AD_____

Award Number: DAMD17-00-1-0535

TITLE: Mitochondria Polymorphism in Neurofibromatosis Type 1

PRINCIPAL INVESTIGATOR: Andreas Kurtz, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, Massachusetts 02114

REPORT DATE: November 2002

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 2002	Annual (1 October 2001 - 1 October 2002)

4. TITLE AND SUBTITLE
Mitochondria Polymorphism in Neurofibromatosis Type 1

5. FUNDING NUNUMBER
DAMD17-00-1-0535

6. AUTHOR(S)
Andreas Kurtz, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
Boston, Massachusetts 02114
email - kurtza@helix.mgh.harvard.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

NF1 is characterized clinically by the development of plexiform and multiple cutaneous neurofibromas. There is no correlation between the numbers, size or prevalence of neurofibromas and the type of mutations in the NF1 gene, suggesting a role for genetic modifiers. Genetic polymorphism in mitochondria could cause variability in the observed tumor phenotype in NF1. Here, we analyzed somatic mitochondrial DNA mutations in cutaneous and plexiform neurofibromas to determine if certain mutations are found predominantly in tumors. We found somatic mutations in 9 of 18 plexiform neurofibromas and 5 of 13 cutaneous neurofibromas. All mutations detected were in the hypervariable D-loop region, where origin of replication and transcriptional regulators are located. Most mutations appeared to be homoplasmic in the tumors. In addition, pre-existing soamtic mtDNA mutations were detected in healthy skin of NF1 patients. Our analysis found that these pre-existing somatic mtDNA mutations accumulate in the tumor, suggetsing a selection for the mutated mitochondria in all cell types present in neurofibromas. In a second ongoing set of experiments we analyze the proportion of germ line mitochondrial DNA variants in a cohort of 500 NF1 patients with high numbers and low numbers of cutaneous neurofibromas.

14. SUBJECT TERMS	15. NUMBER OF PAGES
tumor genetics, mitochondria, DNA polymorphism	40
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Neurofibromatosis type 1 (NF1) is the most common inherited predisposition for tumor development. The predisposing mutation was localized to the NF1 gene coding for neurofibromin, a widely expressed 2818 amino acid protein with ras-GAP activity (1-3). Clinical features of NF1 include pigmentation abnormalities, multiple tumors, skeletal lesions and specific learning dysfunctions (4,5). Tumors in NF1 comprise dermal neurofibromas, peripheral nerve sheath tumors and their malignant derivatives, and less frequently, astrocytoma, ependymoma, meningioma, rhabdomyosarcoma, pheochromocytoma, myeloid leukemia, and others (6).

Despite a complete disease penetrance for mutation carriers, expressivity is highly variable, spanning in severity from hundreds of tumors per patient to individuals who display no or very few tumors (7-9). This variability has triggered a number of studies on the effects of hormonal, environmental, and genetic modifiers in NF1, and a major role for genes other than the *NF1* gene in disease expressivity using quantitative and binary traits for scoring was demonstrated (10).

The high frequency of neurofibromas in some patients with NF1 together with a heterogenous phenotype suggest an increased somatic mutation rate (high second hit frequency) in a subpopulation of patients, and in subsets of cells of the same patient. An increased somatic mutation rate in cellular subpopulations would be indicated by somatic mosaicism of NF1. Mosaic expression of mutated NF1 has been described (11-17).

Taken together, it appears that genetic modification in the *NF1* gene itself is not sufficient to explain the variability of NF1 symptoms. Here we propose that mitochondria (mt) variability is a major candidate for modification of the clinical tumor phenotype in NF1. Mitochondria mutation, proliferation and structural aberrations are the cause for a number of diseases with variable and heterogeneous manifestation (18). Alteration of mitochondrial function and adaptation has recently been implied in tumorigenesis and apoptosis. A number of studies have linked mtDNA polymorphism and mutations to cell apoptosis in leukemia and lipomatosis, and amplification of mtDNA in oncocytic tumors. Recently, somatic mutations of mtDNA have been identified in human colorectal cancer, breast and other cancers (for references see attached manuscript,19). Importantly, it has been shown that mitochondria associate with neurofibromin (20), and with microtubules which are highly dependent on mitochondrial energy metabolism.

Mitochondria generate cellular energy in the form of ATP by oxidative phosphorylation (OXPHOS) and thus they are essential components for eucaryotic cells. Three aspects of mitochondrial OXPHOS are of potential importance for tumorigenesis: (i) energy production, (ii) generation of reactive oxygen species (ROS), and (iii) regulation of programmed cell death, or apoptosis. Most cells contain hundreds of mitochondria and each mitochondrion contains between 1 and 100 mt genomes. Mitochondrial DNA features a high mutation rate which, together with its multiplicity, accounts for the often perplexing phenotypes observed. When a mutation in mtDNA arises, cells initially contain a mixture of wild type (wt) and mutant mtDNA (heteroplasmy). Replicative segregation leads to homoplasmy in which each cell contains only a single mitochondrial subclass. Cells in which a threshold proportion of mutated mitochondria is reached are functionally impaired.

There is now ample evidence of mitochondrial involvement in increasing cancer risk due to oxidative stress, DNA damage, apoptosis regulation (18). In this project we investigated the possibility that mitochondrial components are involved in the pathogenesis of NF1 and that this component is a major contribution for the observed variability. This study consists of two components:

In aim 1 we study the correlation between the genotype of mitochondrial DNA (mtDNA) and the clinical phenotype in NF1. A cohort of NF1 patients will be recruited for this study and falls in two groups: A group with a high tumor burden and a group with low tumor burden. Altogether, 400 patients and 75 controls will be needed to find a meaningful correlation between mtDNA variation and tumor burden. The mtDNA will be analyzed for 20 known variable sites.

In aim 2 we study somatic mutations in mitochondrial DNA in tumors of NF1 patients. Altered mitochondria may have a significant replicative advantage in affected tissue and might thus be amplified in tumors. Certain mtDNA mutations might thus promoter tumor development. In this aim cutaneous and plexiform neurofibromas were resected from NF1 patients and the entire mtDNA analyzed and compared with paired blood mtDNA from the same patients.

Body

Aim 1: We are currently continuing recruiting patients to this study. At this point, about 350 patients have been recruited, which fall at a ratio of approximately 1:1 into either of the two groups – NF1 patients with a low number of cutaneous neurofibromas and with a high number of cutaneous neurofibromas, respectively. In addition, DNA samples from 75 healthy control subjects have been collected. We are optimistic that the required number of 400 patients will be recruited by the end of this year.

From all the recruited patients blood samples were obtained and DNA was isolated. 100 of the samples have been tested for mtDNA variation. The other samples are currently being analyzed.

This is a blinded study. Thus, at the moment we can not associate the mtDNA variations found with either of the two patient groups. In addition, unblinding at this point would not provide useful and statistically relevant information. The study will be unblinded after all samples have been analyzed.

Aim 2: We have recruited 19 NF1 patients with plexiform neurofibromas and 13 NF1 patients with cutaneous neurofibromas for this part of the study. We obtained 1 tumor sample and a paired blood sample from each of the NF1 patients with plexiform nfs. We obtained 2 or more tumors and a paired blood sample from each of the NF1 patients with cutaneous neurofibromas. From three of the patients with cutaneous neurofibromas two additional unaffected skin samples were obtained, along with the cutaneous neurofibromas and blood samples.

DNA was isolated from all samples. Except for 1 patient with plexiform neurofibromas, all DNAs were of sufficient quality to analyze the entire mitochondrial genome for mutations by temporal temperature gradient electrophoresis (TTGE).

Mutational analysis detected somatic mtDNA mutations in 9 of the 18 plexiform neurofibromas and in 5 of 13 cutaneous neurofibromas. All mutations occurred in the hypervariable D-loop regions. Most tumors were homoplasmic or nearly homoplasmic for the

mutated mtDNA, indicating accumulation of the mutated mitochondria and supporting our initial hypothesis.

A surprising finding was the homoplasmic state of mutated mitochondria in many of these mixed cell tumors. In addition, separate tumors from the same patient all harbored the same mitochondria genotype. This indicates that either all tumor cells derive from a single stem cell with a certain mitochondria genotype present, or normal cells in the body harbor a pre-existing mutated mitochondria. To analyze the second hypothesis, we analyzed unaffected skin samples and cutaneous neurofibromas. Mutated mitochondria were readily detected in these samples at a heteroplasmic state. Taken together, our data show that mitochondrial mutation pre-exist in normal tissues of NF1 patients and accumulate in tumors, suggesting a selective advantage for the mutated mitochondria in tall cells of he tumors.

