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TITLE: Growth Mechanisms of Schwann Cell Tumors in NF2

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Patients with neurofibromatosis 2 (NF2) develop multiple schwannomas that, although benign, cause significant morbidity and mortality. In addition NF2 patients have numerous, small, Schwann cell tumorlets in the cauda equina. Previous studies have demonstrated inactivation of both NF2 alleles already occurs in the Schwann cell tumorlet stage, suggesting that additional genetic or epigenetic events are necessary for the development of frank schwannomas. In order to identify the mechanisms that promote this process, we compared gene expression of these two Schwann cell lesions in a single NF2 patient. We employed laser capture microdissection and cDNA microarray analysis, using the Affymetrix platform. 130 differentially expressed genes were identified (p 0.01, >2 fold) and RT-PCR was used for validation of 5 of these genes. Analysis of the Gene Ontology and KEGG pathway terms associated with the upregulated genes (79 genes) identified many genes with known receptor, growth factor or signaling function. There was over-representation of genes involved in the phosphoinositide 3-kinase/Akt (PI3K/Akt) pathway in schwannomas and overexpression of several receptors that may function as autocrine loops for this pathway. Identification of autocrine loops that promote growth in NF2-associated schwannomas may aid in the development of targeted therapies for NF2 patients.
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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 Schwann cell tumorlets in the cauda equina that do not grow and are clinically silent. 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. 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 tumors [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 collected tissues from 5 NF2 patients, including schwannomas, tumorlets and normal peripheral nerves from that underwent autopsy. This large collection of tissues ensured sufficient number for both the microarray and for confirmation (tissues not included in the original class definition study). Although initially two of the four autopsy cases were selected for microarray analysis (based on the quality of total RNA) cases from one autopsy were found not suitable for hybridization. Microarray analysis was therefore performed on lesions from a single NF2 patient, in which 4 schwannomas and 4 tumorlets (in duplicates) were analyzed. All other cases were used for confirmation and validation of differentially expressed genes of interest by RT-PCR.
The small size of tumorlets, made it necessary to use laser dissection in order to avoid contamination of tumorlet samples with adjacent normal nerve (figure 1, appendix). Although laser dissection was not necessary in most schwannomas, in order to assure a uniform, standardized procedure, we performed laser capture on all specimens, including the schwannomas. Frozen tissues were cut on a cryostat at a thickness of 9 µM and a temperature of -25°C, placed onto Superfrost ® microscope slides (Fisher Scientific, Cat. No. 12-544-7), and stored at -80°C. Each case was stained with hematoxylin and eosin and laser captured in duplicate using the PixCell II Laser Capture System (Arcturus Engineering). The slides were fixed in acetone for 5 minutes, dehydrated through a series of increasingly concentrated ethanol solutions, stained with hematoxylin and eosin, rehydrated through ethanol washes and placed on dry ice. Total RNA was isolated using the PicoPure™ RNA isolation kit (Arcturus, Cat. No. KIT0202) and stored at -80°C until RNA amplification. Two rounds of amplification were performed, using the RiboAmp™ RNA Amplification Kit (Arcturus, Cat. No. KIT0201). 3 µg of amplified RNA was used for cDNA synthesis using the Superscript First Strand Synthesis Kit for RT-PCR (Invitrogen), and purified using the Qiagen PCR Purification Kit (Qiagen). cDNA was transformed into mRNA and biotin-labeled with the BioArray High Yield™ RNA Transcript Labeling Kit (Life Sciences Engineering, Inc., Farmingdale, NY). Labeled mRNA was purified using the RNEasy RNA Purification Kit (Qiagen), passing the eluate through the purification column twice in order to obtain a higher yield. Purified samples were quantified and analyzed using the Agilent 2100 bioanalyzer (Agilent Technologies), and only mRNA samples of high quality and concentration (>0.6 µg/µL) were taken to the Massachusetts General Hospital Center Affymetrix Microarray Core Facility, where they were independently reassessed by Core Facility personnel for sufficient RNA quality and quantity (figure 2, appendix). Fragmented biotin-labeled mRNA was hybridized to an Affymetrix HG-U133A chip, which screens over 39,000 transcripts and variants. The final set of samples that were hybridized includes 16 samples from a single NF2 patient: 4 schwannomas and 4 tumorlets, submitted in duplicates. Samples have been submitted in batches, each batch containing both tumorlets and schwannomas, in order to avoid procedure or operator biases.

