 polymorphism is a pentanucleotide microsatellite that we genotyped with native PAGE and ethidium bromide staining, after digesting the PCR product with *Hpa*II to reduce the polymorphic fragment to the 60–120 bp size range (Futreal et al., 1991). The *TP53* intron 6 polymorphism is an *Msp*I PCR-RFLP (polymerase chain reaction-based restriction fragment length polymorphism) (McDaniel et al., 1991). D17S841, 1863, 1800, 1301, and 784 are microsatellites that were genotyped using standard radioactive PCR under conditions listed in the Genome Database. The 1F10 *Pst*I RFLP was genotyped by DNA PCR using primers we designed [1F10X2: 5′-TTT ACC CTC GGA TAC TGG TGT TGC, and 1F10X3: 5′-GAG TAC CTT GGT GGA GGC CCA CTC] at 65°C annealing, followed by digestion with *Pst*I. The uncut allele is 245 bps in size, and presence of the *Pst*I site cuts the product into fragments of 130 and 115 bps (separated on PAGE, visualized with ethidium bromide). For markers at which the patient was heterozygous, DNA from primary tumor material was also genotyped. For tumors that displayed *NF1* LOH, we tested flanking markers to examine the boundaries of the LOH segment. Genotypes and LOH were confirmed by repeat analysis, in particular the RFLPs, to control for partial enzyme digestion.

NF1 Mutation Detection

The protein truncation test, thought to detect 60–75% of germline mutations, was used to screen

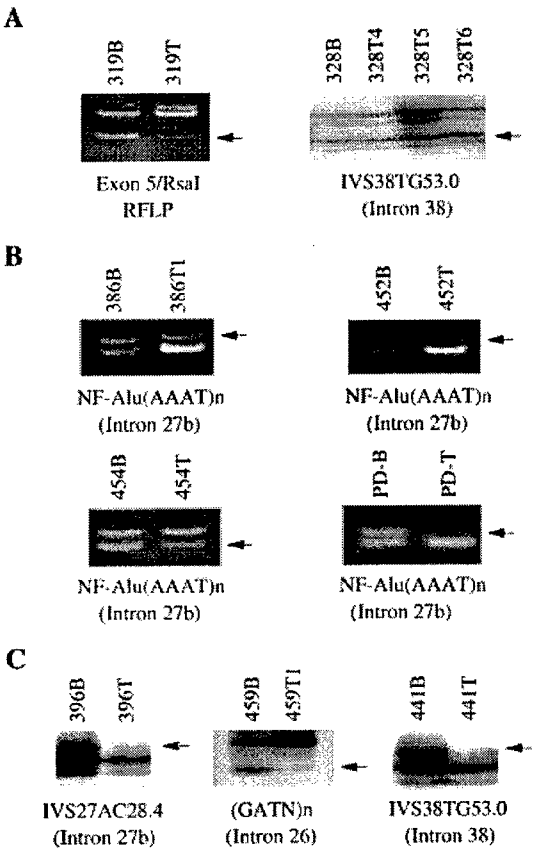

Figure 1. Loss-of-heterozygosity (LOH) analysis. As shown, two dermal neurofibromas (**A**), four plexiform neurofibromas (**B**), and three MPNSTs (**C**) demonstrate *NF1* allele loss (B = blood, T = tumor), whereas two dermal tumors (328T4, 328T6) do not show LOH. The arrow marks the allele that is decreased in intensity, representing LOH.

NF1 mRNA from blood and tumor samples via overlapping RT-PCR fragments, when such material was available (Heim et al., 1995; Park and Pivnick, 1998). Positive PTT signals led to individual examination of the encoded exons by PCR with heteroduplex analysis and SSCP (Abernathy et al., 1997). Individual exons showing electrophoretic abnormalities were directly sequenced using the ABI Big Dye Terminator kit and ABI 377 or 373 automated sequencer, to reveal the mutations.

TP53 Mutation Detection

For all tumors, exons 4–9 were individually amplified (primer sequences and conditions from Oncor, Inc.), and the PCR products were analyzed by heteroduplex analysis and SSCP as above. These six exons are the most commonly mutated exons in *TP53*. PCR products with abnormal electrophoretic patterns were directly sequenced as above. Two positive controls [point substitutions, one in an MPNST (Nigro et al., 1989) and one in an osteosarcoma (data not published)] were used to establish the sensitivity of these assays (particularly SSCP) at detecting even single base changes.

RESULTS

The 15 dermal tumors were obtained from 8 patients. The plexiform tumors and MPNSTs all came from separate patients. Genotyping identified LOH in 2/15 (13%) dermal neurofibromas (2 different patients), 4/10 (40%) plexiform neurofibromas, and 3/5 (60%) MPNSTs, with loss of chromosome 17 markers at least within the *NF1* locus. Figure 1 shows examples of LOH, and an LOH result summary is shown in Figure 2. These results were reproducible, and the level of the residual normal allele was consistent for all loci showing LOH. There is no evidence that any of the LOH regions in dermal or plexiform neurofibromas extend into the p arm (i.e., *TP53* markers). The only MPNSTs that had *TP53* LOH were two of the three MPNSTs that showed *NF1* LOH. *TP53* exon analysis of all tumors failed to reveal any abnormalities, although the two point mutation controls were detected by SSCP, supporting the notion that *TP53* mutations are likely to be absent in exons 4–9 (the "hot spot" exons) in these tumors.

Constitutional mutations were identified in three of the tumors showing LOH, supporting the two-hit hypothesis at *NF1*. In UF452, the germline mutation was found to be C4084T (nonsense mutation R1362X) in exon 23–2 (Fig. 3A), that was maintained in the plexiform tumor sample as assayed by *Taq*I restriction digest analysis (data not shown). In UF459, the germline mutation was found to be a novel single base deletion in exon 21, a frameshift creating a prematurely truncated protein (before the GAP domain) (3683delC). This mutation was also present in the tumor sample (MPNST), indicating that the normal allele was lost in the tumor, fulfilling the two-hit hypothesis (Fig. 3B). UF319's germline mutation is the exon 19b nonsense mutation C3208T (Q1070X at the protein level), and was also retained in the tumor. Germline mutations (some not yet fully characterized) were detected by PTT in several patients whose tumors did not show allelic loss (representing 3 plexiforms, 2 dermal tumors from the same individual, and one MPNST). These aberrant patterns, or the specific mutations, were reproducible in the tumors, also consistent with the two-hit hypothesis (data not shown).

