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13. ABSTRACT (Maximum 200 words) Neurofibromatosis 2 (NF2) is a genetic disorder characterized by the development of bilateral vestibular schwannoma and other nervous system tumors. Both NF2 and neurofibromatosis 1 (NF1) show great clinical variability between individuals with regards to tumor burden, severity of disease and age of onset and death. Despite this overall heterogeneity and unlike NF1, NF2 shows remarkable homogeneity within families, suggesting an effect of the underlying mutation on the resultant phenotype. The hypothesis of this study is that there is a correlation between the highly variable phenotype of neurofibromatosis 2 and the causative genotype. Progress over the last year has included: 1) Substantial revision of the NF2 database and expansion of the consortium to other centers participating in Army funded NF research; 2) Phenotypic analysis of the pediatric subset of subjects entered in the database; 3) Development of alternative methodologies for mutational analysis of the NF2 gene; and 4) Analysis of the extent to which unequal expression of mutant NF2 alleles may limit RNA based analysis of NF2 patients.				
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FOREWORD

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10/27/98
Date

TABLE OF CONTENTS:

Introduction--5

Body--6 to 8

I. Patient ascertainment and database analysis--6 to 7

II. Molecular analysis--7 to 8

Conclusions--9

References--10

Appendices--

Statement of work from 12/1/96 submission

Screen shots of revised database

Letter from Dr. Dilys Parry, National Cancer Institute

Letter from Ms. Ann Masuda, clinical coordinator HEI site

Abstract: "The natural history of neurofibromatosis 2 in 31 affected children"

Abstract: "Mutational analysis of neurofibromatosis 2: alternatives to exon scanning"

Abstract: "Unequal allelic expression of the *NF2* gene in neurofibromatosis type 2"

INTRODUCTION

Neurofibromatosis 2 (NF2) is a genetic disorder characterized by the development of bilateral vestibular schwannoma and other nervous system tumors. Both NF2 and neurofibromatosis 1 (NF1) show great clinical variability between individuals with regards to tumor burden, severity of disease and age of onset and death. Despite this overall heterogeneity and unlike NF1, NF2 shows remarkable homogeneity within families, suggesting an effect of the underlying mutation on the resultant phenotype.

The hypothesis of this study is that there is a correlation between the highly variable phenotype of neurofibromatosis 2 and the causative genotype. We are investigating this hypothesis under three broad areas:

1. The vast majority of NF2 affected individuals harbor point mutations and small frameshifts of the *NF2* gene. What effect does the type and location of mutation have upon the resulting phenotype?
2. Up to one third of the mutations in individuals with NF2 cannot be detected with exon scanning, and these individuals predominantly carry a mild phenotype. What is the pathogenetic mechanism for this phenotype and what implications does it have for the function of the *NF2* protein product as a tumor suppressor?
3. What is the role of the *NF2* gene in atypical and outlying phenotypes of NF2?

The steps we are taking to investigate this hypothesis are:

1. Identification of a large cohort of affected probands and family members from three major collection sites, clinical characterization of the affected individuals and sample collection of affected and unaffected tissues.
2. Molecular genetic analysis of the *NF2* gene in germline specimens using exon scanning and directed sequencing.
3. Development of alternative strategies of mutational detection for germline specimens not found to have abnormalities by exon scanning.
4. Molecular genetic analysis of the *NF2* locus in somatic (tumor) specimens.

These studies may be expected to shed light on the molecular basis for tumor suppression by the *NF2* gene by identification of critical regions of the transcript and alternative mechanisms of inactivation of this protein. They will also make the molecular diagnostics possible for increasing numbers of both probands and at risk individuals and will clarify the diagnosis of variant phenotypes. Finally, the elucidation of genotype phenotype relationships will aid in the prognostication and management of this devastating disorder.

BODY

This section is organized according to the Statement of Work presented in the original proposal. A copy of the Statement of Work is placed in the appendix for reference.

I. Patient ascertainment and database analysis:

A. Revision of the database

Because of concerns raised by NF Peer Review Panel, a statistician, Dr. Zoreh Davenport was added to the project. Dr. Davenport and other members of the consortium made many suggestions regarding the database and it was subsequently extensively revised. Screen shots of the current database, which exists in FileMaker Pro format for both Mac and PC platforms, are placed in the appendix.

