VESTIBULAR SCHWANNOMA: DISSECTING THE PATHOGENIC PROCESS AND CLINICAL APPLICATIONS

Dissertation

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By

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* * * * *

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ABSTRACT

Vestibular schwannomas continue to cause significant morbidity including hearing loss, facial nerve paralysis, brainstem compression and death. Although the gene responsible for maintaining tumor suppression in vestibular schwannomas has been identified, understanding of the mechanism of action of this Neurofibromatosis type 2 (NF2) gene has not yet been sufficient to allow application to clinical treatment.

This dissertation presents three related investigations. The first is a characterization of the promoter sequence of the NF2 gene, which may be important both in mutation analysis and in tissue specific direction for modeling and treatments. The second investigation is the cDNA microarray analysis of expression of genes in vestibular schwannomas when compared to a normal nerve tissue. The third set of experiments examine one specific pathway, the retinoblastoma pathway, identified in the second investigation, and the role it plays in the development of vestibular schwannomas.

The first experiment using NF2 promoter-luciferase constructs demonstrated both positive and negative regulatory elements in the 5′ untranslated region of the
NF2 gene. The identified NF2 promoter region is now being tested for tissue specificity and mutational analysis.

The cDNA microarray investigation identified 42 which were up regulated 3-fold or more when compared to a normal adjacent vestibular nerve. Among them, genes important in angiogenesis and cell signaling were identified. Among genes that were down regulated, an apoptosis-related LUCA-15 gene was highly under expressed in schwannomas when compared to the normal nerve.

In the last set of experiments, expression profiles of eight vestibular schwannomas were chosen and genes from the retinoblastoma-CDK pathway were examined. Among them, CDK2 was substantially under expressed in 7 of the 8 tumors examined. Real-time polymerase chain reaction and immunohistochemistry supported these findings.

Taken together, the above data demonstrates that important regulatory elements exist in the 5’ regions of the NF2 gene and that in addition to the NF2 gene, other genes are deregulated within vestibular schwannomas including cell cycle regulators and other putative tumor suppressor genes. These findings provide fertile ground for further exploration aimed toward eventual improved treatment options.
DEDICATION

This dissertation is dedicated to my wife Susan,

my love and support for twenty-five years
ACKNOWLEDGMENTS

I am indebted to many people for their help and advice during the preparation of this dissertation and pursuit of this degree. I give heart felt thanks to Long-Sheng Chang, Ph.D., my mentor, adviser and friend for his tireless efforts to teach and encourage me. He has been invaluable in assisting in all aspects of this work. I also gratefully acknowledge the support and encouragement of Allan J. Yates, M.D., Ph.D., as a role model Clinician/Scientist, Dennis Pearl, Ph.D., who has overseen my statistical training program and Tom Prior, Ph.D., who first allowed me to work with his lab on the NF2 gene mutation analysis which preceded this project. David E. Schuller, M.D., as Chairman of the Department of Otolaryngology, has been a great facilitator allowing me to pursue this study while continuing a busy clinical practice. I also appreciate the assistance of the members of Dr. Chang’s laboratory where these studies have been carried out including Elena M. Akhmametyeva, Yong Wu, John Lasak and Robert Daniels. Beth Miles-Markley has provided support and review of this dissertation, which is greatly appreciated. I should also like to record my thanks to my family for their forbearance and encouragement during this course of study and research. They are terrific.
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**Clinical Management of Vestibular Schwannomas**


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## TABLE OF CONTENTS

Abstract ................................................................................................................. ii

Dedication ............................................................................................................ iv

Acknowledgments ................................................................................................. v

Vita....................................................................................................................... vi

Table of Contents ................................................................................................ xi

List of Tables ......................................................................................................xiii

List of Figures .....................................................................................................xiv

Chapters:

1. Introduction ...................................................................................................... 1
   1.1. Historical Overview .................................................................................. 1
   1.2. Recent Molecular Progress ..................................................................... 5
2. Significance of Research ............................................................................... 21
3. Clinical Studies .............................................................................................. 24
   3.1. Improvement in Diagnosis ..................................................................... 24
   3.2. Improvement in Treatment Options ...................................................... 28
      3.2.1. Preoperative studies ...................................................................... 28
      3.2.2. Intraoperative studies .................................................................... 28
      3.2.3. Postoperative studies .................................................................... 29
4. Characterization of the Human NF 2 Gene Promoter .................................... 31
   4.1. Introduction ............................................................................................ 31
   4.2. Isolation of Human NF2 Genomic DNA ............................................... 32
   4.3. NF2-Luciferase Analysis of the NF2 Promoter ...................................... 42
4.4. Discussion ................................................................. 45
4.5. Conclusions .............................................................. 47