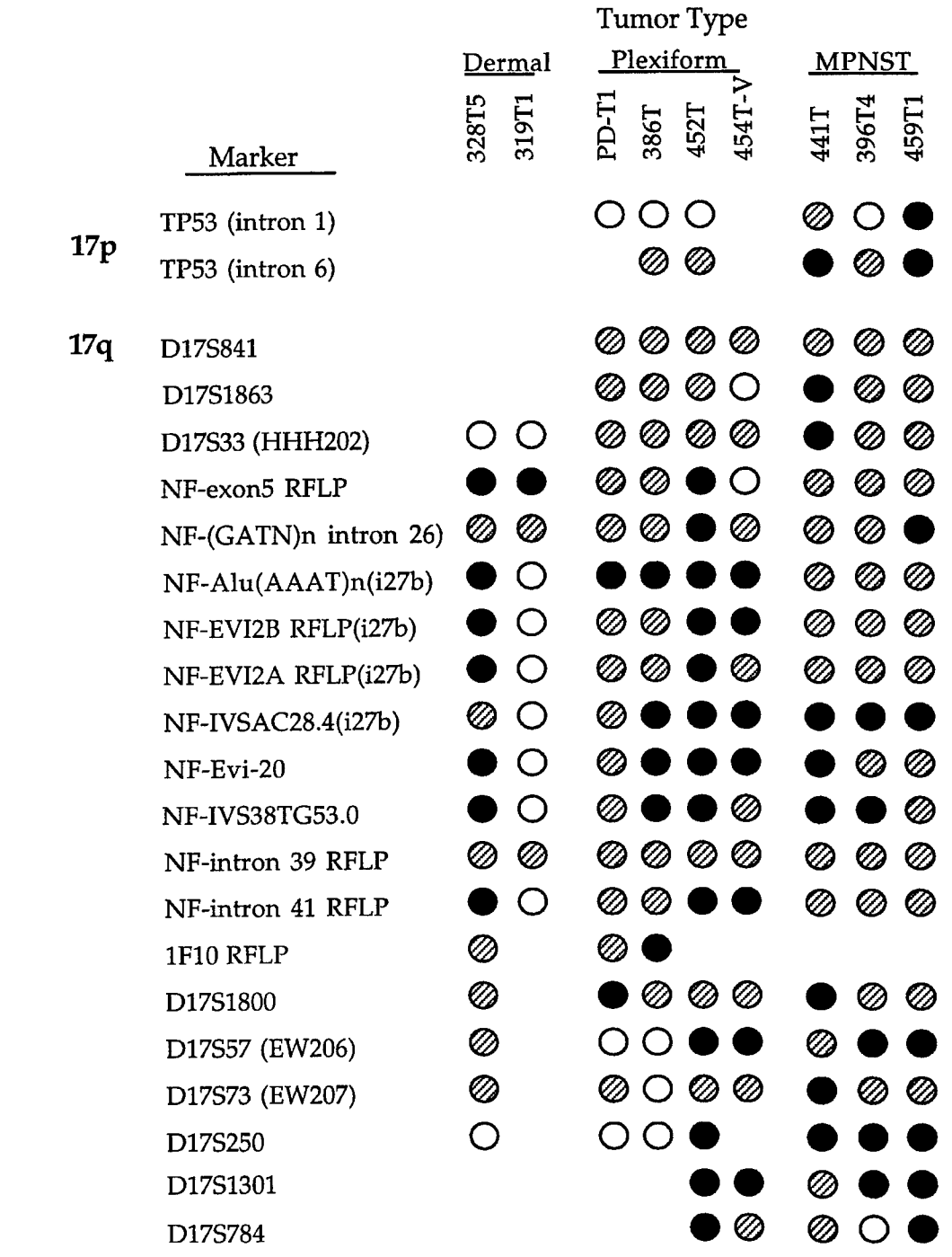

Figure 2. Schematic diagram showing results of loss-of-heterozygosity (LOH) analysis at intragenic *NF1* and flanking markers in the dermal neurofibromas, plexiform neurofibromas, and MPNSTs studied. The markers are listed from pter to qter, in the best estimated order based on published maps. The *TP53* markers are on 17p13.1, and the rest of the markers are on 17q: D17S841–D17S1800 lie in 17q11.2; D17S57, D17S73, and D17S250 lie at 17q12. D17S1301 is not well mapped cytogenetically, but seems to reside in the 17q22–q24 interval based on physical maps. D17S784 is at 17q25 near the telomere. Black circles indicate LOH, striped circles indicate uninformative markers, and open circles indicate heterozygosity (no LOH).

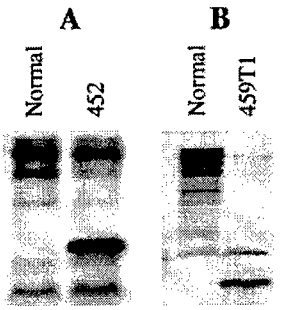

Figure 3. PTT results showing the germline mutations in UF452 (**A**) from leukocytes; and UF459T (**B**) from tumor tissue. The first lane of each panel shows the normal PTT pattern, and the second lane shows the pattern of the patient material.

Parental DNA was analyzed for three patients whose tumors showed LOH (data not shown). For UF328 (a new mutation case), the intron 38 microsatellite showed that the maternal *NF1* allele was lost in the tumor, suggesting that the germline mutation lies on the paternal allele. For PD (also a new mutation case), analysis at D17S1800 (approximately 250 kb downstream of *NF1*) showed that the tumor had lost the maternal allele, with the germline mutation on the paternal allele. Both of these cases are consistent with a preponderance of paternal origin of new *NF1* mutations (Jadayel et al., 1990). In another case, UF319's affected daughter was found to have inherited the allele retained in UF319's dermal tumor, also consistent with the two-hit theory.

Figure 4 shows results of LOH evaluation of six different portions (small, discrete, firm, individual "worm-like" masses) dissected from within a large diffuse plexiform mass of the upper arm of UF454. Diffuse tumors tend to be thin and spread over a large region, often involving the skin and underlying tissue, in contrast to more typical plexiforms (that are more deeply placed and are somewhat better-defined masses). One of the six portions (T-v, Fig. 4) shows a consistent, although subtle, intensity shift, indicative of LOH at informative markers.

DISCUSSION

Our studies discovered *NF1* loss of heterozygosity in all three types of NF1 tumors: dermal and plexiform neurofibromas, and MPNSTs. The region of loss varied among different tumors and in some cases extended to involve most of the long arm of chromosome 17, similar to previous reports in dermal neurofibromas (Colman et al., 1995; Serra et al., 1997). No allelic loss was detected 5′ of *NF1* in benign tumors. Compared to the dermal neurofibromas (both in our study and in others), a larger proportion of plexiform tumors showed LOH, that usually extended through the q arm (in contrast to the typically more limited LOH extent in our series of dermal tumors, this report and Colman et al., 1995). This is consistent with a postulation that plexiform tumors, being more complex and having greater tumorigenic potential, may harbor more genetic changes than do the simpler dermal neurofibromas (supported by recent cytogenetic data: Wallace et al., 2000). This is also consistent with the theory that dermal neurofibromas may require only a somatic *NF1* mutation, whereas the rarer plexiform tumors develop through additional genetic events. These numbers, however, may reflect a sampling effect, because Serra et al. (1997) found loss of the entire 17q in 7/15 dermal neurofibromas. Thus, it will require additional data to more accurately determine the sizes and frequencies of allelic losses in the different tumor types. The percentage of plexiform neurofibromas with LOH in our study is very consistent with previous reports (Daschner et al., 1997; Kluwe et al., 1999).

One possible explanation for lack of allele loss in some tumors is that a more subtle somatic *NF1* mutation occurred (e.g., a point mutation, small deletion, or insertion) that affected neurofibromin production or function, but would not show LOH. A study of bone marrow from 18 NF1 patients with

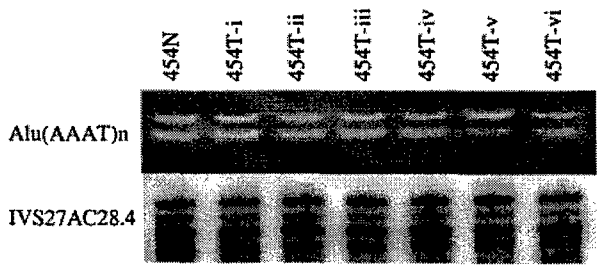

Figure 4. Loss-of-heterozygosity (LOH) analysis in different portions of a single diffuse plexiform neurofibroma. Only the fifth portion of the tumor (454-v) demonstrates LOH when compared to normal tissue (454N), with consistent reduced intensity of the lower alleles for intron 27b markers Alu(AAAT)n and IVS27AC28.4.

malignant myeloid disorders, however, did not support this hypothesis (Side et al., 1997): the protein truncation test failed to identify a somatic *NF1* mutation in the nine (out of 18) patients with no tumor LOH. These results were unexpected, given the sensitivity of the PTT, and the fact that small somatic mutations in other tumor suppressor genes are typically truncating (Bijlsma et al., 1994; Polakis, 1995).

In our study, the two MPNSTs that did not show LOH are somewhat suspect in terms of the tissue used for DNA extraction. UF284's tumor was irradiated before surgery, and thus the DNA present in the sample may be from the residual normal cells (if malignant cells had died). UF158's MPNST, although not irradiated before surgery, was noted to have large regions of necrosis, with the most viable tumor only at the margins. Thus, it is possible that there was a minimal contribution of malignant cell DNA to the UF158T sample.

MPNSTs UF441T and UF459T showed LOH at all informative chromosome 17 loci, suggestive of possible homolog loss as previously seen in NF1 MPNSTs (e.g., Glover et al., 1991). UF396T4 showed only 17q loss, however. The lack of *TP53* mutations (in hot-spot exons 4–9) suggests that *TP53* may play a role only in malignant progression of some plexiform tumors [that is similar to its proposed role late in the genetic pathway in colon cancer (Nigro et al., 1989)], or that other *TP53* aberrations are present but were not detected.

Allelic loss on 17q was found in one of six discrete masses dissected from the diffuse plexiform tumor, with the proximal breakpoint in the *NF1* gene. Presumably, LOH is partially masked in this one tumor fragment (UF454T-v), and fully masked in the others due to admixture of normal cells in the sampled tumor segments, because neurofibromas contain a number of different cell types. This "contamination" by normal cells might also explain the lack of detection of LOH in some of the tumors of other patients. Other explanations for the UF454 observation, however, include: (1) several independent genetic events cooperate in a region to produce diffuse plexiform tumors (i.e., the other portions actually do not have LOH), or (2) LOH had occurred in only that portion of the whole tumor, in which case LOH would not be an initiating event for the whole mass.

Further studies will determine critical pathways in NF1 tumorigenesis; it is possible that this may not always involve somatic inactivation of *NF1*. Also, it remains to be seen whether inactivation of *NF1* alone is sufficient for dermal or plexiform tumorigenesis. Identification of genetic events in plexiform tumors may provide clues about etiology, provide therapeutic targets, and determine which tumors are at risk of malignant transformation.