Key Research Accomplishments

There are three research accomplishments I wish to point out:

1. We have established for the first time that plexiform and cutaneous neurofibromas in NF1 patients harbor somatic mitochondrial DNA mutations. That these mutations are found in most or all different cells of the tumors, that the mutated mitochondria accumulate in the tumors and that all mutations occur in the D-loop region. This finding raises the question on the function of these mutations for neurofibroma growth.
2. We have shown for the first time that somatic mitochondrial DNA mutations exist even in unaffected normal tissues in NF1 patients. These mutated mitochondria are present together with normal mitochondria in a heteroplasmic state. We have also shown that these mutated mitochondria accumulate in tumor tissue, eliminating the normal (germ line) mitochondria from the tumor cells. This indicates a selective advantage for the mutated mitochondria in tumor cells.
3. We have established a large data base for NF1 patients together with accompanying blood DNA samples. The data base contains anonymized information about the clinical phenotype of the patients, especially their tumor burden.

Reportable Outcomes

Reportable are our findings on somatic mtDNA mutations in cutaneous and plexiform neurofibromas as outlined above and in the appendix (manuscript). The data have also been presented at the AACR meeting (San Francisco, 2002) and the Neurofibromatosis meeting (Aspen, 2002).

It remains to be shown whether mtDNA polymorphisms are associated with a severe or mild tumor phenotype in NF1.

Conclusions

We have recruited to date about 350 NF1 patients and 75 control subjects for this study and established a anonymized data base with clinical data. 100 of the NF1 patient DNAs has been analyzed. The 400 samples necessary to obtain statistically meaningful data will be obtained by the end of 2002 and analyzed by the first quarter of 2003.

We have confirmed our initial hypothesis that mutated mitochondria accumulate in neurofibromas, indicating a functional role for neurofibroma development and growth. It remains to be shown what kind of functional consequences the mutations found (D-loop region) might have on a cellular level.

We have surprisingly found that somatic mtDNA mutations pre-exist in normal tissues of NF1 patients and that the mutated mitochondria accumulate in neurofibromas. This is an interesting finding since it raises several important questions: At what point are somatic mtDNA mutations detectable in NF1 and does the proportion increase with age – and related, what is the genotype of mitochondria in neurofibromas in which no mutations were detected by TTGE ? Is there a stem cell disseminating early in development which contains mutated mtDNA, and which gives rise to neurofibromas or are all cells heteroplasmic ? Does the mutation rate in the NF1 gene depend on the presence of mtDNA mutations ? And finally, is there a critical threshold for mutated mitochondria to promote tumorigenesis ?

These appear to be the more acute questions which can be answered by future experimental inquiry.

References

1. Gutmann DH, Wood DL, Collins FS: Identification of the neurofibromatosis type 1 gene product. Proc Natl Acad Sci 88(21):9658-9662, 1991

2. Bernards A. Neurofibromatosis type 1 and Ras-mediated signaling: filling in the GAPs. Biochim Biophys Acta 1242:43-59, 1995

3.Marchuk DA, Saulino AM, Tavakkol R, Swaroop M, Wallace MR, Andersen LB, Mitchell AL, Gutmann DH, Boguski M, Collins FS. cDNA cloning of the type 1 neurofibromatosis gene: complete sequence of the NF1 gene product. Genomics 11: 931-940, 1991.

4. Gutmann DH, Collins FS. von Recklinghausen neurofibromatosis. In: Scriver et al, eds. The Metabolic and Molecular Bases of Inherited Disease. 7th ed. New York: McGraw-Hill, 667-696, 1995

5.National Institutes of Health Consensus Development Conference. Neurofibromatosis: conference statement. Arch. Neurol. 45: 575-578, 1988.

6. Cohen B, Rothner D. Incidence, types and management of cancer in patients with neurofibromatosis. Oncology 3:23-38, 1989.

7. Samuelsson B, Akesson HO. Relative fertility and mutation rate in neurofibromatosis. Hereditas. 1988;108(2):169-71.

8.Riccardi VM. Neurofibromatosis: clinical heterogeneity. Curr Probl Cancer. 1982 Aug;7(2):1-34.

9. Carey JC, Lant JM, Hall BD. Penetrance and variability in neurofibromatosis: a genetic study in 60 families. Birth Defects 15:271-281, 1979.

10. Easton DF, Ponder MA, Huson SM, Ponder BAJ. An analysis of variation in expression of neurofibromatosis (NF) type I (NF1): evidence for modifying genes. Am. J. Hum. Genet. 53: 305-313, 1993.

11. Upadhyaya M. Ruggieri M. Maynard J. Osborn M. Hartog C. Mudd S. Penttinen M. Cordeiro I. Ponder M. Ponder BA. Krawczak M. Cooper DN. Gross deletions of the neurofibromatosis type 1 (NF1) gene are predominantly of maternal origin and commonly associated with a learning disability, dysmorphic features and developmental delay. Human Genetics. 102(5):591-7, 1998.

12. Rasmussen SA, Colman SD, Ho VT, Abernathy CR, Arn PH, Weiss L, Schwartz C, Saul RA, Wallace MR. Constitutional and mosaic large NF1 gene deletions in neurofibromatosis type 1. J. Med. Genetics 35:468-471, 1998.

13. Lazaro C, Ravella A, Gaona A, Volpini V, Estivill X. Neurofibromatosis type 1 due to germ-line mosaicism in a clinically normal father. New Eng. J. Med. 331:1403-1407, 1994.
14. Zlotogora J. Mutations in von Recklinghausen neurofibromatosis: an hypothesis. Am. J. Med. Genetics 46:182-184, 1993.
15. Ainsworth PJ, Chakraborty PK, Weksberg A. Example of somatic mosaicism in a series of de novo neurofibromatosis type 1 cases due to a maternally derived deletion. Hum Mutation 9:452-457, 1997.
16. Colman SD, Rasmussen SA, Ho VT, Abernathy CR, Wallace MR. Somatic mosaicism in a patient with neurofibromatosis type 1. Am J Hum Genet. 1996 Mar;58(3):484-90.
17. Wu BL, Boles RG, Yaari H, Weremowicz S, Schneider GH, Korf BR. Somatic mosaicism for deletion of the entire NF1 gene identified by FISH. Hum Genetics 99:209-213, 1997.
18. Wallace DC. Mitochondrial disease in man and mouse. Science 283:1482-1488, 1999.
19. Maria Lueth, Lan Kluwe, Rosemary Foster, Victor-Felix Mautner, James Gusella, Melanie Hartmann, Duan-Jun Tan, Reinhard E. Friedrich, Pablo Hernaiz Driever, Andreas Kurtz, Lee-Jun C. Wong. Somatic mitochondrial DNA mutations in Neurofibromatosis Type 1 associated Tumors. 2002 (submitted).

Appendices

Submitted manuscript 'Somatic mitochondrial DNA mutations in Neurofibromatosis Type 1 associated Tumors' by Maria Lueth, Lan Kluwe, Rosemary Foster, Victor-Felix Mautner, James Gusella, Melanie Hartmann, Duan-Jun Tan, Reinhard E. Friedrich, Pablo Hernaiz Driever, Andreas Kurtz, Lee-Jun C. Wong.

Somatic mitochondrial DNA mutations in Neurofibromatosis type 1[1]-associated tumors

Maria Lueth, Lan Kluwe, Rosemary Foster, Victor-Felix Mautner, James Gusella, Melanie Hartmann, Duan-Jun Tan, Reinhard E. Friedrich, Pablo Hernaiz Driever, Andreas Kurtz, Lee-Jun C. Wong[2]

Institute for Molecular and Human Genetics, Georgetown University, Washington DC, 20007 [M. L., D.-J. T., L.-J. C. W.], Humboldt University, Virchow Klinikum Charite, Berlin, Germany [M. H., P. H. D.], University Hospital Eppendorf, Hamburg, Germany [L. K.], Klinikum Nord Ochsenzoll, Hamburg, Germany [R. E. F., V.-F. M.], and Masschusetts General Hospital, Harvard Medical School, Charlestown, USA [R. F., J.G., A. K.]

[1]This study is supported by USMRC Neurofibromatosis Research Program award NF990008 to A.K.