5. cDNA Microarray Analysis of Vestibular Schwannomas ............. 49

5.1. Introduction .............................................................. 49
5.2. Vestibular Schwannoma Phenotypes ................................... 50
5.3. cDNA Microarrays ...................................................... 51
5.4. Methods ................................................................. 54
  5.4.1. Tissue procurement .............................................. 54
  5.4.2. Filter hydbridization ............................................. 54
  5.4.3. Microarray gene filter analysis ............................... 55
  5.4.4. Real time-PCR .................................................... 56
  5.4.5. Immunohistochemistry ........................................ 58
5.5. Results ......................................................................... 59
  5.5.1. Clinical .............................................................. 59
  5.5.2. Microarray .......................................................... 60
  5.5.3. Real-time PCR ..................................................... 67
  5.5.4. Immunohistochemical staining .............................. 69
  5.5.5. Mutation analysis ............................................... 74
5.6. Discussion ............................................................... 76
5.7 Conclusions ............................................................... 82

6. Retinoblastoma Protein-CDK Deregulation in Vestibular Schwannomas .... 83

6.1. Introduction .............................................................. 83
6.2. Methods ...................................................................... 86
6.3. Results ........................................................................ 89
  6.3.1. Clinical .............................................................. 89
  6.3.2. cDNA Microarray Analysis ................................... 90
  6.3.3. Real time-PCR ..................................................... 95
  6.3.4. Immunohistochemistry ........................................ 95
6.4. Discussion ............................................................... 98
6.5. Conclusions ............................................................... 101

7. Summary ........................................................................ 102

7.1. Discussion ............................................................... 102
7.2. Future Studies .......................................................... 103
  7.2.1. Analysis of promoter mutations ............................ 103
  7.2.2. Phenotypic determinants ..................................... 104
  7.2.3. Clinical outcomes study ..................................... 104

Bibliography ........................................................................ 106
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1. Human NF2 mutation detection summary</td>
<td>33</td>
</tr>
<tr>
<td>5.1. Primer sequences for real time-PCR</td>
<td>58</td>
</tr>
<tr>
<td>5.2. Clinical manifestations of vestibular schwannomas</td>
<td>61</td>
</tr>
<tr>
<td>5.3. Genes overexpressed in vestibular schwannoma</td>
<td>64</td>
</tr>
<tr>
<td>5.4. Genes underexpressed in vestibular schwannoma</td>
<td>65</td>
</tr>
<tr>
<td>5.5. ERM-associated genes in vestibular schwannomas</td>
<td>66</td>
</tr>
<tr>
<td>5.6. Comparison of cDNA microarray and real-time PCR</td>
<td>68</td>
</tr>
<tr>
<td>5.7. Mutation analysis of the ( \text{NF2} ) gene in schwannomas</td>
<td>75</td>
</tr>
<tr>
<td>6.1. Primer sequences for CDK2 real time-PCR</td>
<td>88</td>
</tr>
<tr>
<td>6.2. pRb-CDK pathway gene panel</td>
<td>92</td>
</tr>
<tr>
<td>6.3. Differential gene expression of the pRB-CDK pathway</td>
<td>93</td>
</tr>
<tr>
<td>6.4. Analysis of CDK2 expression in vestibular schwannomas by real time-PCR</td>
<td>96</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Vestibular schwannoma intraoperative view</td>
<td>18</td>
</tr>
<tr>
<td>1.2 Magnetic resonance image of small and medium vestibular schwannoma</td>
<td>19</td>
</tr>
<tr>
<td>1.3 Magnetic resonance image of large vestibular schwannoma</td>
<td>20</td>
</tr>
<tr>
<td>4.1. Southern hybridization of genomic BAC 18345</td>
<td>37</td>
</tr>
<tr>
<td>4.1a. 5' hybridization</td>
<td>37</td>
</tr>
<tr>
<td>4.1b. Mid NF2 hybridization</td>
<td>38</td>
</tr>
<tr>
<td>4.1c. 3' hybridization</td>
<td>38</td>
</tr>
<tr>
<td>4.2. Diagram of human ( \text{NF2} ) gene</td>
<td>40</td>
</tr>
<tr>
<td>4.3. Isolation of 5' Flanking DNA of human ( \text{NF2} ) gene</td>
<td>41</td>
</tr>
<tr>
<td>4.4. Promoter fusion constructs with luciferase reporter</td>
<td>44</td>
</tr>
<tr>
<td>5.1. cDNA microarray filter</td>
<td>62</td>
</tr>
<tr>
<td>5.2. Anti-osteonectin antibody stained schwannoma</td>
<td>70</td>
</tr>
<tr>
<td>5.3. Anti-rhoB antibody stained schwannomas</td>
<td>71</td>
</tr>
<tr>
<td>a. Positive</td>
<td></td>
</tr>
<tr>
<td>b. Negative</td>
<td></td>
</tr>
<tr>
<td>5.4. Anti-merlin antibody staining</td>
<td>72</td>
</tr>
<tr>
<td>a. Schwannoma</td>
<td></td>
</tr>
<tr>
<td>b. Control cranial nerve</td>
<td></td>
</tr>
<tr>
<td>5.5. Anti-neurofilament antibody staining</td>
<td>73</td>
</tr>
<tr>
<td>a. Schwannoma</td>
<td></td>
</tr>
<tr>
<td>b. Bielshowsky stain axons</td>
<td></td>
</tr>
</tbody>
</table>
5.6. Diagram of RhoB and ERM interaction .......................................................... 81
6.1. pRb-CDK pathway during G₁ to S progression ........................................ 85
6.2. Anti-CDK2 antibody staining ....................................................................... 97
CHAPTER 1