B. Databasing and collection of samples

Databasing and collection of samples was significantly impacted by the revision of the database, since computer entry could not begin until this was completed. Anticipated and actual numbers of patients entered are shown in table 1.

Site	New subjects			Outstanding subjects databased	
	Anticipated	Actual	Sample collection	Anticipated	Actual
MGH	25	8	3	50	12
HEI	25	17	10	43	1
Hamburg	12	24	24*	0	NA

Table 1. Subjects enrolled in NF2 project. Anticipated figures are taken from Table 8, page 14 in the original grant proposal. *Hamburg patients are undergoing initial mutational analysis in Germany, not at the MGH site.

After submission of our initial grant, the NCI project on NF2 was substantially cut for internal funding reasons. Because they are no longer enrolling patients, they have elected to not participate as a full consortium member (see letter in appendix from Dr. Dillys Parry). However, this change will be offset by the launch of a second Army funded initiative at the HEI on the natural history of vestibular tumors in NF2. Through this work, other centers have been recruited to the consortium and will contribute additional patients.

C. Phenotypes of severely affected patients

Because NF2 is classically considered a disease of adults, we examined the clinical characteristics of the children entered in the study. A total of 31 children meeting the NIH criteria for NF2 prior to age 17 years were ascertained. 24 had been diagnosed clinically and 7 were diagnosed using molecular methods. Only 3 of 31 patients (10%) presented with hearing loss. 13 children (42%) presented with skin tumors or ophthalmologic abnormalities, the significance of which was often realized because of a positive family history. Vestibular tumors had been detected in 31 of 33 children at ages 1 through 16 and in 6 the tumors were greater than 2 cm at first evaluation. Other intracranial or spinal tumors had been detected in 28 children. The clinical course of these children was highly variable, dependent on both tumor burden at the time of diagnosis and surgical outcome when tumor resection was attempted. Molecular analysis of these patients showed the overwhelming majority to have truncating mutation, as would be expected from our previous studies.

II. Molecular analysis

A. Alternative mechanisms of mutational analysis

A rapid cDNA based assay was developed as an alternative mechanism of mutational analysis for mildly affected patients. Using the current cohort of 105 affected unrelated individuals who have undergone mutational analysis using standard techniques thorough the consortium, a group of control and experimental subjects was constructed. The control group consisted of 14 of the 18 individuals with known splice site mutations. The experimental group consisted of 23 of the 44 predominantly mildly affected patients whose mutations were not detected using exon scanning. RNA was extracted from lymphoblastoid cell lines and cDNA was synthesized by reverse transcription. Amplification of the entire *NF2* coding region was carried out in six overlapping segments.

No control splice site samples produced unexpected size variants and no uniform change was evident in the samples, confirming the lack of alternative splicing in this tissue which has confounded the analysis of tumor tissue. Expected size alterations were seen in 12 of the 14 splice site controls; expression of the splice mutation could not be seen in 2 specimens, consistent with the studies described below showing under expression or lack of expression of mutated *NF2* alleles.

Of the 23 unfound samples tested, 3 (13%) produced an alteration in one or more of the 6 cDNA segments. In 2 of the 3 samples, the altered allele amplified at a much

less robust level than did the wild-type allele. All aberrant bands were isolated, re-amplified and sequenced; corresponding genomic DNA changes were then sought. Changes in this group included deletion of exons 2 through 8 (GUS16218), duplication of exons 3 and 4 (GUS16983) and insertion of intronic material between exons 13 and 14 (GUS8873), perhaps due to activation of a cryptic splice site.

B. Allelic expression of the *NF2* gene

In collaboration with Dr. Lee Jacoby, a polymorphism in the 3' untranslated region of the *NF2* gene that is informative in about one-third of individuals was developed. This polymorphism permitted standardized assessment of the relative expression of *NF2* transcripts in lymphoblastoid cell lines. RNA from 22 unrelated *NF2* patients known to be heterozygous for a germline *NF2* mutation because of previous molecular analysis was studied. A control group consisting of 14 unaffected controls was amplified in parallel. Unequal allelic expression of 1.8 fold to greater than 100 fold was detected in 15 of 22 *NF2* patients, but no control sample. Underexpression of the mutant allele was documented for all six nonsense or frameshift mutation, 3 of six splice mutation (consistent with the results in section II.A.) and one of four missense mutation. Equal expression of the mutant allele was seen in all in-frame deletions, two splice site mutations and three missense mutations.