[2]To whom requests for reprints and correspondence should be addressed, at Institute for Molecular and Human Genetics, P.O. Box 571477, Georgetown University Medical Center, M4000, 3800 Reservoir Rd, NW, Washington, DC 20057-1477, Tel: 202 784 0760, Fax: 202 784 1770, Email: wonglj@georgetown.edu

[3]The abbreviations used are:

NF1	neurofibromatosis type 1
PNS	peripheral nervous system
mtDNA	mitochondrial DNA
TTGE	temporal temperature gradient gel electrophoresis
PCR	polymerase chain reaction
ROS	reactive oxygen species
ND2	NADH Dehydrogenase Subunit 2
STR	short tandem repeat

PAH phenylalanine hydroxylase

SCA 1 spinocerebellar ataxia type 1

SCA 3 spinocerebellar ataxia type 3

MSI microsatellite instability

[4] The internet address: http://www.mitomap.html

Abstract

Neurofibromatosis type 1 (NF1)[3] is the most commonly inherited disease predisposing to tumor formation due to mutations in the *Nf1* gene. Cutaneous and plexiform neurofibromas are the most frequent tumors in NF1. These benign tumors arise often simultaneously at many different locations in the peripheral nervous system (PNS). The tight association of neurofibromin function with energy metabolism, its ras-GAP function , the heterogeneous clinical expressivity, and the finding of somatic mtDNA mutations in other tumors have prompted us to investigate somatic mtDNA mutations in NF1 associated neurofibromas. MtDNA alterations in the entire mitochondrial genome were analyzed by temporal temperature gradient gel electrophoresis (TTGE) followed by direct DNA sequencing. Somatic mtDNA mutations were found in 10 out of 26 (38.4%) cutaneous neurofibromas and in 9 out of 18 (50%) of plexiform neurofibromas. A total of 32 somatic mtDNA mutations were found. Several plexiform neurofibromas from individual patients have multiple homoplasmic mtDNA mutations. In cutaneous neurofibromas, the same mtDNA mutations were always found in tumors from different locations of the same individual. Furthermore, a progressive change in mutant mtDNA content was demonstrated between blood, skin distant from the tumor, skin overlaying the tumor and the neurofibroma. These results suggest that cells carrying mtDNA mutations are disseminated in the body, and that the mutated mitochondria accumulate in all cell types of the tumor during tumorigenesis.

Introduction

Neurofibromatosis type 1 (NF1) is an autosomal dominant disorder in humans with an estimated prevalence range from 1/2190 to 1/7800 (1). There are no known ethnic groups in which NF1 does not occur or is unusually common. Clinical features of NF1 include café-au-lait spots, axillary freckling, skeletal abnormalities, learning disabilities, and iris hamartomas (Lisch nodules) (2). The most prevalent clinical manifestation of the NF1 disease phenotype is the development of benign dermal and plexiform neurofibromas throughout the peripheral nervous system. At low frequency, benign neurofibromas can progress to highly malignant peripheral nerve sheath tumors (MPNSTs). Other tumor types less frequently associated with NF1 include optic gliomas, myeloid leukemias, phaeochromocytomas, and astrocytomas. Mutations in the *NF1* gene are the underlying cause for the complete disease penetrance but there is marked variable expressivity of the disease phenotype even among members of the same family (3, 4) who share a specific *NF1* mutation. Genotype-phenotype correlation studies have not clearly established significant links between different classes of *NF1* mutations and any particular clinical manifestation. For example, the timing and extent of tumor development are not correlated with any specific mutation type (5, 6) although tumor burden usually increases with age. The wide variability of the NF1 disease phenotype may be due in part to modifier genes at other loci which influence the development and progression of the disorder.The *NF1* gene spans over 300kb of genomic DNA encoding an mRNA of 11-13 kb containing at least 60 exons (2, 7). The genetic defects in NF1 patients range from large deletions to missense and nonsense mutations with approximately 82% of all reported mutations causing truncation of the

protein (8, 9).The *NF1* gene encodes a protein of 2818 amino acids, neurofibromin, that contains a ras-GTPase activating (GAP) domain. Loss of neurofibromin function leads to elevated levels of Ras-GTP and subsequent deregulation of Ras signaling pathways in cells types implicated in NF1 pathogenesis, specifically those involved in tumorigenesis (2, 10-12). Loss of heterozygosity at the *NF1* locus has been shown in both benign dermal and plexiform neurofibromas and in MPNSTs suggesting that neurofibromin normally functions as a tumor suppressor. Neurofibromas are complex tumors comprising Schwann cells, fibroblasts, mast cells, and nerve cells. Molecular analysis of the neurofibroma component cell types has demonstrated that the Schwann cell is the target for the somatic mutation event in the *NF1* gene that leads to tumor development. The wide distribution of cutaneous and plexiform neurofibromas throughout the peripheral nervous system (PNS) may arise from loss of neurofibromin function in many target cells due to distinct somatic mutation events. Alternatively, one or a few precursor cells may became null for *NF1* during early embryogenesis and may disseminate in the PNS during development, giving rise to multiple tumors of clonal origin.

The role of mitochondria in tumor development has gained much attention with recent reports of somatic mitochondrial DNA (mtDNA) mutations in ovarian, esophageal, breast, and colorectal human cancers (15-22). Mitochondria contain multiple copies of circular double stranded DNA molecules that have a high degree of sequence variations among different individuals (23). In addition to energy production, mitochondria play a crucial role in programmed cell death and cell malignancy (24-27). The continuous generation of reactive oxygen speciies (ROS) as side products of

normal function is another feature of mitochondria. In the absence of protective histone proteins and effective DNA repair mechanisms, the mitochondrial DNA (mtDNA) becomes the easy target for oxidative DNA damage while accumulation of ROS due to defective mitochondrial behaviour might also contribute to increased nuclear gene mutagenesis (28). In lieu of the importance of mitochondria in the production of ATP through which protein kinase activities are regulated, mitochondria are likely to be linked with abnormal cell growth. Neurofibromin has been found associated with highly energy dependent microtubules (13) and with mitochondria (14), suggesting that the functional state of mitochondria might directly affect neurofibromin activities.

The characteristics of multisystemic manifestation, variable expressivity, and somatic mosaicism of NF1 prompt us to hypothesize that mtDNA variations and/or somatic mtDNA mutations are involved in the heterogeneous and diffuse clinical expression of NF1. Here we report the presence of mtDNA alterations in neurofibromas and their relationship to cutaneous and plexiform neurofibromas and non-tumor tissues.

Materials and Methods

Tissue Samples

Patients with NF1 were recruited through the Departments of Neurosurgery and Neurogenetics, Massachusetts General Hospital, Harvard University, and through the Department of Neurology, Klinikum Nord Ochsenzoll, Hamburg, Germany. Patients were phenotypically characterized for features of NF1, including number, location, and size of cutaneous neurofibromas. Only patients with a clear diagnosis of NF1 according to NIH criteria (29) were included in this study. Cutaneous and plexiform

neurofibromas were removed during routine surgery, , dissected into multiple aliquots, and frozen immediately. Two or more cutaneous neurofibromas resected from different anatomical sites on each individual were obtained from 13 patients. For three of these patients, skin samples were biopsied from an area overlaying the resected cutaneous neurofibroma and from an area distal to the tumor (Table 2, patients 4, 5 and 6). A single plexiform neurofibroma sample was obtained from each of nineteen patients.

The age of patients with cutaneous tumors ranges from 16 to 50 with a mean age of 37.6. The age of patients with plexiform tumors ranges from 8 to 73 with a mean age of 28.8.

DNA Isolation

DNA was isolated from frozen tissues using proteinase K and phenol/chloroform extraction method. DNA was extracted from peripheral blood lymphocytes using a modified non-enzymatic method (30). Total DNA was quantified using fluorescent Hoechst dye H33258 with DYNA QUANT 200 according to manufacturer's protocol and diluted to 5ng/ul to be used in PCR reactions (31).

Mutational Analysis of the Entire Mitochondrial Genome

DNA isolated from 13 pairs of matched blood and cutaneous neurofibroma samples and from 18 pairs of matched blood and plexiform neurofibroma samples was used for mutational analysis of the mitochondrial genome by temperature gradient gel electrophoresis (TTGE).

Thirty-two pairs of overlapping primers were used to amplify the entire mitochondrial genome by PCR (31). The PCR amplified DNA fragments vary from 306 bp to 805 bp in length. The total amplified fragments contain an average of 75 bp overlap at each end of the fragment. The positions and the sequence of the PCR primers, and the PCR and TTGE conditions were as recently described (31). Briefly, the DNA template, after the initial denaturation at 94°C for 5 min, was amplified over of 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds. The PCR products were denatured at 95 °C for 30 sec and slowly cooled to 45 °C for a period of 45 min at a rate of 1.1 °C/min. The reannealed homoduplexes and heteroduplexes were maintained at 4 °C until TTGE analysis was performed on a Bio-Rad D-Code apparatus. Five microliters of denatured and reannealed PCR products were loaded onto a polyacrylamide gel (acrylamide: bis 37.5:1) prepared in 1.2X TAE buffer containing 6 M urea. Electrophoresis was carried out at 145 V for 4-5 hours at a constant 1-2 °C/hour temperature increment (31). The temperature range was determined by computer simulation from the melting curve of the analyzed DNA fragment (MacMelt software, Bio-Rad Laboratories). The gels were stained with 2 mg/L ethidium bromide for 5 min and imaged with a digital CCD gel documentation system (High performance ultra-violet transilluminator, Ultra-Violet Products).