Analysis of the data was performed using Vector Xpression software (InforMax, Inc., Frederick, MD) and Genesifter, a web-based microarray analysis program (VizXlabs, Seattle, WA). Raw expression values were normalized by linear scaling to achieve similar mean array intensity in all scans. Expression values below 20 were excluded in order to avoid background hybridization expression. The remaining probe sets (21060 from initial set of all differentially expressed genes 22,000) were then subjected to a standard t-test using for the identification of differentially expressed transcripts at unadjusted p-values of 0.01. Genes with a fold change below 2.0 were excluded from further analysis. At unadjusted p-values of 0.01, 130 genes were differentially expressed, with 79 showing increased expression in schwannomas and 51 showing decreased expression in schwannomas (figure 3, appendix).

**Task 2:**

Validation was performed by using quantitative real-time PCR on the test samples and on an independent set of schwannoma and tumorlets (obtained from 4 other NF2 autopsies). 1 µL (0.6-1.5 µg/µL) of amplified mRNA was converted to cDNA and quantified using a Rediplate Picogreen dsDNA Assay plate (Molecular Probes). Each cDNA solution was then diluted to a 0.43 ng/µL starting concentration, mixed with TaqMan ® Universal PCR Master Mix (Applied Biosystems) and the appropriate TaqMan ® Gene Expression Assay (Applied Biosystems) primer/probe set. The quantitative RT-PCR assay used an MGB probe (6FAM-labeled). Samples were run in triplicate, using glyceraldehyde-3-dehydrogenase (GAPDH), a housekeeping gene ubiquitously expressed in tissues, as a cross-plate positive comparison control. Standard curves for each gene were run on each plate using a mixture of cDNA from all experimental samples for the standard curve.

Six genes were evaluated for expression in schwannomas and tumorlets using quantitative RT-PCR: KCNK12, SFRP1, and PIK3CG, ITPR2, OXTR and TNFRSF10. These genes were selected for validation because they are known to have growth promoting effect in schwannomas or Schwann cells in culture. The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as a control probe for cross-plate comparison. Primers and probes have been designed for portions of each of these sequences and were ordered from Applied Biosystems. Differential expression between schwannomas and tumorlets was confirmed in all 6 genes. In 4 of the validated genes there is significant correlation between normalized microarray expression data
and expression data obtained via quantitative RT-PCR. In two genes the absence of significant correlation is presumed to be due to the low expression levels in microarray expression (figure 4, appendix).

**Task 3:**

We performed analysis of the Gene Ontology and KEGG pathway terms associated with the 130 differentially expressed genes to identify the main categories of biological themes and the pathways as well as over-representation of genes involved with distinct biological themes and pathways.

The main functional categories of differentially expressed genes were: metabolism (34 genes), signaling (28 genes), receptors and binding (18 genes), cell growth (27 genes), cell death (6 genes) and transcription regulation (9 genes). Biological themes that were over-represented involved cell communication, cell motility, signal transduction and receptor binding (z score, non random enrichment). Analysis of KEGG pathway terms identified over-representation of genes involved in PI3K pathway, in calcium signaling and in regulation of actin cytoskeleton. Analysis of chromosomal clustering of differentially expressed genes found no "hot spots", that is there was no evidence of clustering of differentially expressed genes with any particular chromosome - the genes were evenly distributed.

**KEY RESEARCH ACCOMPLISHMENTS:**

- Microarray expression analysis of schwannomas and tumorlets and identification of differentially expressed genes

- Identification of many growth factors and receptors that may function as autocrine and paracrine loops in promoting growth of tumorlets into schwannomas

- Identification of activation of PI3K pathway in schwannomas when compared to tumorlets. The synergic activation of PI3K pathway and the Rac pathway may result in schwannoma tumorigenesis, overcoming inhibitory signals that managed to keep tumorlets in check.

**REPORTABLE OUTCOMES**

Platform presentation at an international meeting: The results of this study have been presented in the Canadian Association of Neuropathologists, St. John's, Newfoundland, Canada (September 2005).