INTRODUCTION

1.1. Historical Overview

The first account of a likely vestibular schwannoma is credited to Sandifort in 1777 at the time of autopsy (Sandifort, 1777). Although other sporadic autopsy reports may have included such tumors little detail, if any, was available until a thorough description of the clinical symptoms and tumor course was given by Sir Charles Bell in 1830 (Bell, 1830). Bell described the lesion as follows:

"a tumor containing fluid of the color of urine about the size of a pigeon’s egg was discovered...on the left side, bounded by the petrous portion of the temporal bone, the pons varolii, and the left lobe of the cerebellum...and had by its pressure produced considerable indentation of the left side of the pons...the fifth cranial nerve was flattened and thin as if from pressure....but the seventh, both portio dura (facial nerve) and mollis (acoustic nerve), was completely involved and lost in the tumor from a quarter of an inch from its origin to the meatus internus; and into this foramen...could be seen to enter...the membranous portion of the tumor."

Cruveilhier is credited with recognition of hearing loss as an early symptom of vestibular schwannomas in his description of a 26 year old man who eventually died (Cruveilhier, 1842). In 1882, Fredrich von Recklinghausen described five patients with a congenital disposition for multiple peripheral neurofibromas (von Recklinghausen, 1882) and Wishart first reported bilateral acoustic neuromas
(Wishart, 1882). The latter condition came to be known as central von Recklinhausen's disease until the clear distinction in the two disease entities was later delineated.

Vestibular schwannomas were only described postmortem until advances in the areas of anesthesia and antisepsis allowed an attempt at surgical intervention. The disciplines of neurology and surgery were combined in 1891 when Starr, a New York neurologist, and McBurney, a New York surgeon, collaborated in the first known attempted surgical removal of a vestibular schwannoma. Unfortunately, they reported that

“after opening the suboccipital plate with a mallet and gouge, the cerebellum swelled massively, so much so that it became necessary to shave off the excess.”

No tumor was actually removed and the patient expired 12 days later (McBurney and Starr, 1893).

Successful removal was reported by Sir Charles Balance operating upon a patient of the neurologist Beevor in 1893 (Ballance, 1917). Ballance reported using his finger for dissection of the tumor from the cerebellopontine angle. Although the patient’s life was spared, he subsequently lost an eye due to corneal ulceration and infection from the ensuing facial nerve palsy.

Cushing, considered by most to be the father of neurosurgery, and Dandy, his pupil, initiated significant advances in the removal of vestibular schwannomas (Cushing, 1917; Dandy, 1918). Harvey Cushing of Johns Hopkins University, and later Harvard University, educated the medical profession at the time about the signs and symptoms of vestibular schwannomas and firmly established the field of neurosurgery as a specialty area within the surgical field. He reported the removal of 30 vestibular schwannomas between 1902 and 1917 with detailed descriptions of the clinical course of his patients (Cushing, 1932). Surgical extirpation was
complicated by a lack of antibiotic availability or transfusions, poorly controlled anesthesia, inadequate instrumentation and hemostasis, poor illumination and a lack of optical magnification. Furthermore, the patients being operated upon usually had large tumors accompanied by brainstem compression, hydrocephalus, and multiple cranial nerve deficits; they were in dire circumstances prior to surgical intervention. Consequently, the surgical mortality rate was approximately 80%. Cushing’s eventual reduction of the surgical mortality rate to 4% was therefore a remarkable accomplishment (Cushing, 1932). He advocated a wide bilateral suboccipital craniotomy, where both sides of the base of the cranium were opened simulataneously, and extensive debulking of the tumor. Lack of knowledge of the side of tumor location necessitated the bilateral approach. The onset of tinnitus in one hear followed by hearing loss in the same ear were not initially appreciated as the first early symptoms which would have helped localize the tumor. To aid in the successful removal, Cushing developed surgical clips and electrocautery for controlling bleeding, providing a clearer view of the critical structures of the brainstem in the field (Cushing, 1911; Cushing, 1928).