On TTGE analysis, a single band shift represents a homoplasmic DNA alteration, and a multiple-banding pattern represents a heteroplasmic mutation (32). Any DNA fragments showing different banding patterns between the matched blood and tumor sample pairs were sequenced to identify the exact mutations.

Sequence Analysis

Direct DNA sequencing of the purified PCR product using the original PCR primers and a BigDye terminator cycle sequencing kit (Perkin Elmer) and analyzed on ABI 377 (Applied Biosystem) automated sequencer. The results of DNA sequence analysis were compared with the published Cambridge sequence (33) using Mac Vector™ 7.0 (Oxford Molecular Ltd., Oxford, England) software. Sequence alterations found in both tumor and blood mtDNA were scored as germline variations and checked against the Mitomap database[4] (http://www.mitomap.html). Those alterations not recorded in the database were categorized as novel mtDNA polymorphisms. Any mtDNA sequence differences found between a tumor sample and its corresponding blood sample were scored as somatic mtDNA mutations specific to the tumor.

Results

Somatic mtDNA mutations in cutaneous and plexiform neurofibromas

MtDNA from pairs of matched tumor and normal blood samples was analyzed in parallel by TTGE using multiple PCR products comprising the entire mitochondrial genome. Parallel analysis of a single PCR product from matched blood and tumor samples allowed the rapid detection of nucleotide alterations due to changes in the banding patterns of the products. A single band shift represents a homoplasmic DNA alteration and a multiple-banding pattern represents a heteroplasmic alteration (32). Any mtDNA PCR products showing different banding patterns between matched blood and tumor samples were sequenced to identify the exact mutations. These analyses were carried out on a total of thirteen cutaneous neurofibromas and eighteen plexiform neurofibromas and representative results are shown in Figure 1.

In panel A, TTGE analysis of the D-loop region mtDNA PCR product showed a band shift in plexiform neurofibroma T173 compared to its corresponding blood sample B174, suggesting the presence of a homoplasmic mutation in the tumor sample. Direct sequencing of the blood and tumor mtDNA PCR products revealed 5 homoplasmic tumor specific nucleotide substitutions in this region, including 3 novel ones, A16163G, C16186T, and C16221T. The detected T16189C and T16519C alterations have been reported previously (17, 20, 22).

Panel B illustrates a similar analysis of plexiform neurofibroma T191 and its corresponding blood sample B192, showing a complex shift in the TTGE pattern. Sequencing of the analyzed PCR products revealed two homoplasmic to heteroplasmic changes T199C and G207A. Interestingly, a heteroplasmic change in the proportion of T204C mutation between B192 and T191 was also detected, revealing a shift in the degree of heteroplasmy at T204C in the tumor sample .

Panel C illustrates an analysis of two separate cutaneous neurofibromas (T104 and T105) from a single individual, which revealed a change in a short poly C sequence at nucleotides 303-309 in the conserved sequence block which shifted from C8/C9 heteroplasmy in the blood sample (B106) to near homoplasmy of C8. Similar progressive alteration in the percentage of heteroplasmy was observed in other sets of tumors. The complete results of our analysis of NF-1 associated cutaneous and plexiform neurofibromas are lsted in Table 1.

We analyzed cutaneous neurofibromas from a total of 13 patients. MtDNA from two distinct cutaneous tumors of an individual were analyzed against blood mtDNA of the same individual. Five out of 13 (38.4%) patients with cutaneous neurofibromas had somatic mtDNA mutations in their tumors (Table 1A), and all of them occurred in the hypervariable D-loop region. These samples comprise 5 sets of two independent tumors resected from distinct anatomical sites on a single individual.

Surprisingly, the separate tumors from a single NF1 patient harbored always the same somatic mtDNA mutation, as was the case for tumor samples T104 and T105 (Figure 1, panel C). Blood mtDNA from patient 1 (Table 2, B106) is heteroplasmic for 303-309 C8/C9. Both of its tumors T105 and T104 showed apparently homoplasmic C8, although sequence analysis may not be able to reveal difference in very low percentage of heteroplasmy. Analysis of mtDNA samples from tumors T107 and T108 showed a shift from poly C 303-309 C7 homoplasmy in the matched blood mtDNA sample B109 to C7/C8 heteroplasmy in each of the tumors. Tumors T119 and T120 both harbor the same homoplasmic T16304C mutation when compared to its blood DNA B121, which is homoplasmic for the wild type T16304. Tumor tissue from different

parts of the same tumor also showed the identical mtDNA mutations with comparable degrees of plasmy throughout the tumor (table 3, patient 5). Among the 18 pairs of mtDNA from plexiform neurofibromas analyzed, 9 (50%) showed somatic mtDNA mutations (Table 1B). Four of the plexiform tumors with mtDNA mutations harbored a single alteration (4/9=44.4%) while the remaining five cases (5/9=55.5%) had more than 1 mutation. For example, in one plexiform tumor (T173) we defined 9 distinct somatic mtDNA mutations. A total of 27 somatic mtDNA mutations were identified. All mutations were found in the D-loop region and all were nucleotide substitutions that occurred only once. , Also detected were insertions or deletions in the np303-309 poly C region. This region has been reported to be the somatically unstable mutation hot spot of breast cancer (20, 34). All nucleotide substitutions were T to C and A to G transitions, which is consistent with oxidative DNA damage. The majority (14 out of 27, 51.8%) of the somatic mtDNA mutations in the plexiform tumors were alterations from homoplasmic state in blood mtDNA to homoplasmic state in tumor mtDNA. In six cases, a shift from mtDNA homoplasmy in blood to mtDNA heteroplasmy in the corresponding tumor was found. In four cases, a shift from heteroplasmy in blood mtDNA to homoplasmy in tumor mtDNA was detected. Three pairs of matched samples cases were at heteroplasmic state in both blood and tumor but there were detectable quantitative changes in the degree of heteroplasmy in the blood and tumor mtDNA samples as assessed by quantitative comparison of the nucleotide peak amplitudes in the corresponding sequence profiles (Table 1B).

These findings show that separate cutaneous neurofibromas taken from the same individual harbour identical somatic mtDNA mutations. MtDNA mutations in

cutaneous and plexiform neurofibromas are either homoplasmic or provide a high proportion of mitochondria in heteroplasmic tumors, indicating that mutated mitochondria accumulate in most cells in the tumor.

Somatic mtDNA mutations preexisting in normal skin accumulate in cutaneous neurofibromas

NF1 patients usually have multiple cutaneous neurofibromas throughout the PNS which are complex tumors composed predominantly of Schwann cells and fibroblasts, with a minority of neurons, monocytes and endothelial cells (35). As noted above, homoplasmy for somatic mtDNA mutations was detected in a number of these cutaneous neurofibromas of mixed cell type (Table 1A, tumor samples T104, T105, T119, and T120). Additionally, multiple cutaneous neurofibromas resected from distinct anatomical sites on an affected individual shared identical somatic mtDNA mutations (Tables 1A and 2). Taken together, these results suggest that either all the tumor cells derive from the same stem cell, or the tumor-specific mtDNA profile is already prevalent in most relevant cell types of the body but accumulates in all cells of the tumor. To examine the second possibility, we analyzed mtDNA isolated from normal skin obtained from NF1 patients. we studied mtDNA from three additional sets of multiple cutaneous tumors from various locations from a single individual along with normal skin tissue overlaying the resected tumors, and distal from the tumors. We focused our mtDNA somatic mutation analysis of these matched skin and tumor samples on the D-loop and its surrounding region since the previous data obtained from studying 10 cutaneous and 18 plexiform neurofibromas revealed that all the somatic mtDNA mutations occurred in

the D-loop region. The results of our analyses are shown in Table 2 (patients 4, 5, and 6).

One of these sets (patient 6) did not show any somatic mtDNA D-loop mutations in the tumor and skin samples. (Table 2). The other two sets of tumors displayed somatic mtDNA D-loop mutations. A progressive change in mutant mtDNA content was demonstrated between blood, skin distal from and overlaying the tumor, and the neurofibroma (Table 2). In addition, the same mutation found in skin was also present in different parts of the same tumor or tumors from different locations of the same individual.

Correlation of somatic mtDNA mutations with clinical features in plexiform neurofibromas

We were interested in examining whether there was any association between the presence of mtDNA mutations in plexiform neurofibromas and the NF1 disease phenotype of the affected individuals. We defined somatic mtDNA mutations in 9 of the 18 plexiform neurofibroma samples analyzed and observed no sex or age difference between the individuals who did or did not have somatic mtDNA alterations (Table 3). The *NF1* germline mutation had been identified in 6 of 18 patients with a defined somatic mtDNA mutation in an analyzed plexiform neurofibroma (Table 3). With some exceptions, all patients with somatic mtDNA mutations in an associated plexiform neurofibroma developed Lisch nodules and abnormal pigmentation. The number of mtDNA mutations coincided with early development of cutaneous neurofibromas in cases T173, T189 and T179 (Table 3).