C. Examination of the role of cell culture conditions in unequal expression of alleles

A current report suggests that cell culture conditions, namely the phase of cell growth, may influence the degree of unequal expression of the *NF1* tumor suppressor (Cowley et al., in press). Because the results described in sections A and B suggest that underexpression of mutated alleles may limit the ability of a cDNA based screen to detect causative mutations at the *NF2* locus, we examined the effects of cell growth phase on expression of *NF2* alleles. Cell lines were grown under standardized conditions, and harvested at both log and saturation growth phases. Examination of 3 splice bearing lines (GUS16039, GUS16209 and GUS16097) and 2 nonsense bearing lines (GUS17690 and GUS16206) failed to show any effect of the growing condition on the underexpression of the mutated allele.

CONCLUSIONS:

1. Revision of the NF2 database will provide more comprehensive and reliable access to datapoints for analysis (see appendix). Slight modification of the original consortium, with inclusion of additional centers should maintain our ability to ascertain adequate numbers of patients. Interaction between this study and the recently funded Army grant on the natural history of vestibular schwannoma should greatly facilitate the power of both studies. Although databasing goals were not met this year, all sites have expressed a strong continuing commitment to this and other Army funded NF2 research (see appendix).
2. Analysis of a subset of patients entered in our database illustrates the utility of this multi institutional approach to determine natural history features not readily apparent in single institution studies. In this study of the pediatric population we found that children frequently harbor large vestibular tumors, but are more likely to come to medical attention because of skin tumors and ocular abnormalities. Molecular diagnosis of at risk children (offspring of a parent with bilateral vestibular tumors) is an important tool for early recognition, but of equal utility is a careful dermatological and neurological evaluation. Early detection of tumors while they are still relatively small may lead to a greater array of therapeutic options; whether this will result in better final outcomes and longer life span for these patients will be an important issue in this and other Army funded research on NF2. An abstract describing these findings was presented at the 27th annual meeting of the Child Neurology Society in Montreal, Quebec, October 21 through 24, 1998 (see appendix).
3. cDNA based screening is inexpensive and rapid, and did in fact reveal some of the mutations not detected by standard, genomic based exon scanning. Because of our finding of underexpression of the mutated allele in samples bearing a wide range of mutations, and the lack of our ability to influence this underexpression by manipulating the growing conditions of the cells, we have concluded that cDNA based screening may have limited potential to detect causative mutations. An abstract describing this methodology is being presented at the 48th annual meeting of the American Society of Human Genetics and the satellite meeting of the National Neurofibromatosis Foundation in Denver, Colorado, October 27 through 31, 1998 (see appendix). An abstract describing the phenomenon of unequal expression at the NF2 locus was presented at NNFF International Consortium for the Molecular Biology of NF1 and NF2 in Aspen, Colorado, June 7 through 10, 1998 (see appendix).

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Statement of Work

YEAR ONE

Patient ascertainment:

Approximately 1 proband will be identified per month by each of the on-site clinical coordinators and their data will be entered into the database and appropriate samples collected.

One half of exiting outstanding patients will be entered or up dated in the data base (table 8).

Blood sample collection will be completed on one half of existing outstanding patients.

Molecular analysis:

Alternative mechanisms of analysis will be applied to samples from 50 of the 114 patients in whom exon scanning has failed to reveal causative alteration to estimate the ascertainment rate of these methods.

At the end of this year a report will be prepared on alternative mechanisms of mutational analysis which addresses detection rates and severity grading.

YEAR TWO

Patient ascertainment:

Approximately 1 proband will be identified per month by each of the on-site clinical coordinators and the data will be entered into the database and appropriate samples collected.

In the first half of the year specimens from the remaining outstanding patients will be collected.

In the second half of the year the remaining outstanding patients will be entered into the database.

Molecular analysis:

In the first half of the year the sequence basis for the abnormalities of the patients above will be determined.

In the second half of the year exon scanning will be completed on the outstanding patients.

At the end of this year a report will be prepared on more detailed genotype phenotype relationships in this disease using the data in the data base.

YEAR THREE

Approximately 1 proband will be identified per month by each of the on-site clinical coordinators and the data will be entered into the database and appropriate samples collected.