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RESEARCH ARTICLES

Culture of Cytogenetically Abnormal Schwann Cells From Benign and Malignant NF1 Tumors

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Dermal and plexiform neurofibromas are benign peripheral nerve sheath tumors that arise in neurofibromatosis type 1 (NF1). NF1 patients also have an increased risk of malignant peripheral nerve sheath tumors (MPNSTs), thought to arise in a subset of plexiform neurofibromas. Plexiform neurofibroma pathogenesis is poorly understood, despite the serious clinical problem posed by these tumors. The Schwann cell is hypothesized to be the cell type initially mutated and clonally expanded in plexiform neurofibromas. To test this hypothesis and search for genetic alterations involved in tumorigenesis, we established Schwann cell cultures from plexiform and dermal neurofibromas. Cytogenetic abnormalities were identified in 4/6 plexiform cultures (including one from a plexiform with a sarcomatous component) and 0/7 dermal neurofibroma Schwann cell cultures. There were no consistent chromosomal regions involved in the abnormal karyotypes, suggesting that plexiform tumors are heterogeneous and may bear a variety of primary and/or secondary genetic changes. This is the first study to show successful culturing of genetically abnormal Schwann cell lineages from plexiform neurofibromas. Thus, we present the strongest evidence yet to support the theory that the Schwann cell is the central component in the development of plexiform neurofibromas. This is a key finding for NF1 research, which will lead to further studies of the genetic and biochemical pathogenesis of these Schwann cell tumors. *Genes Chromosomes Cancer 27:117–123, 2000.*

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common autosomal dominant condition characterized by multiple benign neurofibromas, café-au-lait spots, Lisch nodules, and increased risk of malignancy. Dermal neurofibromas (cutaneous or subcutaneous, which usually develop during adolescence and adulthood) are primarily of cosmetic significance. These small tumors, rich in extracellular matrix and sparsely populated by Schwann and mesenchymal cells, involve terminal nerves and have virtually no risk of malignant transformation. In contrast, plexiform neurofibromas are usually congenital, typically involve deep or named nerves, can become very large, and usually cause serious functional impairment. Since plexiform tumors often occur on critical nerves and are not discrete masses, surgical removal is rarely successful, and patients suffer compromised function and may ultimately succumb to their disease. Plexiform tumors develop in 10%–20% of NF1 patients, and these highly cellular masses are believed to progress to highly transformed malignant peripheral nerve sheath tumors (MPNSTs) in an estimated 6% of cases (Gutmann and Collins, 1995).

NF1 is associated with germline *NF1* gene mutations (chromosome band 17q11.2), resulting in absent or abnormal neurofibromin (Gutmann and Collins, 1995). However, NF1 tumor pathogenesis remains poorly understood. Studies have shown *NF1* loss of heterozygosity (LOH) in some benign (Colman et al., 1995; Sawada et al., 1996; Daschner et al., 1997; Serra et al., 1997) and malignant NF1 tumors (Skuse et al., 1989; Menon et al., 1990; Glover et al., 1991; Xu et al., 1992; Legius et al., 1993; Lothe et al., 1993, 1995; Shannon et al., 1994; Martinsson et al., 1997; Side et al., 1997). Thus, cumulative data support the tumor suppressor hy-

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pothesis for NF1 and suggest that at least some neurofibromas contain a clonal cellular element.

Schwann cells are the hypothesized progenitor of neurofibromas, in part because of predominance (40%–80% of cells) and similarities with non-NF1 schwannoma (Peltonen et al., 1988). In addition, neurofibroma Schwann cells have invasive and angiogenic properties, suggesting that these are genetically altered cells (Sheela et al., 1990; Muir, 1995). Although NF1 malignancies have been studied cytogenetically (Riccardi and Elder, 1986; Glover et al., 1991; Jhanwar et al., 1994), there is a lack of such analyses in plexiform neurofibromas, probably due to the heterogeneity of routine primary neurofibroma cultures and the inability of Schwann cells to proliferate in standard culture conditions. To overcome these difficulties and address the issue of tumor cell type, techniques were developed to expand and enrich Schwann cells from NF1 tumors. Cytogenetic studies of these cells subsequently revealed somatic changes in 4/6 plexiform cultures, showing that the tumor cells were successfully cultured and that the Schwann cell is genetically altered in at least some plexiform neurofibromas.

MATERIALS AND METHODS

Culture of Schwann Cells From NF1 Tumors

Resected tumor tissues were washed with Leibowitz L-15 medium containing penicillin and streptomycin. Capsular material was removed and viable tumor isolated. Using cross-scalpels, tumor tissue (1 cm^3) was minced and incubated at 37°C overnight in 10 ml of L-15 medium containing 10% bovine serum, 1.25-U/ml Dispase (Collaborative Research), 300-U/ml collagenase (type XI; Sigma Chemical), and antibiotics. The tissue was dispersed by trituration and strained through a 20-μm mesh nylon screen. The filtrate was diluted with L-15 and centrifuged (500 × g, 5 min). The cell pellet was resuspended in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum and antibiotics, and cells were seeded into tissue culture flasks (~10^6 cells/75 cm^2). After 4 days, cultures were detached by trypsinization and passaged 1:4. Two flasks of cells in standard culture were later harvested and stored in liquid nitrogen. Based on a modification of Rutkowski et al. (1995), two flasks were grown on plastic precoated with laminin (10 μg/ml), prepared as described in Muir (1994), in medium containing recombinant human glial growth factor-2 (rhGGF-2; 25 ng/ml). rhGGF-2 treatment caused rapid proliferation of Schwann-like cells that quickly outgrew patches of fibroblastic cells. During subsequent passage, Schwann-like cells were enriched further by differential detachment using mild trypsinization and shaking. The combination of preferential laminin attachment, differential detachment, and selective mitogen treatment with rhGGF-2 yielded highly enriched (> 99%) Schwann-like cell cultures within 2–4 passages (Fig. 1). These cultures were expanded, withdrawn from rhGGF-2 as indicated, and harvested for analyses. rhGGF-2 was generously provided by M. Marchionni (Cambridge Neuroscience).

Immunocytochemical Characterization of Schwann Cell Phenotype

NF1 tumor cultures were examined for immunoreactivity with antibodies to the Schwann cell antigens S-100 (Dako) and the low-affinity nerve growth factor receptor (p75; hybridoma 200–3-G6–4; ATCC). Cultures grown on chamber slides (Nunc) were fixed with 2% paraformaldehyde for 20 min, then washed with PBS containing 0.5% Triton X-100. Nonspecific antibody binding was blocked with PBS containing 0.1% Triton and 10% normal serum (blocking buffer) for 1 hr. Primary antibodies were diluted in blocking buffer and applied to wells for 2 hr at 37°C. Bound antibodies were labeled with peroxidase-conjugated secondary antibodies (1/500, Dako) for 1 hr at 37°C and chromogenic development was accomplished with 3,3′-diaminobenzidine-$(HCl)_4$ (0.05%) and hydrogen peroxide (0.03%) in PBS. Bromodeoxyuridine (BrdU) incorporation in vitro and immunolabeling of BrdU-DNA were performed as described previously (Muir et al., 1990).

Cytogenetic Analysis

Metaphase cells were harvested by standard cytogenetic methods following short (1–3 days) treatment with rhGGF-2. To further increase mitotic activity, cells were trypsinized, allowed to reattach, and harvested within 16 hr. Mitotic arrest was performed by either addition of 50 μl of ethidium bromide (1 mg/ml) and 30-μl Colcemid (10 μg/ml) per 5 ml of culture medium for 1 hr, or by addition of 50-μl ethidium bromide (1 mg/ml) and 40 μl of dilute Colcemid (5 μg/ml) per 5 ml of culture medium for 16 hr (overnight). Cells were released using trypsin to create a cell suspension and exposed to either a 0.075-M KCl hypotonic solution or a one part 0.075-M KCl to one part 0.8% sodium citrate hypotonic solution for 20 min. Cells were subsequently fixed by three washes with