Germline Sequence Variations

In our analyses of somatic mtDNA mutations in NF1-associated tumors, we detected numerous sequence variations in multiple blood mtDNA samples (Table 1). When the mtDNA sequences from blood was compared with that of the published Cambridge sequence, numerous germline sequence variations were revealed (Table 4). A total of 63 distinct germ-line variations have been identified from the sequenced fragments. These do not represent all the sequence variations which may be present in the analyzed blood, since only the mtDNA PCR products of regions that showed somatic mutations by TTGE in the paired tumor sample were sequenced. Nine of the variations detectable in our studies are novel, and the remaining have been reported in the Mitomap database. Many of the mtDNA germline variations reported here occurred in multiple individual samples. Among them, A73G and T16519C are common polymorphisms while A263G and 303-315insC represent polymorphisms in the Cambridge sequence (33, 36). Although germ-line variations are generally considered silent, missense mutations such as the novel A265V alteration in the mitochondrial protein ND2 may have a functional effect. The subtle changes may accumulate over time and predispose to tumor development.

Discussion

This is the first comprehensive mutational analysis of the entire mitochondrial genome to demonstrate that somatic mtDNA mutations are present in cutaneous and plexiform neurofibromas associated with neurofibromatosis type1. A total of 16 distinct mtDNA alterations were detected in the analyzed tumors. Five of these mutations are novel, whereas the majority of these mutations have been reported as somatic mtDNA alterations in other tumor types (Table 1). The percentage of neurofibromas with somatic mtDNA mutations is similar to those found in glioblastoma and medulloblastomas but lower than those in lung, breast, and oral cancers (15-22). All of the mtDNA somatic mutations identified in our study occured in the hypervariable D loop region of the mitochondrial genome. This is unique since numerous studies on lung, breast, ovarian, bladder, head and neck, glioblastoma, and oral cancers showed that 20-70% of somatic mtDNA mutations were found in coding regions (Table 5). The pathological significance of mutations in non-coding regions of the mitochondrial genome is currently unknown. It is possible that mutations in the conserved sequence block, origin of replication and transcriptional regulatory sequences may affect the number of mitochondria per cell or the total amount of mitochondrial transcripts and mature proteins. Ultimately, the overall oxidative phoshorylation activity of the mitochondria may be affected. The finding that all mutations identified in NF1 associated tumors are in the non-coding D loop region may be related to the fact that both, the cutaneous and plexiform neurofibromas are benign tumors. Notably, in our cohort of NF1 patientsthere is no obvious correlation between the severity of the

disease phenotype and the presence of somatic mtDNA mutations in the analyzed tumors.

One surprising finding was the identification of 9 distinct somatic homoplasmic mtDNA mutations in a single plexiform neurofibroma (T173). One obvious suspicion is that the tumor sample T173 and its corresponding blood sample B174 may in fact have been isolated from two different individuals. To rule out this possibility, we performed genome wide identity on samples T173 and B174.We detected identical alleles at 5 polymorphic sites: the short tandem repeat in intron 3 of the *PAH* gene (chromosome 12), the CTG repeats of the myotonin protein kinase gene (disease gene for myotonic dystrophy, chromosome 19), the CAG repeats of the androgen receptor gene (X chromosome), and in the *SCA 1* (chromosome 6) and *SCA 3* (chromosome 4) genes (data not shown). These results substantiate that plexiform neurofibroma T173 does indeed harbor 9 somatic mtDNA mutations, 4 of which are novel. The cause for this uncommonly large number of tumor specific somatic mtDNA mutations is not clear. It is possible that the point mutations in the origin of H-strand replication and the termination-associated sequence regulate the mtDNA synthesis and transcription in the tumor. Alarge number (>6) of somatic mtDNA mutations also occurred in approximately 5-10% of medulloblastomas, breast, and lung cancers. As outlined in Table 5, there is an average of about 1-3 somatic mtDNA mutations per tumor (15, 17, 20, 22) . In our analysis of NF1-associated tumors, we detected average of 1 somatic mtDNA mutation in cutaneous neurofibromas, and an average of three mutations in plexiform neurofibromas.

Multiple mutations may or may not occur simultaneously. In order to reach a homoplasmic state, there must be some mechanism for advantageous selection and a sufficient number of cell divisions. Mutations in the origin of replication (D-loop region) may provide a replicative advantage of these mutant mtDNAsThis is supported by the observation that multiple mutations in tumors of the same patient were almost always found in a homoplasmic state (Table 1, tumors T159 and T173). Furthermore, the same homoplasmic mutations are found in tumors resected from different anatomical sites of a single individual (Table 2, tumors T119 and T120).

The most common somatic mtDNA mutations identified in our studyt are insertions or deletions in the poly C region at nucleotides 303-309.Microsatellite instablility (MSI) was not detected in any of the other 10 short tandem repeat regions in the mitochondrial genome (data not shown). These observations implied that the variability in the 303-309 region is due to a mutational hotspot rather than a true microsattelite instability. Study of short tandem repeats in nuclear genes will be necessary to elucidate the mechanism of MSI in NF1.

To rule out the possibility that some mutations may not be detectable by TTGE, we randomly chose 6 samples that did not show TTGE positive banding patterns and sequenced 10 coding regions containing stretches of 6-8 homopolynucleotides. No mutations were found. We believe that the somatic mtDNA mutations observed in NF1 are not due to PCR artifact or random MSI.If they were PCR or sequencing artifacts, the changes in the mutant proportion would appear randomly and not be progressive. The mutations in the nucleotide 303-309 region were not observed in mtDNA from 40 normal muscle tissues isolated from individuals ranging in age from 0 to 65 years (unpublished

observation). Thus, the somatic mutations detected in the nucleotide 303-309 region are probably due to tumor specific genomic instability. One interesting observation is that mutations at nucleotide position (np) 204 and 207 occurred 3 times in 3 unrelated patients. This result suggests that the np204 and np207 are either mutation hot spots or the mutant mitochondria have selective growth advantage.

The presence of the same somatic mtDNA mutation in distinct cutaneous neurofibromas from a single individual, and the observed homoplasmy of the somatic mutation are unexpected and argues against the independent occurrence of each of the mutations in the separate tumors from distant sites. Instead, these results indicate the presence of preexisting mitochondrial DNA variations. To test this hypothesis, we analyzed cutaneous neurofibromas and unaffected skin biopsies from distinct sites of the same patient for the presence of mutations in the D-loop region of the mitochondrial genome. We readily detected mtDNAmutations in unaffected tissue confirming the hypothesis that the mtDNA mutations are present in normal cells before tumors develop. Furthermore, the progressive increase in prevalence of the mutant mitochondria, with lowest proportions in skin samples and highest, close to homoplasmic proportions in any of the distinct tumors of the same individual suggests a selective advantage for cells carrying these mtDNA mutations.

The detection level for heteroplasmy using TTGE is around 5%, high enough to detect differences in mitochondria between the major cell types in these tumors, Schwann cells and fibroblasts. The homoplasmy for somatic mtDNA mutations in several of the mixed cell neurofibroas as demonstrated in our study may also be explained by the pre-existence of a low level of heteroplasmy for mtDNA mutations in

healthy tissue. Taken together, our results support the hypothesis that somatic mtDNA mutations occur early during development generating a low heteroplasmic state. A random drift of mitochondria composition in one direction, possibly influenced by age and changes in the genetic backgroundlike loss of the *NF1* gene, would give rise to enrichment of the pre-existing mtDNA mutants that may enhance local cell growth and promote neurofibroma development in the diffuse pattern observed clinically.

Acknowledgement

We thank Dr. Mia macCollin for valuable discussions and suggestions.

Figure Legend

Fig. 1. Detection of somatic mtDNA mutations in plexiform and cutaneous neurofibromas by TTGE and sequence analysis.

A, Comparison of PCR amplified mtDNA D-loop region from plexiform neurofibroma T173 and paired blood sample B174. Sequencing reveals multiple homoplasmic nucleotide substitutions in plexiform neurofibroma mtDNA.

B, Comparison of mtDNA D-loop region from plexiform neurofibroma T191 and paired blood sample B192. Sequencing revealed two changes T199C and G207A, from homoplasmic in normal to heteroplasmic in tumor and one heteroplasmic to heteroplasmic change T204C in the same region.

C, Comparison of mtDNA D-loop region from two cutaneous neurofibromas T104 and T105 from the same individual and paired blood sample B106. Sequencing reveals a gradual change from a heteroplasmic 303-309 C8/C9 to a homoplasmic C8 in both cutaneous neurofibromas.