Dandy, a student of Cushing’s, was credited with the first unilateral suboccipital approach in 1925 (Dandy, 1925). Only the base of the cranium on the effected side was opened with the unilateral approach, signifying a significant advancement in tumor removal. Subsequently, Towne proposed x-ray films of the petrous pyramid to view the widening of the porus acousticus (Towne, 1926) and Dandy proposed pneumoencephalography (injecting air into the spinal column to provide contrast around the tumor in the area between the brainstem and the inner ear) for improved localization of intracranial tumors. Dandy advocated complete tumor removal because of the problem of recurrence and reported that he had never seen an acoustic tumor recur after total removal. The mortality rate for Dandy’s
series of surgical removal of vestibular schwannomas was 22%, which reflected his more aggressive approach requiring total tumor removal (Revilla, 1948).

The next pioneer in the management of vestibular schwannomas was William F. House. He combined the use of the operating microscope and otologic surgical techniques with neurosurgical techniques to revive the translabyrinthine approach and also proposed the middle cranial fossa approach for tumor removal (House, 1961). The translabyrinthine approach gave access to the area of the brainstem (cerebellopontine angle) without requiring any significant retraction of the brain. Although the translabyrinthine approach was first described in 1904, and performed in 1912 (Panse, 1904; Fraenkel and Hunt, 1904; Quix, 1912), several reports followed detailing the difficulty of the dissection, poor hemorrhage control and cerebrospinal fluid leaks leading to meningitis. (Marx, 1913; Von Schmiegelow, 1915; Zange, 1915). These factors, together with the harsh criticism of the prominent neurosurgeons of the day, pushed the approach into obscurity until it was resurrected in 1961 by House (House, 1964). Dandy termed the translabyrinthine approach “a wholly impractical suggestion” (Dandy, 1925). Cushing’s advice regarding the translabyrinthine approach was:

“If the otologist has ambitions to treat these lesions there is no possible route more dangerous or difficult than this one which has been proposed by Panse, Quix, and Schmiegelow... A proposal of this sort I am sure would never occur to an otologist who has general surgical training before he engaged in the particular surgery of his specialty” (Cushing, 1928).

Despite stern opposition, William F. House persevered and is considered the father of modern neurotology for his contributions to the surgical treatment of vestibular schwannomas. To date, the mortality rate for surgical resection of vestibular schwannomas has been reduced to 0.5% (Welling et al., 1999). Improved
neuroanesthesia, antibiotics, instrumentation, intraoperative monitoring and earlier diagnosis all played a vital role. Preservation of the facial nerve has become a routine goal of tumor removal, whereas previously facial nerve paralysis was accepted as the norm. Also, hearing preservation has been increasingly successful. Brackmann et al. reported that 71% of 24 consecutive patients had hearing preserved at or near preoperative levels (Brackmann, 1964).

In summary, an overview of the historical advances in the recognition and treatment of vestibular schwannomas during the past century reveals that major progress occurred when two or more disciplines joined efforts to address the problem at hand. The combined efforts of neurologists and surgeons in the 1890’s led to premortem diagnosis and the first attempts at surgical removal of vestibular schwannomas; this helped define the role of neurosurgery as a specialty. The combined otologic and neurosurgical techniques of House and Hitselberger (the neurosurgeon with whom House worked), in the 1960’s led to improved diagnostic and therapeutic outcomes leading to the birth of the subspecialty of neurotology.

By combining recent advances in molecular biology techniques, the diagnosis and management of vestibular schwannomas is again likely to be moved significantly forward, with the ultimate outcome being novel therapeutic approaches which will lead to further decline in mortality and morbidity from these tumors. A brief review of the recent advances in the molecular biology of vestibular schwannomas which have prepared the stage for this dissertation are as follows.

1.2. Recent Molecular Progress

Schwann cells

Schwann cell precursors arise from the neural crest and differentiate under the influence of neuregulins, platelet derived growth factor (PDGF), and thyroid
hormone (T3). Early in embryogenesis Schwann cell differentiation and growth is dependent upon paracrine secretion of ß-neuregulins and endothelins from nearby axons. Later, however, Schwann cells survive without the stimulation of axonal secretion by way of an autocrine feedback loop, which utilizes insulin-like growth factors, platelet derived growth factors, and neurotropin 3, together with laminin and lysophosphtidic acid (Parkinson et al., 2002). Schwann cells also stimulate axonal growth and the development of the mesenchymally derived epineurial sheaths of connective tissue (Mirsky et al., 2002). This is an important functional role in nerve regeneration.

Understanding the molecular mechanisms that lead to the eventual death of the Schwann cell may provide important clues pertaining to the formation of schwannomas. Evidence suggests that Schwann cell death may occur through the proteosome pathway, which is caspase mediated (Pasquini et al., 2002). The development of schwannomas represents dysfunction in one or more of the Schwann cell regulatory pathways, but how this is mediated and which pathways are involved are yet to be elucidated.