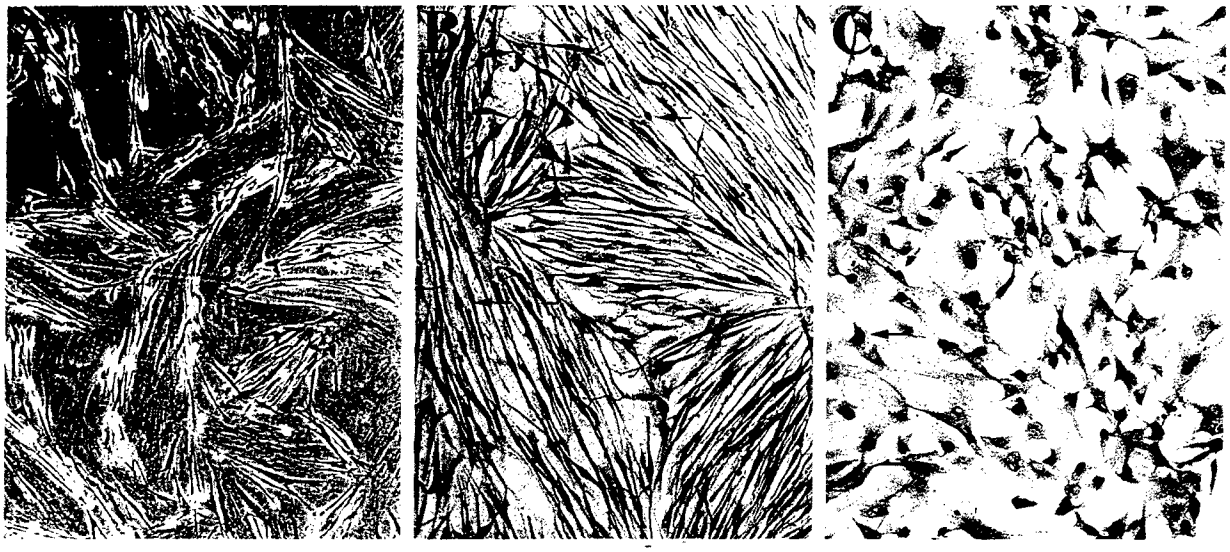

Figure 1. Characterization of SC$^+$ (pNF94.5) and SC$^+$ (pNF92.1) cultures. **A:** Phase contrast photomicrograph of pNF94.5 as a mixed primary culture (passage 1) composed of Schwann cells (phase-bright cell bodies) and fibroblastic cells (asterisk). After Schwann cell enrichment, SC$^+$ (pNF94.5) (passage 3) was withdrawn from rhGGF-2 and grown for 48 hr in medium containing serum and BrdU. **B:** Double immunolabeling for BrdU-DNA (arrows) and S-100 shows SC$^+$ (pNF94.5) mainly contains quiescent Schwann cells (BrdU-negative and S-100–positive cells). **C:** The same double immunolabeling of SC$^+$(pNF92.1) demonstrates that cells of Schwann lineage were highly proliferative in response to serum only after withdrawal of rhGGF-2.

Carnoy's fixative (3:1 methanol:glacial acetic acid). Metaphase spreads were prepared using standard cytogenetic slide making procedures and artificially aged at 54°C for 16 hr. Chromosomes were G-banded (GTG) following modifications of the procedure described by Seabright (1971) and were analyzed by conventional light microscopy. Imaging and karyotyping was performed via standard computer imaging techniques (Applied Imaging).

RESULTS

Primary cultures of the dissociated neurofibromas were composed of spindle-shaped Schwann-like cells and flattened fibroblastic cells. Figure 1A shows plexiform culture SC$^+$ (pNF94.5) in initial stages of Schwann cell enrichment. Schwann cells from neurofibromas generally fail to proliferate under standard culture conditions and are consequently overgrown by fibroblastic cells (Muir, 1995). However, for many neurofibromas, subculture in the presence of the Schwann cell mitogens rhGGF-2 and laminin resulted in highly enriched Schwann cell populations largely devoid of contaminating fibroblastic cells. Only tumor cultures highly enriched in Schwann cells (> 95% S-100–positive cells) were used in this study. Table 1 lists the Schwann cell-enriched (SC$^+$) tumor cultures studied. SC$^+$ cultures from dermal neurofibromas were quiescent or scarcely proliferative in the absence of rhGGF-2. However, this was true for only one-half of the SC$^+$ cultures from plexiform tumors. SC$^+$ (pNF94.5) is an example of a plexiform Schwann cell culture that showed little DNA synthesis in the absence of rhGGF-2 (Fig. 1B). On the other hand, 3/6 plexiform cultures became enriched with a dominant Schwann cell population that continued to expand in serum-containing medium without rhGGF-2. It is important to note, however, that Schwann cell enrichment was vastly improved in the presence of rhGGF-2, a further indication that these cells were of Schwann lineage. BrdU-labeling of one such culture, SC$^+$ (pNF92.1), showed extensive proliferation in the absence of rhGGF-2 (Fig. 1C). SC$^+$ (pNF95.11b) and SC$^+$ (pNF95.6) were the other serum-responsive plexiform Schwann cell lines (see Table 1), but unlike SC$^+$ (pNF92.1), these cultures were derived from classic plexiform tumors with no apparent sarcomatous component. Although different cell morphologies were observed between the different plexiform SC$^+$ cultures, each cell line was mainly homogeneous and immunostained positively for the Schwann cell antigen S-100 (Fig. 1B and C). It is important to note that none of the SC$^+$ cultures were immortalized and all senesced after repeated passage regardless of mitogen stimulation. However, all of the plexiform Schwann cell cultures were expanded beyond the number of passages observed as the limit for Schwann cells derived from normal nerve and the three serum-responsive lines senesced only after protracted expansion (Rutkowski et al., 1995).

TABLE 1. NF1 Neurofibroma Schwann Cell Cultures and Karyotypes

Cell line	Karyotype
Dermal Neurofibromas	
SC^+ (cNF96.5f)	46,XX[18]
SC^+ (cNF96.5g)	46,XX[13]
SC^+ (cNF97.2a)	46,XX[18]
SC^+ (cNF97.2b)	46,XX[5]
SC^+ (cNF97.5)	46,XY[20]
SC^+ (cNF94.5)	46,XY[18]
SC^+ (cNF93.1a)	46,XX[10]
Plexiform Neurofibromas	
SC^+ (pNF95.6)	46,XY[20]
SC^+ (pNF95.11b)	46,XY[20]
SC^+ (pNF94.5)	46,XY,t(1;9)(p36.3;p22)[24]
SC^+ (pNF95.5)	46,XX,t(2;11)(q13;q23)[3]/ 46,XX,del(12)(q13)[1]/ 91,XXXX,del(2)(p13),idic(4)(q?31.2),−20[1]/ 46,XX[15]
SC^+ (pNF95.1)	8/14 cells had unrelated non-clonal abnormalities as described below: 44,XX,dic(16;20)(p13.3;q13.3)dic(17;22)(p13.3;q13.3)[1]/ 46,XX,+X,−9[1]/ 44,XX,dic(1;17)(q44;p13.3),dic(9;16)(p12;q24),+der(16)t(9;16)(p12;q24), −18,add(22)(q11.2)[1]/ ?46,XX,t(16;22)(p13.3;q13.3)[1]/ 45,XX,del(12)(p13),add(17)(p13.3),dic(22;22)(p11.1;q13.33)[1]/ 45,XX,+1,−12,−16[1]/ 45,XX,−8,add(16)(q24),add(20)(q13.3)[1]/ 45,XX,der(9)(9pter → q34::?::15q26 → 15pter),−10,+12,−15[1]/ 46,XX[6]
SC^+ (pNF92.1)[a]	46,XX,t(1;9)(p36.1;q21.2),t(9;12)(p24;q13),t(11;17)(q?14.2;?p13)[2]/ 46,XX,t(2;11)(q21;q13),t(3;19)(p12;q13.1),del(6)(q21.2),add(10)(p15), del(14)(q24),add(22)(p11.1)[2]/ 53 ~ 58,XX + 2,add(2)(p21)×2,add(2)(q37),+6,+7,add(7)(q34),+8, add(11)(p11.2),add(11)(q23), −13,add(14)(p11.1),+16,add(16)(q12.1),+17,+19, add(19)(q13.3),+20,+21,−22[cp3]/ 100 ~ 116,XXXX,−X,idemx?[cp2][b] 44,XX,−6,−17,−18,+21[1]/ 46,XX,add(22)(q13)[1]/ 47,XX,+5[1]/ 46,XX[4]

[a]This plexiform tumor had malignant foci.

[b]These two near-pentaploidy cells included the same structural abnormalities observed in the composite karyotype described immediately above; however, the exact copy number could not be established.

As shown in Table 1, no cytogenetic abnormalities were found in the seven cultures derived from dermal neurofibromas. However, 4/6 plexiform tumor cultures showed karyotypic aberrations. In SC^+ (pNF94.5), all metaphases contained an apparently balanced translocation. The karyotype was 46,XY, t(1;9)(p36.3;p22) (Fig. 2). A culture from a dermal neurofibroma from the same patient showed only a normal karyotype (18/18 cells, data not shown), demonstrating that the translocation was not constitutional. To test whether the chromosome abnormality in SC^+ (pNF94.5) might have been an artifact (selective subculture during Schwann cell enrichment), a second, independent culture of pNF94.5 was also studied. This culture, derived from a separate vial of cells frozen from the first passage, contained a mixture of predominantly Schwann cells and fibroblasts (as in Fig. 1A). The culture was stimulated with rhGGF-2 to induce Schwann cell mitosis and then karyotyped. The t(1;9) was evident in 14/14 metaphases, showing that the translocation is a somatic mutation in the primary tumor and was present prior to Schwann cell enrichment. Two cultures, SC^+ (pNF95.6) and SC^+ (pNF95.11b), showed no cytogenetic rearrangements. However, these cultures have several tumorigenic characteristics in addition to rhGGF-2–independent growth (data not shown), suggesting that underlying genetic abnormalities likely exist that are not detectable at the cytogenetic level.

SC^+ (pNF92.1), a plexiform neurofibroma with sarcomatous foci, showed a complex karyotype with three abnormal clones (see Table 1). Two of 16 cells showed three translocations [t(1;9),t(9;12),t(11;17)].