Reference

1. Friedman, J. M. Epidemiology of neurofibromatosis type 1. Am J Med Genet, *89:* 1-6, 1999.
2. Gutmann, D. H. and Collins, F. S. Neurofibromatosis 1., 8th edition, Vol. 1, p. 877-896. New York: McGraw-Hill, 2001.
3. Carey, J. C. and Viskochil, D. H. Neurofibromatosis type 1: a model condition for the study of the molecular basis of variable expressivity in human disorders. Am J Med Genet, *89:* 7-13, 1999.
4. Carey, J. C., Lant, J. M., and Hall, B. D. Penetrance and variability in neurofibromatosis: a genetic study in 60 families. Birth Defects, *15:* 271-281, 1979.
5. Tonsgard, J. H., Yelaavarthi, K. K., Cushner, S., Shon, M. P., and Lindgren, V. Do NF1 gene deletions result in a characteristic phenotype? Am J Med Genet, *73:* 80-86, 1997.
6. Heim, R. A., Kam-Morgan, L. N. W., Binnie, C. G., Corns, D. D., Cayouette, M. C., Farber, R. A., Aylsworth, A. S., Silverman, L. M., and Luce, M. C. Distribution of 13 truncating mutations in the neurofibromatosis 1 gene. Hum Molec Genet, *4:* 975-981, 1995.
7. Li, Y., O'Connell, P., Breidenbach, H. H., Cawthon, R., Stevens, J., Xu, G., Neil, S., Robertson, M., White, R., and Viskochil, D. Genomic organization of the neurofibromatosis 1 gene (NF1). Genomics, *25:* 9, 1995.
8. Shen, M. H., Harper, P. S., and Upadhyaya, M. Molecular genetics of neurofibromatosis type 1 (NF1). J Med Genet, *33:* 2-17, 1996.
9. Upadhyaya, M., Ruggieri, M., Maynard, J., Osborn, M., Hartog, C., Mudd, S., Penttinen, M., Cordeiro, I., Ponder, M., Ponder, B. A. J., Krawczak, M., and Cooper, D. N. Gross deletions of the neurofibromatosis type 1 (NF1) gene are predominantly of maternal origin and commonly associataed with a learning disability, dysmorphic features and developmental delay. Hum Genet, *102:* 591-597, 1998.
10. Whittinghofer, A. Signal transduction via Ras. Biol Chem, *379:* 933-937, 1998.
11. Bernards, A. Neurofibromatosis type 1 and Ras-mediated signaling: filling the GAPs. Biochim Biophys Acta, *1242:* 43-59, 1995.
12. Weiss, B., Bollag, G., and Shannon, K. Hyperactive Ras as a therapeutic target in neurofibromatosis type 1. Am J Med Genet, *89:* 14-22, 1999.
13. Gregory, P. E., Gutmann, D. H., Mitchell, A., Park, S., Boguski, M., Jacks, T., Wood, D. L., Jove, R., and Collins, F. S. Neurofibromatosis type 1 gene product (neurofibromin) associates with microtubules. Somat Cell Mol Genet, *19:* 265-274, 1993.
14. Roudebush, M., Slabe, T., Sundaram, V., Hoppel, C. L., Golubic, M., and Stacey, D. W. Neurofibromin colocalizes with mitochondria in cultured cells. Exp Cell Res, *236:* 161-172, 1997.
15. Liu, V. W. S., Shi, H. H., Cheung, A. N. Y., Chiu, P. M., Leung, T. W., Nagley, P., Wong, L. C., and Ngan, H. Y. S. High incidence of somatic mitochondrial DNA mutations in human ovarian carcinomas. Cancer Res, *61:* 5998-6001, 2001.

16. Alonso, A., Martin, P., Albarran, C., Aquilera, B., Garcia, O., Guzman, A., Oliva, H., and Sancho, M. Detection of somatic mutations in the mitochondrial DNA control region of colorectal and gastric tumors by heteroduplex and single-strand conformation analysis. Electrophoresis, *18:* 682-685, 1997.
17. Fliss, M. S., Usadel, H., Caballero, O. L., Wu, L., Buta, M. R., Eleff, S. M., Jen, J., and Sidransky, D. Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. Science, *287:* 2017-2019, 2000.
18. Copeland, W. C., Wachsman, J. T., Johnson, F. M., and Penta, J. S. Mitochondrial DNA alterations in cancer. Cancer Investigation, *20:* 557-569, 2002.
19. Hibi, K., Nakayama, H., Yamazaki, T., Takase, T., Taguchi, M., Kasai, Y., Ito, K., Akiyama, S., and Nakao, A. Mitochondrial DNA alteration in esophageal cancer. Int J Cancer, *92:* 319-321, 2001.
20. Tan, D.-J., Bai, R., and Wong, L.-J. C. Comprehensive scanning of somatic mitochondrial DNA mutations in breast cancer. Cancer Res, *62:* 972-976, 2002.
21. Polyak, K., Li, Y., Zhu, H., Lengauer, C., Willson, J. K., Markowitz, S. D., Trush, M. A., Kinzler, K. W., and Vogelstein, B. Somatic mutations of the mitochondrial genome in human colorectal tumours. Nature Genet, *20:* 291-293, 1998.
22. Kirches, E., Krause, G., Warich-Kirches, M., Weis, S., Schneider, T., Meyer-Puttlitz, B., Mawrin, C., and Dietzmann, K. High frequency of mitochondrial DNA mutations in glioblastoma multiforme identified by direct sequence comparison to blood samples. Int J Cancer, *93:* 534-538, 2001.
23. Wallace, D. C., Lott, M. T., Brown, M. D., and Kerstann, K. Mitochondrial and neuroophthalmologic diseases., 8th edition, Vol. 2, p. 2425-2509. New York: McGraw-Hill, 2001.
24. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. Prevention of apoptosis by bcl-2: release of cytochrome c from mitochondrial blocked. Science, *275:* 1129-1132, 1997.
25. Torroni, A., Stepien, G., and Hodge, J. A., et al. Neoplastic transformation is associated with coordinate induction of nuclear and cytoplasmic oxidative phosphorylation genes. J Biol Chem, *265:* 20589-20593, 1990.
26. Liang, B. C. Evidence for association of mitochondrial DNA sequence amplification and nuclear localization in human low-grade gliomas. Mutat Res, *354:* 27-33, 1996.
27. Boultwood, C., Fidler, C., and Mills, K. I., et al. Amplification of mitochondrial DNA in acute myeloid leukaemia. Br J Haematol, *95:* 426-431, 1996.
28. Penta, J. S., Johnson, F. M., Wachsman, T., and Copeland, W. C. Mitochondrial DNA in human malignancy. Mutat Res, *488:* 119-133, 2001.
29. Korf, B. R. Diagnosis and management of neurofibromatosis type 1. Curr neurol Neurosci Rep, *1:* 162-167, 2001.
30. Lahiri, D. and Nurnberger Jr., J. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucleic Acids Res, *19:* 5444, 1991.
31. Wong, L.-J. C., Liang, M.-H., Kwon, H., Park, J., Bai, R., and Tan, D. Comprehensive scanning of the whole mitochondrial genome for mutations. Clin Chem, *in press*, 2002.

32. Chen, T. J., Boles, R., and Wong, L.-J. C. Detection of mitochondrial DNA mutations by temporal temperature gradient gel electrophoresis. Clin Chem, *45:* 1162-1167, 1999.
33. Anderson, S., Bankier, A. T., Barrell, B. G., deBruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Rose, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. Sequence and organization of the human mitochondrial genome. Nature, *290:* 457-465, 1981.
34. Parrella, P., Xiao, Y., Fliss, M. S., Sanchez-Cespedes, M., Mazzarelli, P., Rinaldi, M., Nicol, T., Gabrielson, E., Cuomo, C., Cohen, D., Pandit, S., Spencer, M., Rabitti, C., Fazio, V. M., and Sidransky, D. Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates. Cancer Res, *61*, 2001.
35. Peltonen, J., Jaakkola, S., Lebwohl, M., Renvall, S., Risteli, L., Virtanen, I., and Uitto, J. Cellular differentiation and expression of matrix genes in type 1 neurofibromatosis. Lab Invest, *59:* 760-771, 1986.
36. Andrews, R. M., Kubacka, I., Chinnery, P. F., Lightowlers, R. N., Turnbull, D. M., and Howell, N. Reanalysis and revision of the Cambridge reference sequence for human mitochodrial DNA. Nature Genet, *23:* 147-147, 1999.