**CONCLUSIONS**

Patients with NF2 develop two types of Schwann cell lesions, Schwannomas and Schwann cell tumorlets, and the two lesions are histologically indistinguishable. Although in both schwannomas and Schwann cell tumorlets the wild type allele of the NF2 gene is lost, the two differ greatly in their biological behavior. While tumorlets stay small over the life span of the patient and have no clinical symptoms, schwannomas grow and compress adjacent structures with clinical consequences of increased morbidity and mortality.
A parallel situation was recently described in Von Hippel Lindau disease (VHL), another hereditary brain syndrome in which affected patients often develop cerebellar hemangioblastomas. In addition to cerebellar hemangioblastomas affected VHL patients were found to have many angio-mesenchymal tumorlets in the dorsal nerve roots. As in NF2, loss of heterozygosity for the VHL gene was found already in the angio-mesenchymal lesions, suggesting that, (as in NF2) inactivation of the VHL wild type allele is necessary, but not sufficient for the formation of a symptomatic tumor.  

The hypothesis that there are additional "hits" subsequent to the loss of NF2 allele in schwannomas was proposed by Woods et al but very few schwannomas were found to have genomic alterations (amplifications or deletions) with comparative genomic hybridization. This may be due to technical problems of CGH or alternatively, the underlying events may not be alterations at the DNA level but rather epigenetic events undetectable by CGH. Our findings are more consistent with the second possibility as we did not find over representation of any chromosome for differentially expressed genes (over or under expressed).

Our microarray expression analysis of Schwann cell tumorlets and schwannomas has identified 130 differentially expressed genes. Although the sample size is small and all samples analyzed were only from one patient, the differentially expressed gene set has been validated in a larger panel from multiple NF2 patients by a different method, RT-PCR.

When the genes were analyzed to find common biological themes, of the 130 differentially expressed genes there was over representation of genes involved in signaling, receptor or binding activity (z score). Some of these genes have a known growth promoting effect on Schwann cells in culture or have been found to be overexpressed in schwannomas in previous studies. Examples include potassium channel and fibroblast growth factor. Other differentially expressed genes have a known growth promoting effect in other tumor types, for example insulin growth factor, growth hormone, epiregulin.

Although the function of the NF2 encoded protein, merlin, is still unknown, interactions between merlin and p21-activated kinase 1 (Pak1) suggest that loss of merlin expression results in activation of Pak1 with resulting JNK activation. Other studies have shown activation of JNK through Rac and cdc42. Interestingly, in our study the KEGG pathway analysis of the differentially expressed genes has shown over representation of genes in the PI3K pathway. As cross talk between PI3K pathway and JNK pathway is known to occur, our hypothesis is that the synergic effect of the two pathways overcomes the inhibitory feedback mechanisms that arrest the tumorlets' growth. Thus, the loss of NF2 results in growth and tumorlet formation, but this growth is arrested by inhibitory factors and unless a second set of events occur, the tumor rests in the tumorlet stage and remains small. In some lesions, a second "hit" occurs, resulting in PI3K activation by a number of growth factors and the combined effect of NF2 loss and PI3K activation promotes growth and schwannoma formation.

The synergic effect of multiple "hits" on biological behavior was recently shown in glioblastomas. Mischel et al have shown that glioblastomas that have EGFR amplification and loss of PTEN are significantly less responsive to treatment (EGFR kinase inhibitors) then glioblastomas that have EGFR amplification but retain PTEN activity. This suggests that the combined effect of loss of PTEN (inhibitor of PI3K pathway) and amplification of EGFR (activator of PI3K pathway) on the PI3K pathway overcome the inhibitory effect of the kinase inhibitor. The findings from this study provide insight into the growth of schwannomas in NF2 patients, specifically, into the events underlying the progression from the small asymptomatic Schwann cell tumorlet to the large symptomatic schwannoma, paving the road for further studies of the PI3-kinase pathway in schwannomas. Finally, the identification of important autocrine loops in NF2-associated schwannomas may lead to the development of targeted treatment that will inhibit schwannoma growth.
REFERENCES


Appendix

Figure 1: H&E frozen section.
Tumorlet and adjacent nerve. Laser capture is essential to select only tumorlet cells.

Figure 2: Agilent Bioanalyzer results of amplified and labeled RNA.
The first 6 samples show good quality RNA and were used for GeneChip Analysis. The last three in the panel show RNA degradation and were discarded.
Figure 3: Microarray gene expression data for selected genes.

Graphical representation of expression differences in microarray data for selected genes which were validated by quantitative RT-PCR. From top: PIK3CG, KCNK12 and SFRP1.
Figure 4: Correlation of Microarray data to Taqman data for selected genes (KCNK12 and SFRP1)
Correlation = 0.93
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