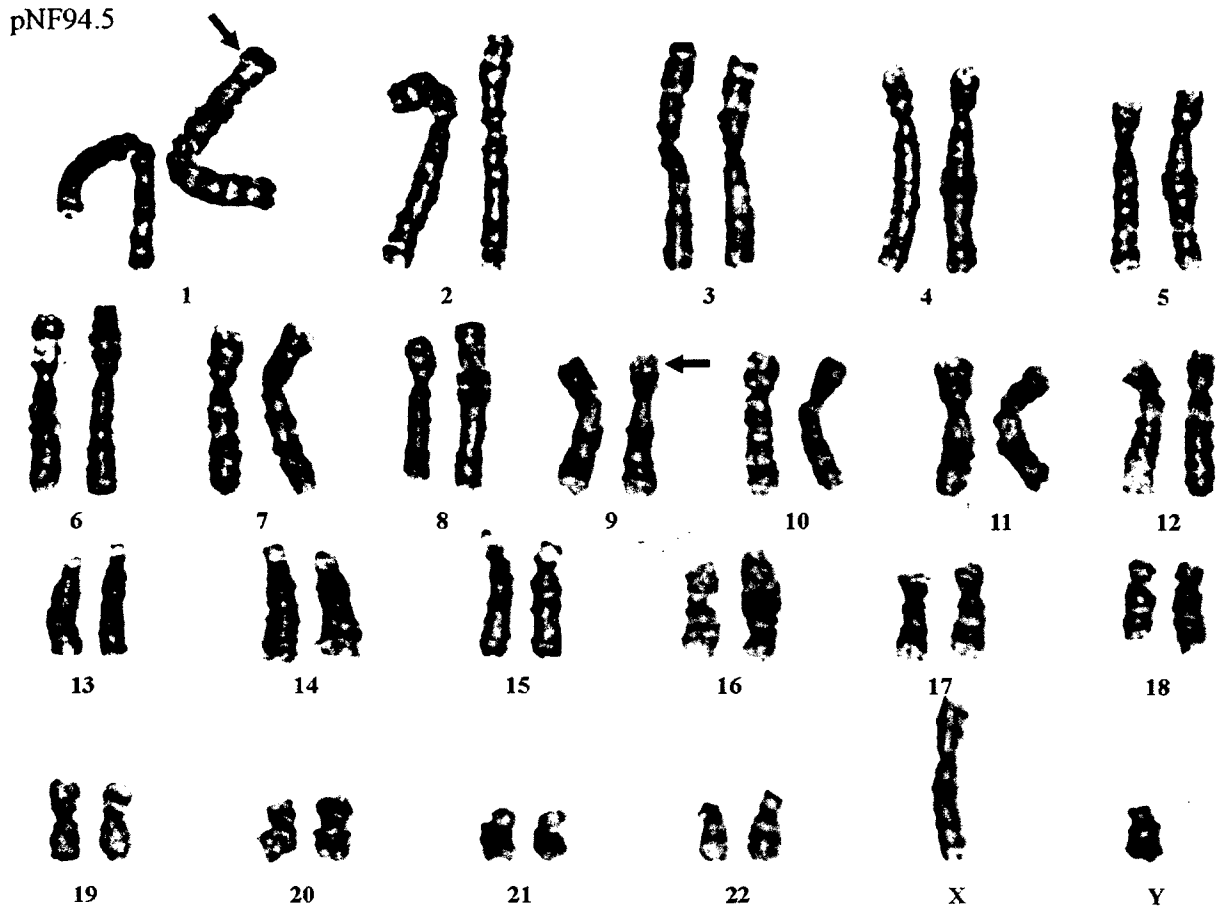

Figure 2. Karyotype of SC+ (pNF94.5) Schwann-enriched culture, showing the t(1;9)(p36.3;p22) (breakpoints indicated by arrows) at a 400-band level.

A second unrelated clone (two cells) was more complex, with translocations, deletions, and unbalanced translocations, although none were the same as in the previous clone. Three cells contained varying abnormalities with 53–58 chromosomes, including multiple marker chromosomes and material of unknown origin attached to several chromosome arms. The remaining five abnormal metaphase cells contained various aberrations ranging from a simple trisomy 5 to near-pentaploidy; the two near-pentaploidy cells also contained some of the same structural abnormalities seen in the three cells above. It should be noted that this tumor was irradiated after its first resection years earlier; our specimen was this tumor's recurrence and it is unclear whether the irradiation therapy may have affected the karyotype or contributed to the development of the malignancy. All other tumors studied here were devoid of malignant foci and the patients had not received radiation or chemotherapy.

Plexiform SC+ (pNF95.5) had aberrations in 5/20 cells (Table 1). Three of these cells had a t(2;11)(q13;q23); one was 46,XX,del(12)(q13), and the other cell was nearly tetraploid with a deletion of 2p and an isodicentric chromosome 4. The other abnormal culture SC+ (pNF95.1) had cytogenetic aberrations in 8/14 metaphases, with each abnormal cell being unique. However, all of the structural abnormalities appeared to involve telomeres. In particular, 9q, 16q, 17q, and 20q were involved; some rearrangements resulted in dicentric chromosomes by end-to-end fusions (telomere associations).

DISCUSSION

The four cases with cytogenetic abnormalities are the first somatic mutations discovered in Schwann cells cultured from plexiform neurofibromas, and the significance is twofold. First, this shows that our culture conditions favor tumor cell growth and enrichment (to homogeneity in at least one case) and strongly implicates the Schwann cell as an abnormal element in neurofibromas that is somatically mutated and clonally expanded. This accomplishment vastly improved our ability to

examine the cytogenetic pattern of this cellular component of initially heterogeneous NF1 tumors. This led to the second significant finding, namely, cytogenetic abnormalities, which suggests that chromosome abnormalities appear to be a factor in plexiform neurofibroma development.

Tumor cytogenetic analysis is often useful in identifying gene regions involved in tumorigenesis. Previous studies of malignant NF1 tumors have revealed complex karyotypes (Riccardi and Elder, 1986; Decker et al., 1990; Glover et al., 1991; Rey et al., 1993; Jhanwar et al., 1994; Mertens et al., 1995; Rao et al., 1996), as seen in SC^+ (pNF92.1). SC^+ (pNF92.1) did not contain the same rearrangements in either of the two clones or in the nonclonal abnormal cells, suggesting that genetic instability might be involved in this tumor. The possibility of genetic instability leading to the aberrations must also be considered, in particular for SC^+ (pNF95.1), which shows telomere breaks, since telomere associations are most frequently reported in chromosome breakage syndromes and tumors (Sawyer et al., 1996). This suggests that mechanisms such as loss of integrity of the DNA repair system might be involved in a proportion of plexiform neurofibromas.

Dermal neurofibromas have been previously karyotyped (as primary tissue), with predominantly negative results (Glover et al., 1991), although one was reported with complex karyotypes in 8/33 cells (Riccardi and Elder, 1986). Our current study found no cytogenetic abnormalities in seven dermal neurofibroma Schwann cell cultures. Combined with the literature, this suggests that either the Schwann cell is not the tumor clonal cell type, or the culturing did not successfully enrich for neoplastic cells, or chromosomal abnormalities are infrequent in the tumor Schwann cell population. Given these tumors' slow and limited growth, and lack of malignant potential compared to plexiforms, the last possibility seems most likely.

Our results support the notion that genetic loci other than *NF1* may be of importance in formation of plexiform neurofibromas and MPNSTs. Since rearrangements were not consistent among the abnormal cases, the idea of a single common pathway in NF1 tumorigenesis is not supported. While there is no direct evidence that the abnormalities are causally related to the tumor development, it is possible that genes in the breakpoint or aneuploid regions (particularly in abnormal clones) contribute to the phenotype. For example, one could speculate that the pNF94.5 1;9 translocation affects the *CDKN2A* gene, which lies at 9p21–22 and is involved in multiple tumor types (Kamb et al., 1994; Martignetti et al., 1999). Another observation suggesting possible involvement of this region in NF1 is a constitutional 9p21 deletion found in a non-NF1 patient with a plexiform tumor (Petty et al., 1993). Likewise, the pNF94.5 1p36 breakpoint might affect the *p73* gene (Jost et al., 1997), which encodes a protein with function similar to p53, which in turn has been implicated in some NF1 malignancies (Nigro et al., 1989; Menon et al., 1990; Glover et al. 1991, Xu et al., 1992; Legius et al., 1994; Lothe et al., 1995).

The data presented here support the long-held hypothesis that plexiform neurofibromas harbor clonally abnormal Schwann cells, a key finding for the NF1 research arena. The Schwann cell subculture method is a valuable tool to provide enriched and expandable tumor material in a manipulatable system for future molecular and cellular experiments. Progress in understanding the genetic and biochemical pathways in benign and malignant NF1 tumor formation will be hastened by the use of these cultures, and the data and cultures will be crucial in developing targets for and testing therapeutic interventions.