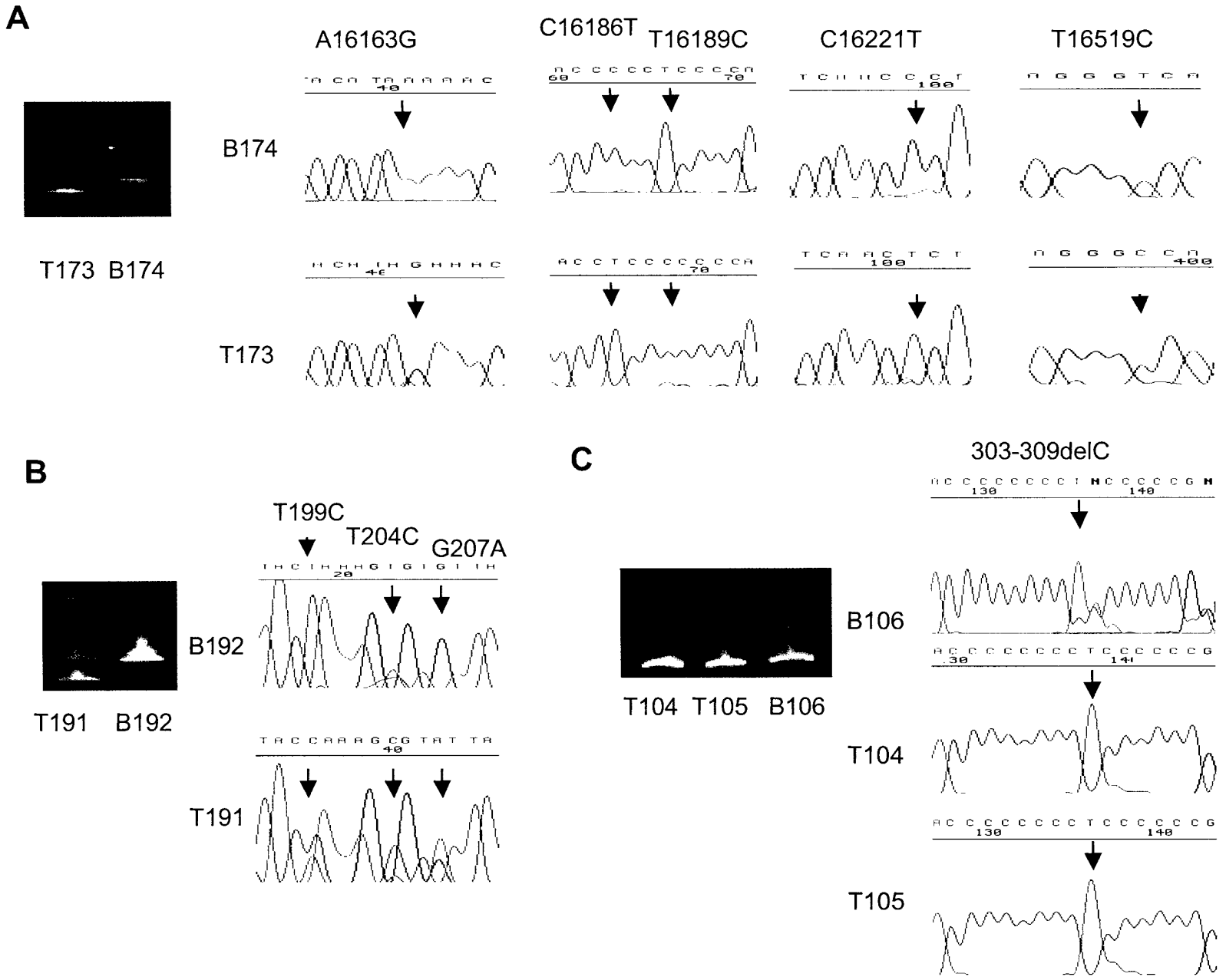
A
A16163G
C16186T
T16189C
C16221T
T16519C
B174
T173 B174
T173
B
T199C
T204C
G207A
B192
T191 B192
T191
C
303-309delC
B106
T104 T105 B106
T104
T105

Table 1 A *Somatic mtDNA mutations in NF1 associated cutaneous neurofibromas*

Case number	Gene/ region	Somatic Mutation	Cambridge Sequence	nl to tu Pattern[a]	Function[c]	Previously reported in tumors[b]	Reference
T104	D-LOOP	303-309delC,C9/8-C8	C	hetero-homo	Conserv.Sequence Block	crc,gastric,eso,ov, brca	15,19,16,28
T105	D-LOOP	303-309delC,C9/8-C8	C	hetero-homo	Conserv.Sequence Block	crc,gastric,eso,ov, brca	15,19,16,28
T107	D-LOOP	303-309insC,C7-C7/8	C	homo-hetero	Conserv.Sequence Block	crc,gastric,eso,ov, brca	15,19,16,28
T108	D-LOOP	303-309insC,C7-C7/8	C	homo-hetero	Conserv.Sequence Block	crc,gastric,eso,ov, brca	15,19,16,28
T119	D-LOOP	T16304C	T	homo-homo	Hypervariable Segment 1	ov	15
T120	D-LOOP	T16304C	T	homo-homo	Hypervariable Segment 1	ov	15

[a] nl: normal (blood); tu: tumor; homo: homoplasmic; hetero: heteroplasmic
[b] crc: colorectal cancer; eso: esophageal cancer; ov: ovarian cancer; brca: breast cancer
[c] Conserv.: Conserved Sequence Block

8

8

T590	D-LOOP	303-309insC,C7/8-C8/7	C	hetero-hetero	Conserv.Sequence Block	crc,gastric,eso,ov, brca	15,19,16,28
T591	D-LOOP	303-309insC,C7/8-C8/7	C	hetero-hetero	Conserv.Sequence Block	crc,gastric,eso,ov, brca	15,19,16,28

Table 1 B *somatic mtDNA mutations in NF1 associated plexiform neurofibromas*

number	region	Mutation	Sequence	Pattern[a]		reported in tumors[b]	Reference
T159	D-LOOP	A73G	A	homo-homo	Hypervariable Segment 2	eso	19
T159	D-LOOP	C16193T	C	homo-homo	Hypervariable Segment 1	novel	this study
T159	D-LOOP	C16278T	T	homo-homo	Hypervariable Segment 1	ov	15
T159	D-LOOP	C16519T	T	homo-homo		lung, glioblastoma	17,22
T165	D-LOOP	T64C	C	hetero-hetero	Hypervariable Segment 2	novel	this study
T171	D-LOOP	303-309delC,C9/8-C8/9	C	hetero-hetero	Conserv. Sequence Block	crc,gastric,eso,ov, brca	15,19,16,28
T173	D-LOOP	C64T	C	homo-homo	Hypervariable Segment 2	novel	this study
T173	D-LOOP	A73G	A	homo-homo	Hypervariable Segment 2	eso	19
T173	D-LOOP	T152C	T	homo-homo	H-strand origin	ov	15
T173	D-LOOP	T195C	T	homo-homo	H-strand origin	lung, glioblastoma	17,22
T173	D-LOOP	A16163G	A	homo-homo	Termin.associated sequ.	novel	this study
T173	D-LOOP	C16186T	C	homo-homo	7S DNA	novel	this study
T173	D-LOOP	T16189C	T	homo-homo	7S DNA	brca	20
T173	D-LOOP	C16221T	C	homo-homo	Hypervariable Segment 1	novel	this study
T173	D-LOOP	T16519C	T	homo-homo		lung, glioblastoma	17,22
T179	D-LOOP	303-309insC,C7/8-C8	C	hetero-homo	Conserv. Sequence Block	crc,gastric,eso,ov, brca	15,19,16,28
T187	D-LOOP	303-309delC,C7/8-C7	C	hetero-homo	Conserv. Sequence Block	crc,gastric,eso,ov, brca	15,19,16,28
T189	D-LOOP	T204C	T	hetero-homo	H-strand origin	gastric, glioblastoma	16,22
T189	D-LOOP	G207A	G	homo-hetero	H-strand origin	brca	20
T189	D-LOOP	303-309insC,C7-C7/8	C	homo-hetero	Conserv. Sequence Block	crc,gastric,eso,ov, brca	15,19,16,28

[a] nl: normal (blood); tu: tumor; homo: homoplasmic; hetero: heteroplasmic
[b] crc: colorectal cancer; eso: esophageal cancer; ov: ovarian cancer; brca: breast cancer
[c] Termin.: Termination associated sequence; Conserv.: Conserved Sequence Block

Table 2 Somatic mtDNA mutations in two separate cutaneous neurofibromas from each of 6 patients, and in paired skin samples of patients 4-6. In all samples from patients 4-6 only the D-loop region was analyzed.

