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13. ABSTRACT *(Maximum 200 Words)*

Patients with Neurofibromatosis 2 (NF2) are affected by multiple schwannomas that can cause significant morbidity and mortality. NF2 patients also develop numerous, small, neoplastic Schwann cell tumorlets in the cauda equina that do not grow and are clinically silent[3-6]. Since *NF2* gene inactivation has been shown to occur in both tumorlets and schwannomas, additional genetic or epigenetic events are required for frank, symptomatic schwannomas to develop[7-11].

The purpose of this study is to find the specific growth-associated factors that drive the progression of some Schwann cell tumorlets to become symptomatic schwannomas, by using oligonucleotide expression array analysis. For this purpose, we have collected frozen specimens including schwannomas, tumorlets and normal peripheral nerves from three NF2 patients. Because optimal RNA quality is of paramount importance for this type of study, we have focused on the development of suitable standardized protocols for the processing, staining and laser capture procedures. In addition we have optimized our protocols for RNA extraction and amplification from laser captured tissue and analyzed the tissues banked for RNA quality and quantity by the Agilent Bioanalyzer system. The collected specimens have been histologically examined by frozen sections and laser capturing is ongoing.

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INTRODUCTION

Patients with Neurofibromatosis 2 (NF2) are affected by multiple schwannomas that can cause significant morbidity and mortality. In addition to the schwannomas, NF2 patients also develop numerous, small, neoplastic Schwann cell tumorlets in the cauda equina that do not grow and are clinically silent[3-6]. Since *NF2* gene inactivation has been shown to occur in both tumorlets and schwannomas, we have posed the hypothesis that additional genetic or epigenetic events are required for frank, symptomatic schwannomas to develop[7-11]. The purpose of this study is to find the specific growth-associated factors that drive the progression of some Schwann cell tumorlets to become symptomatic schwannomas, by using oligonucleotide expression array analysis.

PROGRESS REPORT BODY:

a) Approved Statement of Work:

Task 1: To compare the expression profile of NF2-associated Schwann cell tumorlets and frank schwannomas using microarray analysis and, if necessary, cDNA representational differential analysis (Months 1-10):

a. Laser-capture microdissection of Schwann cell tumorlets [10 samples] and schwannomas [10], RNA extraction and generation of cDNA.

b. microarray hybridization and analysis of data, statistical analysis and priorization.

c. cDNA-representational difference analysis on pairs of Schwann cell tumorlets and schwannomas [3 pairs].

Task 2: To evaluate and validate the candidate growth-promoting factors at the RNA and protein levels on NF2-associated schwannomas relative to tumorlets; NF2-associated schwannomas relative to tumor growth rate; sporadic schwannomas relative to sporadic Schwann cell tumorlets; and schwannomas relative to Schwann cell hyperplasia in murine NF2 models (months 11-36).

a. Northern blotting and RT-PCR, frozen tissues: Schwann cell tumorlets (NF2-associated [10 samples] and murine hyperplasia [10]) and schwannomas (NF2-associated [20], sporadic [30] and murine) [10].

b. Optimization of commercially available antibodies to candidate proteins

c. Generation of monoclonal and polyclonal antibodies to candidate proteins

d. Optimization of newly generated antibodies

e. Western blotting, frozen tissues: Schwann cell tumorlets (NF2-associated [10 samples] and murine hyperplasia [10]) and schwannomas (NF2-associated [20], sporadic [30] and murine) [10]

f. Immunohistochemical analysis, frozen and archival tissues: NF2-associated Schwann cell tumorlets [10 samples], NF2-associated schwannomas [70], sporadic tumorlets [5], sporadic schwannomas [30], murine hyperplasia [10]and murine schwannomas [10].

Task 3: To begin investigation of the mechanisms underlying overexpression of specific differentially expressed, growth-associated molecules (e.g., evaluation of gene amplification, cloning of promoter regions).

b.) Studies and Results

Task 1: We have been actively collecting tissues from NF2 patients, including schwannomas, tumorlets and normal peripheral nerves. For calendar year 2002, we harvested tissues from 2 additional NF2 patients that underwent autopsies. RNA analysis of banked tissues has shown that the RNA quality was best preserved in autopsy tissue that was immersed in OCT and snap-frozen in liquid nitrogen (as opposed to snap freezing on dry ice). Therefor, all harvested tissues in the last case have been immersed in OCT in individual cryo-cassetts and snap frozen in liquid nitrogen. We have collected 20 tumorlets and 4 schwannomas from each of the NF2 patients. Now our collection includes schwannomas, tumorlets and peripheral nerves from 3 NF2 autopsy cases and we are confident to have enough lesions with good quality RNA to achieve statistically relevant results as

well as sufficient number of tissues to be used later as "test tissues" (microarray analysis of tissues not included in the original class definition study) and tissues to be used for immunohistochemical study of gene products identified by the microarray data. The normal nerves are to be used as control reference tissue for the microarray analysis. Total RNA was extracted from representative tissues from each one of the banked autopsies and analyzed to determine which of the cases have RNA quality most suitable for the microarray analysis and which of the cases should be best used for confirmatory immunohistochemical studies at a later point.