Vestibular schwannomas

Vestibular schwannomas are histologically benign tumors of the neural sheath that originate on the superior or inferior vestibular nerve (Figure 1.1). They occur either as bilateral tumors, the hallmark of neurofibromatosis type 2 (NF2), or as sporadic unilateral tumors (Figure 1.2). Unilateral tumors constitute 95% of vestibular schwannomas and bilateral tumors only 5%. Although histologically benign, vestibular schwannomas can be life threatening and carry a significant morbidity associated with compression and damage of adjacent cranial nerves (Figure 1.3). Bilateral schwannomas are particularly debilitating as they are
frequently associated with profound bilateral deafness. Brainstem compression is also more likely with bilateral tumors. Unilateral schwannomas represent approximately 6% of all intracranial tumors. The reported incidence of new vestibular schwannomas ranges from 10 to 25 per million per year (Nestor et al., 1988; Tos et al., 1992).

NF2 is a clinically autosomal dominant disease which may cause symptoms of tinnitus, hearing loss, imbalance, facial paresthesias, facial nerve paralysis, and diplopia. If left unattended, it may eventually result in blindness, hydrocephalus caused by blockage of spinal fluid through the brain, and death from brainstem compression. The disease is highly penetrant. Patients who inherit a mutation within the \textit{NF2} gene have a 95% chance of developing bilateral vestibular schwannomas. Other disease features of NF2 include intracranial meningiomas, ependymomas, spinal schwannomas, and presenile lens opacities (Fontaine et al., 1991; Kaiser-Kupfer et al., 1989; Kanter et al., 1980; Martuza and Eldridge, 1988).

NF2 is now recognized as a distinctly different disease from neurofibromatosis type 1 (NF1) or von Recklinghausen’s disease.

NF1 is one of the most frequent human genetic diseases with a prevalence of 2-3 cases per 10,000 population (Crowe et al., 1956). Cafe'-au-lait macules, multiple peripheral neuromas and neurofibromas, intertriginous freckling, and Lisch nodules develop in most affected NF1 patients. Other important clinical manifestations of NF1 include mental retardation, scoliosis, optic gliomas and a proclivity to develop malignant peripheral nerve sheath tumors. The NF1 gene, which harbors mutations in this disease process, has been localized to chromosome 17.

Likewise, NF2, or central neurofibromatosis, has been identified clinically in individuals who harbor mutations within the NF2 gene. The NF2 gene has been
localized to chromosome 22. NF2 is an extremely debilitating disease and leads to a decreased life expectancy in those afflicted. The prevalence of NF2 is estimated at approximately 1 in 40,000 (Evans et al., 1992). There is no known ethnic predilection. Affected individuals often show eighth-nerve dysfunction beginning in early adulthood; however, occasionally the onset will be delayed into the 5th or 6th decade or occur in early childhood (Mautner et al., 1993). The onset of symptoms or diagnosis occurs approximately 5 years earlier in cases that are maternally inherited rather than paternally inherited (Evans et al., 1992; Kanter and Eldrige, 1978). About one half of the cases have no family history of NF2 and thus represent new germ line mutations. Post-zygotic mutation can result in mosaicism where only a portion of the patients’ cells carry the mutation (Gardner, 1930; Huson, 1994).

The NF2 disorder has historically been subdivided into two groups (Evans et al., 1992). The *Wishart* type is the more severe phenotype with associated spinal tumors and typical onset in the late teens or early twenties (Wishart, 1822). The *Gardner* type has a later onset and less severe presentation with limited associated intracranial tumors (Gardner, 1930). A new and related third group of NF2 patients termed *Segmental/NF2* will be discussed hereafter.

According to current diagnostic criteria (Evans et al., 1992; Short et al., 1994; NIH Consensus Conference, 1991), an individual is considered to have NF2 if he or she has;

1) Bilateral vestibular schwannomas; or

2) A first-degree relative with bilateral vestibular schwannomas and the patient has either a) a unilateral vestibular schwannoma, or b) two of the following: meningioma, schwannoma, glioma, neurofibroma, posterior subcapsular lens opacity, or cerebral calcification; or
3) Two of the following: unilateral vestibular schwannoma, multiple meningiomas, glioma, neurofibroma, posterior subcapsular lens opacity, or cerebral calcification.

The term “vestibular schwannoma” is used in preference to the previously used term “acoustic neuroma” because these tumors are neither neuromas, nor do they arise from the acoustic nerve. This nomenclature is in agreement with the recommendation of the National Institute of Health consensus conference panel in 1991 (NIH consensus conf, 1991).