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Exon 10b of the *NF1* gene represents a mutational hotspot and harbors a recurrent missense mutation Y489C associated with aberrant splicing

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Purpose: To analyze the spectrum and frequency of *NF1* mutations in exon 10b. **Methods:** Mutation and sequence analysis was performed at the DNA and cDNA level. **Results:** We identified nine exon 10b mutations in 232 unrelated patients. Some mutations were recurrent (Y489C and L508P), others were unique (1465–1466insC and IVS10b+2delTAAG). Surprisingly, at the RNA level, Y489C causes skipping of the last 62 nucleotides of exon 10b. Another recurrent mutation, L508P, is undetectable by the Protein Truncation Test. **Conclusion:** As exon 10b shows the highest mutation rate yet found in any of the 60 *NF1* exons, it should be implemented with priority in mutation analysis. ***Genetics in Medicine*, 1999:1(6):248–253.**

Key Words: *Neurofibromatosis type 1, mutation, hotspot, aberrant splicing, polymorphism, MIR*

Neurofibromatosis type 1 (NF1, MIM 16,2200) is one of the most common autosomal dominant disorders, affecting about 1:3500 individuals in all ethnic groups. The main characteristics are cutaneous or subcutaneous neurofibromas, café-au-lait (CAL) skin spots, iris Lisch nodules, and freckling.[1] Other features found in only a minority of patients include scoliosis, macrocephaly, pseudarthrosis, short stature, malignancies, and learning disabilities. As NF1 clinically presents with great variability, even among patients in the same family, the pathogenicity underlying the phenotype must be complex.

The *NF1* gene has been mapped to 17q11.2 and was positionally cloned.[2–4] The *NF1* gene is approximately 350 kb in size, contains 60 exons and codes for a 11- to 13-kb transcript with an open reading frame coding for 2818 amino acids.[5] By RT-PCR, *NF1* mRNA appears to be ubiquitous, but by Western blot, neurofibromin is best detected in neural-crest-derived tissues.

The mutation rate in the *NF1* gene is one of the highest known in humans (reviewed by Huson and Hughes[1]) with approximately 50% of all NF1 patients presenting with new mutations. Despite the high frequency of this disorder in all populations, relatively few mutations have been identified at the molecular level, with most unique to one family. A limited number of mutational "hotspots" have been identified: R1947X (C5839T) in exon 31 and the 4-bp region between nucleotides 6789 and 6792 in exon 37, both implicated in about 2% of the NF1 patients (reviewed by Upadhyaya and Cooper[6]). In this study we report that another mutational hotspot resides in exon 10b. By analyzing altogether 232 unrelated NF1 patients, we identified 9 mutations in exon 10b, indicating that this exon is mutated in almost 4% of NF1 patients. So far this is the highest score obtained for a particular exon in the 9 years since the *NF1* gene was discovered.

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MATERIALS AND METHODS

NF1 patients

For all patients, the diagnosis of NF1 was based on the presence of two or more of the diagnostic criteria proposed by the NIH Consensus Statement in 1988[7] and updated in 1997.[8] The study was approved by the Institutional Ethical Committees and informed consent was obtained from the patients studied. Patients were recruited randomly without bias as they were seen for medical follow up and genetic advice. Patients were recruited as part of a general mutation study. Seventy-three patients were contributed by the Department of Medical Genetics of Gent and by the Service de Genetique, Hopital Erasme, Brussels; 159 patients were contributed by the Department of Pediatrics, Division of Genetics, University of Florida, Gainesville, and by the Departments of Neurology and Medicine, Center for Human Genetics, Duke University Medical Center, Durham.

Nucleic acid extraction

DNA and RNA samples were obtained from 37 unrelated NF1 patients by extraction from EBV-transformed lymphoblastoid cell lines. In addition, DNA and total RNA samples

were obtained from 195 unrelated NF1 patients by extraction from peripheral blood lymphocytes. Total cellular RNA and genomic DNA was isolated as described.[9]

cDNA analysis and in vitro transcription/translation analysis

First strand cDNA was synthesized by random priming[9] and cDNA was amplified using 5 primer pairs for amplification of the total coding region.[10] 4 μL PCR product was used in an optimized in vitro transcription/translation reaction as described.[9,11] An identical truncated peptide fragment of 55 kD was observed in 2 of 37 patients by in vitro transcription/translation of the fragment spanning exons 1 to 12a and the corresponding cDNA was analyzed by cycle sequencing with and without subcloning using 0.15 μM fluorescein isothiocyanate (FITC) labeled primers, designated by nucleotide positions: 5′-CTTCGGAATTCTGCCTCT-3′ (400–418), 5′-CTGATATGGCTGAATGTG-3′ (719–736), 5′-GCCTGTGTCAAACTGTGT-3′ (967–984), and 5′-CACACCCAGCAATACGAA-3′ (1367–1384), and the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham, Arlington Heights, IL). Samples were loaded on a 6% LongRanger gel (FMC, Rockland, ME) containing 7 M urea and analyzed on an ALF automated DNA sequencer. To check for the presence of the missense mutation Y489C in a fraction of the cDNA, RT-PCR fragments were cloned using the pCR-TOPO cloning kit (InVitrogen, San Diego, CA) and 90 individual clones were further analyzed by cycle sequencing.

Genomic DNA analysis

Exon 10b was amplified using the primer pair as described[12] and PCR products were further analyzed by cycle sequencing without subcloning.[9] Genomic DNA from 195 unrelated NF1 patients from which no EBV lymphoblastoid cell line was available was analyzed by heteroduplex analysis or single strand conformation analysis or both, and aberrant migrating PCR fragments were further analyzed by cycle sequencing.[13] Mutations are reported according to the recommendations of the Nomenclature Working Group,[14] with the start site of translation denoted as nucleotide 1 for both cDNA and genomic alterations.

RESULTS

The total coding region of the *NF1* gene was analyzed by the protein truncation test in 37 unrelated NF1 patients from which an EBV lymphoblastoid cell line was available.[10] In two patients, an identical shortened fragment of approximately 55 kD was discerned in the region encompassing the exons 1 to 12a. In both patients, in vitro transcription/translation for the other regions only showed normal sized fragments. By electrophoresis of the RT-PCR fragments from Patient 1 two discrete bands were discerned on a 1.5% agarose gel, i.e., a normal-sized band of 1868-bp and a band that was approximately 60 bp smaller. In Patient 2, however, only a normal-sized band was seen, indicating that the truncated protein of identical size was formed in a different way in this patient. cDNA sequencing in this region indeed revealed a different mutation in both patients. In Patient 2, an insertion of C at nt 1465–1466 in exon 10b was found, immediately resulting in the creation of a stop codon at this site. In Patient 1, a deletion/skipping of the last 62 nucleotides of exon 10b was observed in RNA from both lymphocytes and the EBV-lymphoblastoid cells (Fig. 1B). Here too, the immediate result is formation of a stop codon at this site, explaining the identical picture seen by protein truncation analysis. Further analysis of exon 10b at the genomic level confirmed the presence of an insertion 1465insC in Patient 2. In Patient 1, however, a missense mutation was identified: A1466G, changing the codon for Tyr to Cys (Y489C) (Fig. 1A). Both parents of this sporadic patient did not carry this missense mutation. This missense mutation masquerades as a splicing defect: indeed substitution of A to G at position 1466 of the genomic DNA creates a new splice donor site (CT/GTAAG) (Fig. 1C). Analysis of the normal and mutant se-