Patient #	sample #	Specimen type	Location	Somatic mutation	% of heteroplasmy[a]	
1						
	106	blood		303-309 insC C7/C8	C7~50%	C8~50%
	105	tumor 1		303-309 delC C7/C8	C7~0%	C8~100%
	104	tumor 2		303-309 delC C7/C8	C7~5%	C8~95%
2						
	109	blood		303-309 insC C7/C8	C7~100%	C8~0%
	108	tumor 1		303-309 insC C7/C8	C7~60%	C8~40%
	107	tumor 2		303-309 insC C7/C8	C7~40%	C8~60%
3						
	121	blood		T16304	T~100%	C~0%
	120	tumor 1		T16304C	T~0%	C~100%
	119	tumor 2		T16304C	T~0%	C~100%
4						
	587	blood		303-309 insC C7/C8	C7~50%	C8~50%
	586	skin	distal from tumor	303-309 insC C7/C8	C7~40%	C8~60%
	585	skin	overlaying the tumor	303-309 insC C7/C8	C7~30%	C8~70%
	583	tumor 1	Thorax/Abdomen	303-309 insC C7/C8	C7~10%	C8~90%
	584	tumor 2	Thorax/Abdomen	303-309 insC C7/C8	C7~5%	C8~95%
5						
	594	blood		303-309 insC C7/C8	C7~50%	C8~50%
	588	skin	distal from tumor	303-309 insC C7/C8	C7~40%	C8~60%
	589	skin	overlaying the tumor	303-309 insC C7/C8	C7~40%	C8~60%
	590	tumor 1	Thorax right side	303-309 insC C7/C8	C7~20%	C8~80%
	591	tumor 2, part 1	Thorax/Abdomen	303-309 insC C7/C8	C7~0%	C8~100%
	592	tumor 2, part 2	Thorax/Abdomen	303-309 insC C7/C8	C7~20%	C8~80%
	593	tumor 2, part 3	Thorax/Abdomen	303-309 insC C7/C8	C7~0%	C8~100%
6						
	598	blood				
	597	skin	2cm away from 595	no mutation found		
	596	tumor 1	Thorax right side	no mutation found		
	595	tumor 2	Thorax right side	no mutation found		

[a] Percentage of heteroplasmy was estimated from the sequencing chromatogram. They do not represent the actual proportion. However, the trend of progressive alteration was obvious (patients, 4 and 5). For samples 104 and 105 TTGE gel chromatogram was used to estimate the percentage of mutant heteroplasmy, which was too low to be revealed by sequencing (Fig. 1C).

Table 3 ***Clinical Informations about patients with plexiform neurofibromas***[a]

Case #	sex	Age y	known NF mutation	cutaneous Neurofibromas	subcutaneous Neurofibromas	plexiform Neurofibromas	café-au-lait spots	axillary freckling	groin freckling	Lisch nodules
T157	f	10	no	0	0	1	>6	bilateral	no	no
T159	**f**	**39**	**yes**	**35**	**0**	**1**	**>9**	**bilateral**	**no**	**yes**
T161	m	34	no	>200	30	1	>6	bilateral	bilateral	yes
T163	m	57	no	>300	0	2	>12	bilateral	no	yes
T165	**m**	**17**	**no**	**0**	**0**	**2**	**>12**	**bilateral**	**bilateral**	**yes**
T169	f	11	no	0	2	1	>12	bilateral	no	yes
T171	**f**	**24**	**yes**	**0**	**0**	**2**	**>12**	**bilateral**	**bilateral**	**yes**
T173	**m**	**8**	**no**	**0**	**5**	**1**	**9**	**no**	**no**	**yes**
T175	m	45	no	>300	>50	2	>12	bilateral	bilateral	yes
T177	m	34	no	>2000	0	1	>6	bilateral	bilateral	yes
T179	**f**	**11**	**yes**	**10 to 50**	**0**	**1**	**>12**	**bilateral**	**bilateral**	**no**
T181	f	38	yes	<10	0	1	>12	bilateral	bilateral	yes
T183	m	24	yes	<10	0	1	>12	no inf.	no inf.	no inf.
T185	**m**	**63**	**yes**	**>300**	**0**	**1**	**4**	**no inf.**	**no inf.**	**yes**
T187	**m**	**13**	**yes**	**<10**	**0**	**1**	**>6**	**bilateral**	**bilateral**	**yes**
T189	**f**	**13**	**yes**	**0**	**<10**	**2**	**>6**	**bilateral**	**bilateral**	**yes**
T191	**m**	**73**	**no**	**20**	**0**	**3**	**>12**	**bilateral**	**bilateral**	**yes**
T193	m	29	yes	0	0	1	>6	no inf.	no inf.	no inf.

[a] patients with somatic mtDNA mutations are in bold

Table 4 *Germline sequence variations* [a,b]

A. Novel

Gene/region	Germ-line mutation	Frequency[c]	Significance
D-loop	T10C	1	7S DNA
D-loop	T55C	1	7S DNA
D-loop	T57C	1	Hypervariable Segment 2
D-loop	T408A	1	L-strand promoter
16S	A2706G	1	16S RNA
ND2	C5263T	1	GCC-GTC, **A265V**
COI	G6917A	1	GTG-GGG, V338V
ND4	A11947G	1	ACA-ACG, T396T
D-loop	C16465T	1	

B.Reported

Gene/region	Germ-line mutation	Frequency[c]	Significance
D-loop	T72C	4	Hypervariable Segment 2
D-loop	A73G	11	Hypervariable Segment 2
D-loop	T146C	2	H-strand origin
D-loop	C150T	1	H-strand origin
D-loop	T152C	4	H-strand origin
D-loop	A189G	1	H-strand origin
D-loop	C194T	2	H-strand origin
D-loop	T195C	5	H-strand origin
D-loop	T199C	1	H-strand origin
D-loop	T204C	2	H-strand origin
D-loop	G207A	2	H-strand origin
D-loop	C242T	1	mtTF1 binding site
D-loop	A263G	12	H-strand origin
D-loop	C295T	1	mtTF1 binding site
D-loop	303-309insC	13	Conserved Sequence Block II
D-loop	C462T	1	
D-loop	T489C	1	
D-loop	A508G	1	
D-loop	514insCA	1	
D-loop	514insCACA	1	
D-loop	568insCCC	1	
12s	A663G	2	12S RNA
12s	G709A	1	12S RNA
ND2	G4580A	1	ATG-ATA, M37M
ND2	A4769G	2	ATA-ATG, M100M
COI	T6776C	1	CAT-CAC, H291H
ND4	G11914A	1	ACG-ACA, T385T

Table 4 *Continued*

B.Reported

Gene/region	Germ-line mutation	Frequency[c]	Significance
ND4	G12007A	1	TGG-TGA, W416W
ND5	A12612G	1	GTA-GTG, V92V
ND5	A12693G	1	AAA-AAG, K119K
ND5	C12705T	2	ATC-ATT, I123I
ND6	A14233G	1	ATC-GTC, **I29V**
CytB	T14798C	1	TTC-CTC, **F18L**
D-loop	G16145A	1	
D-loop	C16186T	1	Hypervariable Segment 1
D-loop	C16188T	1	Hypervariable Segment 1
D-loop	T16189C	1	Hypervariable Segment 1
D-loop	T16192T	1	Hypervariable Segment 1
D-loop	C16193T	1	Hypervariable Segment 1
D-loop	C16195T	1	Hypervariable Segment 1
D-loop	C16222T	1	Hypervariable Segment 1
D-loop	C16223T	3	Hypervariable Segment 1
D-loop	C16278T	4	Hypervariable Segment 1
D-loop	C16290T	1	Hypervariable Segment 1
D-loop	C16292T	1	Hypervariable Segment 1
D-loop	C16294T	3	Hypervariable Segment 1
D-loop	C16296T	2	Hypervariable Segment 1
D-loop	T16298C	3	Hypervariable Segment 1
D-loop	T16304C	2	Hypervariable Segment 1
D-loop	A16309G	1	Hypervariable Segment 1
D-loop	T16311C	2	Hypervariable Segment 1
D-loop	T16362C	2	Hypervariable Segment 1
D-loop	G16390A	1	Hypervariable Segment 1
D-loop	T16519C	4	

[a] Total number of distinct germline sequence variations: 63
Novel: 9
Reported: 54
[b] Missense substitutions are in bold
[c] Number of tumors, which carry germline-variation

Table 5 ***Summary of mtDNA mutations in various tumors***

	Our studies						Other studies				
	NF1 cutaneous	plexiform	lung	breast	Medullo-blastoma	oral	ovarian	bladder	Head & neck	Glio-blastoma	lung
No. of tumors	13	18	14	19	10	18	10	14	13	17	14
No. of cases with mtDNA mutations	5	9	10	14	5	14	6	9	6	6	6
% tumor with mtDNA mutation	38	50	71	74	50	78	60	64	46	35	43
Total no. of mtDNA mutations	5	27	26	27	17	27	6	20	9	18	9
Number of mutations/tumor	1	3	2.6	1.93	3.4	1.93	1	2.2	1.5	3	1.5
No. of mutations in D-loop	5	27	17	22	11	19	2	6	6	10	6
% of mutations in D-loop	100	100	65	81	65	70	33	30	67	56	67
Reference	this study	this study	to be published	20	to be published	to be published	15	17	17	22	17