Vestibular schwannomas vary in their origin along the vestibular nerve, but tend to arise at the transition zone between the central myelin and peripheral myelin sheath of the nerve. The actual location of the tumor origin along the two-centimeter course of the vestibular nerve from the brainstem to the vestibular end organ (semicircular canals, utricle and saccule) is important clinically. Tumors originating within the bony confines of the internal auditory canal compress the adjacent auditory nerves early in its growth, whereas tumors which originate in the more spacious cerebellopontine angle may grow to several centimeters without significant symptoms. Cytologically, no differences have been found between spontaneous and familial tumors; however, on histologic examination, approximately 40% of the familial or NF2 tumors appear to have grape-like clusters that can infiltrate the fibers of the individual surrounding facial and cochlear nerves (Sobel, 1993). The lobular pattern does not reflect a multiclonal source of tumors, as NF2 schwannomas have been found to be monoclonal (Jacoby et al. 1990). Embedded cochlear or facial nerve axons in the schwannoma are seen more commonly in NF2 patients, whereas cochlear or facial nerve axons are less commonly seen embedded in the unilateral tumors.
Identification of the **NF2** gene

Twenty-three patients from a large NF2 kindred were studied with linkage analysis and the **NF2** locus localized near the center of the long arm of chromosome 22 (Wertelecki et al., 1988). The NF2 gene was further localized by study of a translocation in the long arm of chromosome 22 in a young woman which resulted in NF2 (Arai et al., 1992). The break point in the long arm of chromosome 22 allowed more detailed localization of the gene.

Following genetic mapping, physical mapping and positional cloning studies led to identification of the NF2 gene. Two investigative teams working independently in 1993, Trofatter et al. and Rouleau et al., identified the NF2 tumor-suppressor gene whose protein product was named **merlin** (for moesin-ezrin-radixin like protein) by the former and **schwannomin** (a word derived from schwannoma, the most prevalent tumor seen in NF2) by the latter (Trofatter et al., 1993; Rouleau et al., 1993). Parenthetically, in order to recognize both teams who identified the NF2 gene, and to retain the significance of the relationship to the protein 4.1 family as well as the easily recognized schwannoma tumors, we recommended adopting the term “schwannomerlin” as the combined name of the protein product of the NF2 gene (Welling and Chang, 1999). In publishing this recommendation we have been able to get the two opposing groups to agree upon one thing – neither group liked our recommendation and both of them continue to promote their original nomenclature. For simplicity, the **NF2** gene product will be referred to as “merlin” in this dissertation.
In addition to vestibular schwannomas, mutations within the \textit{NF2} gene have been frequently identified in meningiomas and occasionally identified in other tumor types including mesotheliomas, breast and colon carcinoma, gliomas, melanomas, and pheochromocytomas (Bianchi et al., 1994, 1995; Lekanne et al., 1994; Ruttleaved et al., 1994; Rustgi et al., 1995; Sekido et al., 1995; Merel et al., 1995; Deguen et al., 1998; Lasota et al., 2001).

The identification of the \textit{NF2} gene has provided new insights into potential \textit{NF2} function

Merlin shares a high degree of homology to the erythrocite protein 4.1-related superfamily of proteins, linking the actin cytoskeleton to the plasma membrane. In particular, three proteins, ezrin, radixin, and moesin, referred to as the ERM family, share a great deal of homology with merlin (Algrain et al., 1993; Trofatter et al., 1993). The proteins belonging to this family all have a similar N-terminal globular FERM domain (F for 4.1 protein) (Chishti et al., 1998) followed by an $\alpha$-helical stretch, and finally a charged carboxyl-terminus. The overall structure of merlin is similar to that of the ERM proteins and to that of protein 4.1, the prototype for the superfamily. The key functional domains of merlin may lie within the highly conserved ERM domains and the unique C-terminus of the protein. Merlin, unlike its ezrin, moesin and radixin, has a growth suppression role, while other ERM family members seem to facilitate growth. Until recently, merlin was the only tumor suppressor known in the protein 4.1 family; recently a second tumor suppressor from the protein 4.1 family, DAL-1, was also identified. DAL-1 suppresses cell proliferation in meningiomas, but not schwannoma cells (Gutmann et al., 2001).

Membrane proteins that have been found to bind to the FERM domain of the ERM include the hyaluronan receptors CD44 and layilin, CD43, sialophorin, and
intercellular adhesion molecules-1 and -2 (ICAM-1 and ICAM-2) (Tsukita et al., 1994; Hynes; 2002, Heiska et al., 1998; Legg and Isacke, 1998; Serrador et al., 1998; Yonemura et al., 1998). The ERMs also bind to actin in the cytoskeleton via a conserved C-terminal domain and possibly via a second actin-binding site in the N-terminal half of the proteins (Turunen et al., 1994; Roy et al., 1997). However, the C-terminal domain of merlin appears to be unique and lacks the conventional actin-binding motif found in the ERM proteins. Recently, in addition to association with the actin cytoskeleton, merlin has been shown to associate with membrane domains which contain lipid rafts and caveolae which are highly enriched in signaling molecules that regulate cellular responses to proliferative and antiproliferative stimuli (Curto et al., 2002; Stickney et al., 2002).