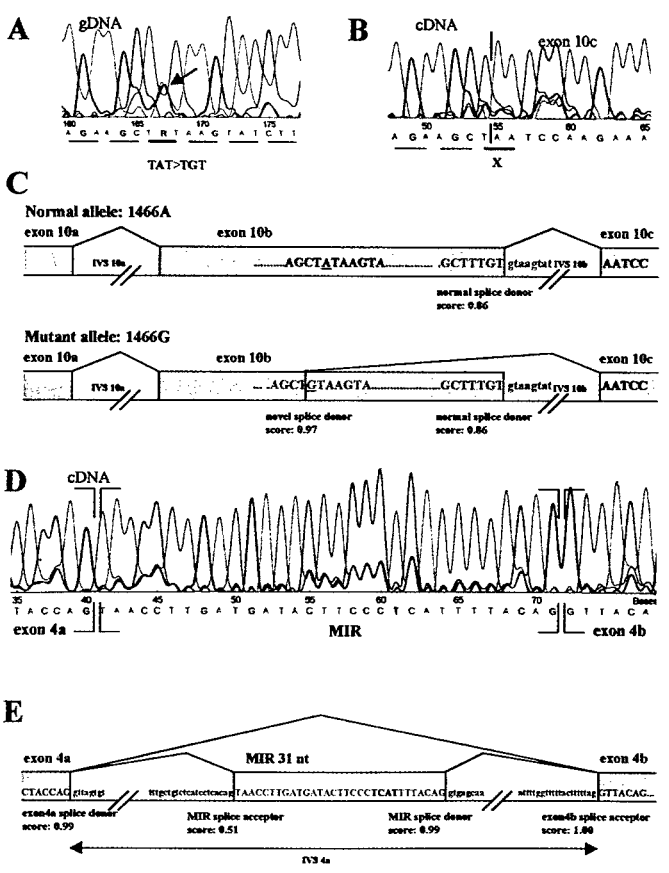

Fig. 1 (A) Cycle sequencing without subcloning of the genomic region of exon 10b in Patient 1 showing transition of A to G at nt 1466 resulting in the formation of a splice donor site CT/gtaag; (B) Cycle sequencing of the mutant cloned cDNA allele in Patient 1. The last 62-bp of exon 10b are skipped and followed by exon 10c, resulting in the formation of a stop codon at amino acid 489; (C) Schematic diagram of the genomic region surrounding exon 10b. Green boxes represent exons, red sequences denote splice donor sequences. By the transition of A to G at nt 1466 a novel splice donor is formed; (D) Cycle sequencing of a cloned RT-PCR fragment encompassing the exons 1 to 12b using sequencing primer 5′-CTTCGGAATTCTGCCTCT-3′ (400–418). Between exon 4a and 4b the presence of the 31-bp MIR-sequence is shown; (E) Schematic diagram of the genomic region surrounding IVS 4a. Green boxes represent exons, red sequences denote splice donor sequences, green sequences denote splice aceptor sequences.

quence using the program for splice site prediction by neural network (available at URL: www-hgc.lbl.gov/projects/splice.html) showed a 0.86 score for the normal exon 10b donor site (GCTTTGT/gtaagtat) and a higher 0.97 score for the new donor site created by the missense mutation Y489C (AGAAGCT/gtaagtat). RT-PCR fragments from an EBV lymphoblastoid cell line of Patient 1 were cloned and 90 individual clones were further analyzed by cycle sequencing to check for the presence of the missense mutation in a fraction of the cDNA. In 50 cDNA clones showing a normal-sized band of 1868 bp on a 1.5% agarose gel, only the wild type sequence was found and in none of them the missense mutation was present. In 40 clones containing a slightly smaller insert (approximately 60 bp) as evidenced by agarose gel electrophoresis, the smaller size was due to the skipping of the last 62 nucleotides of exon 10b along with intron 10b in the mRNA (Fig. 1B). This indicates that the major outcome of the mutation Y489C at the cDNA level is skipping of the last 62 nucleotides of exon 10b. Although Y489C and 1465–1466insC are different mutations at the genomic DNA level, both result in the formation of a premature stop codon at exactly the same position well before the GAP domain of neurofibromin. As the finding of two different mutations at the same spot (i.e., 1465–1466insC and 1466A>G) is in itself indicative of a mutational hotspot,[15] these findings prompted us to analyze exon 10b in a larger patient population consisting of 195 unrelated NF1 patients from which genomic DNA and total RNA was available but no EBV lymphoblastoid cell line. In Patients 3, 4, 5, and 6 the missense mutation Y489C was identified by heteroduplex analysis or single strand conformation analysis or both, followed by cycle sequencing of the aberrant migrating genomic fragments and further analysis at the cDNA level. In Patients 4, 5, and 6, the effect of the missense mutation Y489C at the mRNA level was identical to Patient 1, i.e., skipping of the last 62 nucleotides of exon 10b. Surprisingly, three different sized transcripts were seen after RT-PCR of total RNA extracted from fresh lymphocytes from Patient 3 (no EBV lymphoblastoid cell line was available from this patient). RT-PCR fragments were cloned and further analyzed by cycle sequencing. In the smallest sized fragments, the mutant exon 10b lacking the last 62 bp of the exon 10b was followed by the correct exon 10c but hereafter the exon 11 was neatly skipped out. Skipping of exon 11 was not seen in Patients 1, 4, 5, and 6. In the clones carrying the intermediate-sized transcripts, the mutant exon 10b lacking the last 62-bp was found. In the largest fragments, the normal exon 10b sequence was present as expected. Furthermore, between exon 4a and exon 4b, a 31-bp cryptic exon was inserted in *cis* with the skipping of the last 62 bp of exon 10b and exon 11 in some of the mutant clones (Fig. 1D). This 31-bp cryptic exon was also seen in some mutant clones lacking only the last 62 bp of exon 10b, but also in some normal clones. The same cryptic exon was recently found in a patient showing multiple splicing errors but an unknown constitutional mutation.[16]

In Patient 7, a deletion at the splice donor site of exon 10b was found (IVS10b+2delTAAG) and analysis of RNA extracted from a neurofibroma showed a strong shorter RT-PCR product in addition to the normal sized fragment. By cycle sequencing, it was shown that the shorter transcript was formed by the perfect splicing out of exon 10b. As this splicing alteration resulted in the production of a stable mRNA and the size of exon 10b is a multiple of three, the possibility remains that this allele produces neurofibromin lacking only the 45 amino acids encoded by exon 10b.

In two unrelated NF1 patients (8 and 9; one Caucasian and one African American) an identical missense mutation was identified changing the codon for Leu to Pro (L508P; 1523T>C). This missense mutation that would not be detected by the Protein Truncation Test, does not create/destroy a restriction enzyme site, but shows a specific reproducible shift in single strand conformation polymorphism analysis. Analysis of 153 unrelated Caucasian Americans and 20 unrelated Black Americans ruled out that the alteration is a polymorphism. Another 30 unrelated Caucasian Europeans were analyzed by direct sequencing of exon 10b. In none was the missense mutation L508P found. Table 1 summarizes the effects on RNA splicing of the different germline mutations found in exon 10b.

A novel polymorphism was identified at a CpG dinucleotide in intron 10a: IVS10a-32C>T. We have tested 61 unrelated NF1 patients and 72 normal control persons for this polymorphism and no statistically different values were obtained between the two populations. Taking both populations together, we obtained allele frequencies of 0.68 for the IVS10a-32C allele and 0.32 for the IVS10a-32T allele. In four NF1 patients heterozygous for this polymorphism, we analyzed by RT-PCR the outcome of the IVS10a-32T allele with respect to the splicing of exon 10b and did not observe an effect on the splicing. As the number of polymorphisms at the 5′ end of the *NF1* gene is limited, this novel polymorphism, which can be detected by *Mae*II digestion, may be a useful tool in segregation analyses.

DISCUSSION

In this study we identified nine novel exon 10b mutations in 232 unrelated NF1 patients: Y489C or 1466A>G (5X), L508P (2X), 1465–1466insC (1X), and IVS10b+2delTAAG (1X). Our findings indicate that exon 10b is mutated in almost 4% of the NF1 patients, although so far, only one missense mutation has been reported in exon 10b, K505E.[16] However, only two studies analyzed the complete coding region of the NF1 gene including exon 10b, and the total number of patients analyzed in both studies is small, i.e., 36 patients.[10,16] No other studies specifically looked at exon 10b. Mutational analysis is hampered by the large number of exons, the wide variety of mutations, and the presence of several pseudogenes. The majority of mutations identified hitherto reside in the GAP-related domain, encoded by the exons 21 to 27a and in the exons downstream of this GAP-related domain. The exons lying at the 5′ end of the gene have not been studied exhaustively by many groups. One reason may be that for most exons at the 5′ end the available intronic sequence information was limited to the

Table 1
Summary of the germline mutations found in exon 10b and their effects on RNA splicing

Patient	gDNA mutation	Tissue(s) analyzed • cDNA observations
P1	Y489C; 1466A>G	EBV lymphoblastoid cell line and fresh lymphocytes
		• skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
P2	1465–1466insC	EBV lymphoblastoid cell line
		• formation of a stop codon at AA 489
P3	Y489C; 1466A>G	Fresh lymphocytes
		• skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
		• skipping of 62 nt at cDNA nt 1466–1527 AND skipping of exon 11
		• insertion of a cryptic 31 nt exon, between exon 4a and exon 4b resulting in formation of a stop codon at nt 516 of the cDNA, both on the normal and mutant allele
P4	Y489C; 1466A>G	Fresh lymphocytes
		• skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
P5	Y489C; 1466A>G	Fresh lymphocytes
		• skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
P6	Y489C; 1466A>G	Fresh lymphocytes
		• skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
P7	IVS10b+2delTAAG	RNA from a plexiform neurofibroma (blood RNA not available)
		• skipping of exon 10b
P8	L508P (1523T>C)	No RNA available
P9	L508P (1523T>C)	Fresh lymphocytes
		• no effect on splicing of exon 10b

intron-exon boundaries, thus hampering the search for optimal primer pairs that can be used to amplify these exons, in the absence of sequences derived from pseudogenes.

The novel mutations 1465insC and Y489C are different at the genomic DNA level, but both result in the formation of a premature stop codon at the mRNA level at exactly the same position through different mechanisms. Y489C illustrates that, although the mutation is reported according to the recommendations of the Nomenclature Working Group, nomenclature can be misleading. Indeed, by nature Y489C belongs to the group of splicing error mutations and not to the group of missense mutations. This unexpected finding further stresses the importance of documenting the outcome of a mutation at the mRNA level.