In the first half of the year tumor specimens will be collected on all outstanding probands. In the second half of the year family members will be collected.

In the first half of the year the sequence basis of the abnormalities of exon scanning will be determined.

In the second half of the year tumor analysis will be completed.

At the end of this year a report will be prepared on the use of tumor specimens to determine genotype-phenotype relations.

MAIN MENU

Record #

☐ Main ☐ Patient Data ☐ Presentation ☐ Hearing ☐ Neurology ☐ Radiology
☐ Ophthalmology ☐ Derm/Pet Nerve ☐ Non Genetic ☐ Family
☐ Surgery ☐ Tumors ☐ Mal Genetic

PATIENT DATA

Record #

DEMOGRAPHICS

☐ Main ☐ Patient Data ☐ Presentation ☐ Hearing ☐ Neurology ☐ Radiology
☐ Ophthalmology ☐ Derm/Pet Nerve ☐ Non Genetic ☐ Family
☐ Surgery ☐ Tumors ☐ Mal Genetic

Name: Address: City: State: Zip:
 Country: DOB: Gender: ☐ Male ☐ Female ☐ Age:
 Date of Birth: Date of Death: Entry Site:
 Date First Visit: Date Last Visit:

ENTRY SITE

☐ Main ☐ Patient Data ☐ Presentation ☐ Hearing ☐ Neurology ☐ Radiology
☐ Ophthalmology ☐ Derm/Pet Nerve ☐ Non Genetic ☐ Family
☐ Surgery ☐ Tumors ☐ Mal Genetic

CREATED **MODIFIED**

PRELIMINARY

Name: Address: City: State: Zip:
 Country: DOB: Gender: ☐ Male ☐ Female ☐ Age:
 Date of Birth: Date of Death: Entry Site:
 Date First Visit: Date Last Visit:

Record Serial Number

GLS # Other ID
 Institution ID #

CLIENT DATE AND TIME

Date: Time:
 Date: Time:

PRESENTATION

Record #

PRESENTING SYMPTOMS

☐ Tremor ☐ Hearing Loss ☐ Balance Disorder ☐ Visual Change ☐ Other...
☐ Other Cranial Nerve ☐ Focal Motor ☐ Skin Tumor ☐ Asymptomatic + Family Member

First MD CONTACT

Date: Age:
 Type of MD: Initial DX:

Current Diagnoses

Date: Type of MD:
 Date: Type of MD:

PRESENTING SYMPTOMS

☐ Tremor ☐ Hearing Loss ☐ Balance Disorder ☐ Visual Change ☐ Other...
☐ Other Cranial Nerve ☐ Focal Motor ☐ Skin Tumor ☐ Asymptomatic + Family Member

First MD CONTACT

Date: Age:
 Type of MD: Initial DX:

Current Diagnoses

Date: Type of MD:
 Date: Type of MD:

HEARING

Record #

HEARING LOSS

☐ Main ☐ Patient Data ☐ Presentation ☐ Hearing ☐ Neurology ☐ Radiology
☐ Ophthalmology ☐ Derm/Pet Nerve ☐ Non Genetic ☐ Family
☐ Surgery ☐ Tumors ☐ Mal Genetic

Date: Age:
 Type of MD: Initial DX:

Current Diagnoses

Date: Type of MD:
 Date: Type of MD:

HEARING LOSS

☐ Main ☐ Patient Data ☐ Presentation ☐ Hearing ☐ Neurology ☐ Radiology
☐ Ophthalmology ☐ Derm/Pet Nerve ☐ Non Genetic ☐ Family
☐ Surgery ☐ Tumors ☐ Mal Genetic

Date: Age:
 Type of MD: Initial DX:

Current Diagnoses

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OPHTHALMOLOGY		DATE / /	
REASON FOR REFERRAL ☐ Refraction ☐ Contact Lens ☐ Cataract ☐ Glaucoma ☐ Macular Degeneration ☐ Diabetic Retinopathy ☐ Other	EXAMINER ☐ Ophthalmologist ☐ Optometrist ☐ Nurse Practitioner ☐ Physician Assistant ☐ Other	CLINICAL HISTORY ☐ History of Trauma ☐ History of Infection ☐ History of Systemic Disease ☐ History of Medication Use ☐ History of Surgery	PHYSICAL EXAMINATION ☐ Visual Acuity ☐ Visual Fields ☐ Intraocular Pressure ☐ Fundus Examination ☐ Slit Lamp Examination ☐ Other
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DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20892