Identification of NF2 mutations in schwannomas and other tumors

The correlation between clinical expression and specific NF2 mutations in vestibular schwannomas and other NF2 associated tumors has been attempted. A number of somatic mutations in vestibular schwannomas from sporadic unilateral tumors and NF2 tumors have been characterized (MacCollin et al., 1993; Bianchi et al., 1994; Deperez et al., 1994; Irving et al., 1994; Jacoby et al., 1994, 1996, 1997; Sainz et al., 1994, 1995, 1996; Twist et al., 1994; Bourn et al., 1995; Merel et al., 1995; Kluwe et al., 1996; Parry et al., 1996; Welling et al., 1996; Rutledge et al., 1996; Irving et al., 1997; Gutmann et al., 1998; Stokowski and Cox, 2000). In the tumors from NF2 patients that we isolated, point mutations accounted for the majority of mutations, whereas small deletions accounted for the majority of mutations in the unilateral tumors. Mutations likely to truncate the NF2 protein have been reported to cause a more severe phenotype (Irving et al., 1994; Jacoby et al., 1994; Parry et al., 1996), while missense mutations or small in-frame insertions have
been reported to associate with a mild phenotype (Bourn et al., 1995; Merel et al., 1995; Rutledge et al., 1996; Welling et al., 1996; Gutmann et al., 1998). We recently identified mutations within the coding regions of the \( NF2 \) gene in cystic schwannomas (Welling et al., 2002). Cystic schwannomas account for only 4% of all vestibular schwannomas, but have a particularly aggressive nature and respond poorly to both surgical and radiation treatments. Previously mutations within the \( NF2 \) gene had not been described in cystic tumors.

Phenotypic variability within NF2 families with the same mutation has also been reported (Mautner et al., 1996). Although some missense mutations of the \( NF2 \) gene have been associated with a milder phenotype, severe phenotypes have also been identified. Missense mutations within the \( \alpha \)-helical domain appear to be associated with a less severe phenotype than mutations within the conserved ERM domains (Guttman et al., 2001). Likewise, large deletions may give rise to mild phenotypes as well (Bruder et al., 2001). Given the heterogeneity of clinical response to various mutations, other yet unknown regulatory factors likely play an important role in the clinical manifestations in the subtypes of schwannomas.

It should also be noted that not all vestibular schwannomas examined carry an identifiable mutation in the coding region of the \( NF2 \) gene (Sanson et al., 1993; Bourn et al., 1994; Irving et al., 1994; Jacoby et al., 1994; MacCollin et al., 1994; Twist et al., 1994; Merel et al., 1995; Kluwe et al., 1996; Welling et al., 1996; Zucman-Rossi et al., 1998). Additional mechanisms for inactivation of the \( NF2 \) gene in some NF2 patients may exist. The possibility of a modifier gene has been suggested (Bruder et al., 1999). Also, the possibility of mutation or methylation in the regulatory region of the \( NF2 \) gene has been suggested as a possible mechanism of gene inactivation (Kino et al., 2001). We recently reported the complexity of the \( NF2 \) gene’s post-transcriptional regulated-alternative splicing and differential
polyadenylation and suggested that these may also be means of inactivation of the
NF2 gene (Chang et al., 2002).

Merlin is essential for embryonic development and a tumor suppressor in
Schwann cells

Heterozygous Nf2 knockout mice develop a variety of malignant metastatic
tumors with osteosarcomas at a high frequency and fibrosarcoma and hepatocellular
carcinoma at an increased, but lower frequency (McClatchey et al., 1998). Nearly all
of the tumors exhibit loss of the wild-type Nf2 allele, indicating that merlin has a
classical tumor suppressor function. However, none of the heterozygous Nf2 mice
develop tumoral or non-tumoral manifestations of human NF2. Homozygous Nf2
mutant mouse embryos fail in development at approximately day 7 of gestation and
die immediately prior to gastrulation, displaying poorly organized extra-embryonic
ectoderm (McClatchey et al., 1997). By using Cre-mediated excision of exon 2 in
Schwann cells targeted by a myelin P0 promotor, conditional homozygous Nf2
knockout mice have been produced and display characteristics of NF2 including
schwannomas, Schwann cell hyperplasia, cataract, and osseous metaplasia
(Giovannini et al., 2000). Although these results argue that loss of merlin is
sufficient for schwannoma formation in vivo, none of the lesions observed in these
mice was found in the vestibular nerve. This is in contrast to those vestibular
schwannomas commonly found in patients with NF2. In addition, meningiomas, a
frequent manifestation of the human NF2 disease, were not observed in the
conditional homozygous Nf2 knockout mice, suggesting that meningioma progenitor
cells may not be permissive to the P0 promotor. Kalamarides et al. were successful
at creating a mouse meningioma model by biallelic inactivation of the Nf2 gene in
arachnoidal cells. An adenoviral Cre-mediated excision of Nf2 exon 2, was carried
out in arachnoidal cells by direct injection. Thirty percent of mice with arachnoidal cell Cre-mediated excision of Nf2 exon 2 developed a range of meningioma subtypes histologically similar to the human tumors (Kalamarides et al., 2002). These results suggest that mice with conditional \textit{Nf2} gene inactivation in leptomeningeal cells are prone to the development of meningiomas. The \textit{Nf2} gene therefore appears to play an important role as a growth regulator for leptomeningeal cells.