It is intriguing that in 1 of the 5 patients carrying the mutation Y489C, analysis of cloned RT-PCR products showed that some mutant alleles skipped the last 62 bp of exon 10b as well as the complete exon 11. Exon 10c was retained in these clones. Skipping of exon 11 was not seen in the other patients. The cause of the exon 11 skipping in Patient 3 is unknown and may not even be due to a sequence change in the *NF1* gene, or it could be caused by a point change in an intron that renders this exon skipping more likely to occur. As we do not have an EBV lymphoblastoid cell line from this patient, we were not able to study whether this different splicing is tissue-specific. Noteworthy is that, by splice site prediction using neural networks (available at: URL: www-hgc.lbl.gov/projects/splice.html), no splice acceptor sequence can be defined for exon 11, not even after using an acceptor score cutoff of 0.05. This might indicate that splicing of exon 11 is mediated through mechanisms different from splicing of those exons with a well-defined splice acceptor sequence.

The cryptic 31-bp exon between exon 4a and exon 4b was found on normal as well as on mutant cloned cDNA fragments of Patient 3. From ongoing experiments we know that this cryptic 31-bp exon is formed, albeit typically at a low level, in different cell types in NF1 patients as well as in normal persons, and hence does not appear associated with a mutation in the *NF1* gene per se.[17] In some neurofibromas, the cryptic 31-bp exon can be found on both alleles as well (unpublished results). A search of sequence databases showed that this 31-bp sequence was 100% homologous with a part of the *NF1* intron 4a. Using the splice site prediction by neural network program we found that this sequence is flanked by a splice acceptor sequence (score 0.51) tttgctgtctcatcctcacag/TAACCTTGATGATACTTCCC and a very strong splice donor sequence (score 0.99) TTTACAG/gtgagcaa (Fig. 1E). The mechanisms by which this splice acceptor/donor pair is up- or downregulated is unknown, nor is it known whether this transcript is translated in vivo. The 31-bp cryptic exon is part of a fragment of the ancient class of Mammalian-wide Interspersed Repeat

Table 2
Summary of the clinical findings in the patients carrying a mutation in exon 10b of the NF1 gene

Trait	P1	P2	P3	P4	P5	P6	P7	P8	P9
Sex	F	F	M	M	F	F	F	M	M
Age (Yr)	3	7.5	39	9	48	23	18	80	17
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Black American
CALs	>6 and >5mm	>6 and >5mm	>6 and >15mm	>6 and >5mm	>6 and >15mm	>6 and >5mm	>6 and >15mm	>6 and >15mm	>6 and >15mm
Cutaneous NFs	No	Yes (subcutaneous)	Yes	No	Yes	Yes	Yes	Yes	Yes
Plexiform NF	Yes: neck and left hemithorax	Yes: paravertebral	No	No	No	Several larger NFs that may be plexiforms	Yes: of left hand	No	Yes: periorbital
Freckling	Yes	ND	Yes	Yes	ND	Yes	Yes	Yes	Yes
Optic glioma*	No	ND	No	No	No	No	No	ND	ND
Lisch nodules	No	ND	Yes	Yes	Yes	No (no slit lamp exam)	Yes	ND	Yes
Osseous lesion	Hypoplasia of the left thoracic pedicles from thoracic vertebrae I–VII; thoracic scoliosis	Scoliosis requiring osteosynthesis	No	No	Scoliosis; no bone dysplasia	Scoliosis	No	No	No
First-degree relative with NF1	No	Yes	Yes	No	No	No	No	ND	No
Other manifestations	Macrocephaly	Macrocephaly	Complex partial epilepsy; mentally functioning at a lower level	Macrocephaly, learning disabilities	Seizures	Xanthogranulomas	Short stature, learning disabilities	None	Learning disabilities; macrocephaly

M = male, F = female.
*Presence/absence of optic pathway glioma as evidenced after MRI evaluation; ND, not determined.

elements (MIR) (as defined in Genbank sequence AC004222, nt 22263 to 22346 and confirmed by A.F.A. Smit and P. Green, [personal communication, The Genome Center, Washington, DC, 1998] using the program RepeatMasker [Available at: http://ftp.genome.washington.edu/RM/RepeatMasker.html]). Further research into the direct outcome of this germline mutation at the mRNA level in different tissues and cell types in the different affected persons from this family, and comparison with the nonaffected family members as a control may shed more light on this event.

The missense mutation L508P was identified in two unrelated NF1 patients and absent in 203 unrelated control persons from different ethnic origin. Proline is a very rigid amino acid and its presence creates a fixed kink in a polypeptide chain. Although both amino acids are hydrophobic and the amino acid change is conservative, the introduction of a nonaliphatic imino group into the polypeptide may have dramatic consequences for protein structure. Moreover, leucine at this position is conserved in the mouse (L10370), rat (D45201), Fugu rubripes (AF064564), and drosophila (L26501). We presume that this missense mutation is pathogenic and may point to a region of the protein with a hitherto unknown function.

NF1 genotype-phenotype correlation studies have not been extensive or successful except for the consistent correlation between the deletion of the entire *NF1* gene and flanking region and a distinctive phenotype including severe developmental impairment, dysmorphic features, and large numbers of neurofibromas.[18–20] Detailed clinical data together with the genotypes of the patients were submitted to the NNFF International *NF1* genetic mutation database, to allow NF1 genotype-phenotype correlation studies in the future. In 4 of 9 patients scoliosis was present (Table 2). It is worth noting that in our patients with a mutation in exon 10b, a high proportion (5 of 9) harbored plexiform neurofibromas (Table 2). Patient 3, who does not have plexiform neurofibromas himself, has a child with a plexiform neurofibroma on the lip.

In conclusion, a novel mutational hotspot has been identified in exon 10b of the *NF1* gene: a mutation in exon 10b was found to be present in 9 of 232 (3.9%) unrelated NF1 patients. By cDNA-SSCP analysis of the total coding region in 71 Spanish NF1 patients, an identical skipping of nt 1466–1527 of exon 10b caused by the mutation Y489C was found in two patients (E. Ars and C. Lazaro, personal communication, Medical and Molecular Genetics Center–IRO, Barcelona, Spain). The finding of another missense mutation in exon 10b (K505E) in 1 of 14 unrelated American patients[16] further underscores this finding. Taking these data together, 12 exon 10b mutations were reported in 317 unrelated NF1 patients (~3.8%) and is, thus far, the highest score obtained for an *NF1* exon. This is particularly significant given that exon 10b only encodes 1.7% of the open reading frame. Our results indicate that this exon should be implemented with priority in *NF1* mutation analysis.

Acknowledgments

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Health Center Institutional Review Board

PO Box 100173
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MEMORANDUM

DATE: May 18, 2000

TO: Margaret R. Wallace
Box 100296

FROM: R. Peter Iafrate, Pharm.D.
Chair, IRB-01

SUBJ: IRB Protocol #41-1992 **Expires on 6/2/01**

TITLE: "GENETIC STUDIES OF NEUROFIBROMATOSIS 1" [SUBTITLE: GENETIC STUDIES OF TUMORIGENESIS IN NEUROFIBROMATOSIS] [SUBTITLE: THE ROLE OF CUMULATIVE GENETIC DEFECTS IN NF1 TUMORIGENESIS] [SUBTITLE: MUTATION AND GENETIC ANALYSIS OF NEUROFIBROMATOSIS]

Re-approval of this research project was granted on **5/17/00**. **Enclosed is the dated, IRB-approved Informed Consent Form** that must be used for enrolling subjects into this project. You have approval for **12 months** only.

You are responsible for obtaining renewal of this approval prior to the expiration date. Re-approval of this project must be granted before the expiration date or the project will be automatically suspended. If suspended, new subject accrual must stop. Research interventions must also stop unless there is a concern for the safety or well being of the subjects. **You must respond to the continuing review questions within 90 days or your project will be officially terminated.**

The IRB has approved exactly what was submitted. Any change in the research, no matter how minor, may not be initiated without IRB review and approval, except where necessary to eliminate hazards to human subjects. If a change is required due to a potential hazard, that change must be promptly reported to the IRB.

Any severe or unanticipated side effects or problems, and all deviations from federal, state, university or IRB regulations must be reported, in writing, within 5 working days.

Upon completion of the study, you are required to submit a summary of the project to the IRB office.

Research records must be retained for three years after completion of the research; if the study involves medical treatment, it is recommended that the records be retained for eight years.

If VAMC patients will be included in this project, or if the project is to be conducted in part on VA premises or performed by a VA employee during VA-compensated time, review by the VA Subcommittee for Research is required.

You are responsible for notifying all parties about the approval of this project, including your co-Investigators and Department Chair. If you have any questions, please feel free to contact the IRB-01 office at (352) 846-1494.

Cc: IRB File
Pharmacy
VA Research Center
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US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
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REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 10 Jun 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
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