October 26, 1998

Mia MacCollin, M.D.
Neuroscience Center, MGH--East
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Dear Mia:

As I indicated to you in previous conversations, increasing demands on my time from other obligations in our Division have made it impossible for me to maintain a commitment to seeing new NF2 probands and families. For this reason, I am unable to enroll new patients in your genotype-phenotype study. However, I would be delighted to continue to collaborate with you on the analysis of the 37 probands whose DNA we sent you previously. I am especially interested in your continuing to search for the molecular basis of the NF2 mutation in patients whose mutation you were not able to identify on your earlier studies. I would also be glad to address any phenotypic issues that may arise with regard to these patients, utilizing our already extensive database on these subjects.

Sincerely,

A handwritten signature in cursive script, reading "Dilys M. Parry", is positioned above the typed name.

Dilys M. Parry, Ph.D.
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HOUSE EAR INSTITUTE

Research and Education . . . so all may hear

October 19, 1998

Mia MacCollin, MD
Neuroscience Center, MGH-East
Building 149, 13th Street
Charlestown, MA 02129

Dear Mia:

The following is a progress report from House Ear Institute for the USAMRMC grant "Genotype Phenotype Relationships in NF2." Progress is reported for the period September 30, 1997 through September 29, 1998.

- I. # of new NF2 probands identified** 17
of blood samples sent to MGH 1
of blood samples at HEI 9 (can part of it be sent to you?)
of blood samples needed 7
- II. Pre-existing NF2 probands** 132 (reported in Table 8 of grant. Accurate number is 187).
of probands databased 1 (partial)
of blood samples sent to MGH Not sure (at least 2 - Gail Umphrey and Patrick Dillon)

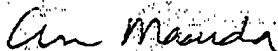
III. Obstacles preventing goals being met

House Ear Institute began 3 studies on NF2 (including "Genotype Phenotype Relationships of NF2") simultaneously while preparing to submit a grant to study NF2 (this is the 4th study). Because all NF2 patients qualify for at least 2 of the studies, HEI planned to ask patients (at the same time) to participate in all of the studies for which they qualify. This required that all informed consent forms be IRB-reviewed and approved, questionnaires be created and approved, and database programs be created and approved. Delays in each of the studies resulted in the inability for HEI to meet their goal for the Genotype Phenotype grant.

IV. HEI plans in the coming year

HEI is committed to meeting its goals for the Genotype Phenotype grant. We are currently in the process of contacting all of the HEI NF2 patients and asking them to participate in our research projects on NF2. The Clinical Coordinator will follow-up on patients consenting to the Genotype Phenotype grant and obtain blood samples and send them to Massachusetts General Hospital and obtain database information. We realize that we will need to accomplish 2 years worth of work within 1 year, but we are determined to meet our goals for this project.

Sincerely,



Ann Masuda, M.S., CCC-A
Clinical Coordinator and Research Audiologist
Clinical Studies Department

THE NATURAL HISTORY OF NEUROFIBROMATOSIS 2 IN 31 AFFECTED CHILDREN Mia

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Neurofibromatosis 2 (NF2) is a genetic disorder leading to multiple nervous system tumors. Although classically considered a disease of adults, many NF2 patients are retrospectively found to have had at least subtle signs of the disorder in childhood. We report the natural history of 31 children meeting the NIH criteria for NF2 prior to age 17 years. Only 3 of 31 patients (10%) presented with hearing loss. 13 children (42%) presented with skin tumors or ophthalmologic abnormalities, the significance of which was often realized because of a positive family history. Vestibular tumors have been detected in 31 of 33 children at ages 1 through 16 and in 6 the tumors were greater than 2 cm at first evaluation. Other intracranial or spinal tumors have been detected in 28 children. The clinical course of these children was highly variable, dependent on both tumor burden at the time of diagnosis and surgical outcome when tumor resection was attempted. 2 patients died during the period of follow-up at ages 2 and 20 years. Although NF2 affected children frequently harbor large vestibular tumors, they are more likely to come to medical attention because of symptoms of skin tumors and ocular abnormalities. Further work is needed to define an optimal intervention strategy for these patients.