Characterization of the NF2-associated lesions has continued at the histological level. We have cut frozen sections, which were stained with hematoxyllin and eosin and histologically evaluated the presence or absence of tumorlets. The microscopic size of the tumorlets makes it often impossible to distinguish grossly between a tumorlet and a fold or kink in a normal nerve, thus requiring microscopic confirmation. Because NF2 patients are prone to develop both schwannomas and meningiomas, which again, may be indistinguishable, based on gross appearance alone, histological verification of all schwannomas was also performed. In three cases we had to use immunohistochemistry (S100 and EMA) to better define lesions with intermediate histological features of schwannomas and meningiomas. All three were confirmed by immunohistochemistry to be meningiomas and dropped from the microarray study. This type of careful histological examination of each lesion, although time consuming and tedious, guarantees that the micro-array profiling will be performed on a homogeneous, well-characterized set of schwannomas and tumorlets. The histological evaluation and classification of lesions is complete for 2 of the autopsies (confirming 42 tumorlets and 12 schwnnomas) and is ongoing for the third (most recent) autopsy.

We have established the protocol for laser capturing tissue from cryostat sections that included the staining protocol and the number of pulses, power and size of the laser beam to be used for laser capture. We have also established the protocols for RNA extraction of laser-captured material and RNA amplification protocol that yield optimal quality and sufficient quantity of RNA. The RNA was checked with the Agilent 2100 Bioanalyzer, which allows the detection and analysis of very small quantities of RNA (nanograms). In addition, we are in the process of testing the quality of RNA in selected amplified samples by an Affimetrix test chip that establishes the ratio between 3' and 5' of RNA in the sample. Finally, in a few selected cases, total RNA and amplified RNA from laser-captured tissue (from the same specimens), will be analyzed by Affymetrix GeneChips to compare the gene expression profile.

Laser dissection is essential in the case of tumorlets because the lesions are very small and embedded in of in adjacent normal nerve. Laser dissection is not necessary in schwannomas. However, in order to assure a uniform, standardized procedure, we are performing laser capture on the schwannomas and peripheral nerves included in the study. Therefor all tissues (schwannomas, tumorlets, peripheral nerves) selected for microarray-analysis will be laser captured and the RNA extracted and amplified using the same protocols. Affymetrix's new algorithm of data analysis software (MAS 5.0) works best if each data point of the data consists of duplicated or more experiments. The software can then provide p-values based on a quite standard (i.e., well-accepted) statistical procedures. We are therefor using duplicates for all samples. Although that increased the labor and time in the initial phase of the study, we believe it will facilitate the interpretation and analysis of the data.

In addition, some of the amplified RNA from each sample will be used for confirmatory analysis (in selected genes, based on microarray gene expression data) using the Real Time Quantitative PCR (Taqman, ABI Prism 7700 System). This system monitors PCR at every cycle by using a fluorogenic probe. It provides amplification plots of the sample as well as of standards (serial dilutions with known starting copy number). Therefor providing an evaluation of the copy number of the sample from the amplification plot (based on the threshold cycle that its plot crosses a defined fluorescence threshold). This is a very sensitive and reproducible technique, and provides confirmation to microarray expression data. We are developing the protocols for the Taqman procedure.

I am decreasing my effort to 50% on this project. However, this change does not change my role or degree of involvement in this project.

KEY RESEARCH ACCOMPLISHMENTS:

- Acquisition of large numbers of tumorlets, schwannomas and normal peripheral nerves from NF2 patients
- Development and optimization of protocols for the harvesting, processing, laser-capturing, extraction and amplification of laser-captured RNA.
- Histological verification of the tissues collected on frozen sections
- Laser capturing of specimens (ongoing).
- Development and optimization of protocols for the Taqman Real Time PCR (ongoing).

Our efforts have been directed in insuring sufficient numbers of lesions and optimal RNA quality for the microarray analysis. The availability of a large number of histologically well-characterized tissues and the development and optimization of standardized protocols are essential initial steps for this study. The microarray results and the correct interpretation of the data analysis are dependent on the uniform, careful histological verification of specimens and on the quality of RNA used.

REPORTABLE OUTCOMES

Non Applicable

CONCLUSIONS

Non Applicable

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