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Montreal, Quebec, October 22, 1998*

Mutational analysis of neurofibromatosis 2; alternatives to exon scanning
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Neurofibromatosis 2 (NF2) is a rare autosomal dominant syndrome characterized by benign tumor growth in the nervous system. Mutational analysis by exon scanning is both expensive and cumbersome, and fails to detect causative changes in the majority of mildly affected patients. Because both intronic changes affecting splicing and multi-exon deletions have been reported to produce a mild phenotype, we developed a rapid cDNA based assay and tested its efficacy on a large cohort of individuals. Exon scanning methodologies were applied to genomic DNA from 105 affected unrelated individuals and detected a total of 61 causative mutations (24 nonsense, 14 frameshift, 18 splice site, 5 non truncating alterations); in 44 samples no mutation could be detected. RNA extracted from lymphoblastoid cell lines from 22 of the 44 individuals and a single individual commercially scanned was then reverse transcribed and amplified in six overlapping segments comprising the entire coding region of the NF2 gene. Control material consisting of cDNA derived from 14 of the 18 splice site patients was analyzed in parallel. Putative mutations, consisting of size alterations in the resulting amplification products were directly sequenced from re-amplified cDNA products. 3 of 23 samples (13%) produced one or more alterations in segment sizes, which corresponded to deletion of exons 2 through 8, duplication of exons 3 and 4, and insertion of intronic material between exons 13 and 14. 2 of 3 alterations amplified at a substantially less robust level than the wild-type allele, suggesting decreased expression of the mutated allele. No control splice site sample produced unexpected size variants, confirming the lack of alternative splicing in this tissue. However, expression of the splice mutation could not be documented in 2 of the 14 samples. Although cDNA screening may be an inexpensive method of revealing some mutations not observed in genomic DNA exon scans, underexpression of mutated alleles may limit its utility. Other methodologies for detecting mutations in mildly affected NF2 patients must be developed.

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Title: Unequal Allelic Expression of the *NF2* Gene in Neurofibromatosis Type 2

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Neurofibromatosis type 2 (NF2) is a severe genetic disorder caused by alteration of the *NF2* gene on chromosome 22. A number of germline and somatic mutations have been reported, confirming that inactivation of the *NF2* gene product, merlin or schwannomin, underlies NF2; however, mutational analyses by current exon scanning approaches have identified less than two-thirds of the expected mutations. The mutational basis of disease is particularly difficult to establish for mildly affected patients, or for those with outlying phenotypes such as unilateral schwannomas or multiple meningiomas. We describe here a new polymorphism in the 3' untranslated region (3'UTR) of the *NF2* gene, which is informative in about one-third of individuals. Using this polymorphism we have examined *NF2* allelic expression in lymphoblastoid cells from 24 unrelated NF2 patients, including six with unidentified mutations, and from 14 unrelated, unaffected controls. Unequal allelic expression (2-fold to greater than 100-fold) was observed for 16 of the *NF2* mutations. In most cases, the *NF2* mutant allele could be phased with the 3'UTR polymorphism by family studies, loss of heterozygosity in a tumor from the patient, or by analysis of cloned cDNAs. Underexpression of the *NF2* mutant allele was documented for six of six nonsense or frameshift mutations, three of six splice mutations, and one of four missense mutations. In contrast, equal expression or slight overexpression of the *NF2* mutant alleles was observed for two in-frame deletions, a splice mutation causing an in-frame exon deletion, a splice alteration extending the normal open reading frame by three codons, and three missense mutations. Equal *NF2* allelic expression was also observed in all 14 control mRNAs. Thus the types of *NF2* mutations that were predicted to truncate the protein product, which are the more frequent types of alteration seen in NF2 patients and NF2-associated tumors, were associated with underexpression of the mutant allele. In contrast, the less frequent *NF2* mutations creating in-frame alterations were equally expressed or slightly overexpressed in the mRNA. The ability to detect both *NF2* alleles using this 3'UTR polymorphism should facilitate the detection and characterization of germline and somatic *NF2* mutations. The effect of specific types of mutations on unequal expression may explain the genotype-phenotype relationships in this disorder. Finally, further work is underway to determine if unequal expression of mutated alleles may provide a simple and rapid test for persons desiring molecular diagnosis for NF2.

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