**Merlin regulates actin cytoskeletal-mediated processes and cellular proliferation**

Over-expression of the \textit{Nf2} gene in mouse fibroblasts or rat schwannoma cells can limit cell growth (Lutchman and Rouleau, 1995; Sherman et al., 1997; Gutmann et al., 1998) and suppress transformation by a ras oncogene (Tikoo et al., 1994). The growth control of certain Schwann cells and meningeal cells is abrogated by the loss of \textit{NF2} function, suggesting that \textit{NF2} deficiency disrupts some aspect of intracellular signaling that leads to a signal to proliferate, albeit slowly.

ERM proteins have been shown to be involved in cellular remodeling involving the actin cytoskeleton (Bretscher et al., 2000). Like the ERMs, merlin is expressed in a variety of cell types where it localizes to areas of membrane remodeling, particularly membrane ruffles, although its precise distribution may differ from ERMs expressed in the same cells (Gonzalez-Agosti et al., 1996). Schwannoma cells from NF2 tumors have dramatic alterations in the actin cytoskeleton and display abnormalities in cell spreading (Pelton et al., 1998). These results suggest that merlin may play an important role in regulating both actin cytoskeleton-mediated processes and cell proliferation.
To date, several proteins that are likely to interact with merlin have been identified. These include the ERMs, hyalurin receptors CD44 and layilin, F-actin, microtubules, βIII-spectrin, β1-integrin, NHERF, SCHIP-1, hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) and p21 activated kinase 2 (PAK2), Rac1 and Paxillin. (Takeshima et al., 1994; Hynes, 2002; Sherman et al., 1997; Sainio et al., 1997; Huang et al., 1998; Murthy et al., 1998; Obremski et al., 1998; Scoles et al., 1998, 2000; Xu and Gutmann, 1998; Goutebroze et al., 2000; Herrlich et al., 2000; Morrison et al., 2001). Presently, how these protein-protein interactions related to the tumor suppressor activity of merlin is largely not understood. The association of merlin with CD44 and β1-integrin raises the possibility that merlin might function as a molecular switch in the signaling pathways. CD44 is a transmembrane hyaluronic acid receptor implicated in cell-cell and cell-matrix adhesion, cell motility, and metastasis (Sherman et al., 1994; Herrlich et al., 2000). Recently, Morrison and colleagues provide elegant evidence that merlin mediates contact inhibition of growth through signals from the extracellular matrix (Morrison et al., 2001). At high cell density, merlin becomes hypo-phosphorylated and inhibits cell growth in response to hyaluronate, a mucopolysaccharide that surrounds cells. Merlin’s growth-inhibitory activity depends on specific interaction with the cytoplasmic tail of CD44. At low cell density, merlin is phosphorylated, growth permissive, and exists in a complex with ezrin, moesin, and CD44. These data indicate that merlin and CD44 form a molecular switch that specifies cell growth arrest or proliferation.

Merlin is regulated by phosphorylation

Rac1, a member of the RhoGTPase family, has recently been demonstrated to be a mediator of phosphorylation of merlin thereby inactivating its growth
suppressor mechanism. Among the Rac/Cdc42 effectors, protein 21 activated kinase 2 (Pak2) has been shown to phosphorylate merlin at serine 518 and inactivate its function (Xiao et al., 2001; Shaw et al., 2001; Kissil et al., 2002). These findings are interesting because Rac1 has recently been shown to effect integrin-mediated cell cycle progression. In particular, Rac1 can regulate both the transcription and translation of a key regulatory factor of the cell cycle, cyclin D1 (Mettouchi et al., 2001).

Understanding the regulatory pathways and function of merlin are key to development of mechanistically based treatments.
Figure 1.1. Vestibular schwannomas seen intraoperatively through the posterior fossa view. The 7th and 8th nerves are seen anterior to the tumor protruding from the porous acusticus.
Figure 1.2. A) Magnetic resonance image T1 coronal with gadolinium showing a left vestibular schwannoma limited to the internal auditory canal and B) a right schwannoma tumor extending to cause slight brainstem compression.
Figure 1.3. Large vestibular schwannoma in A) axial, B) sagittal and C) coronal MRI demonstrating significant brainstem compression and ventricular enlargement (arrow) from obstructive hydrocephalus.
CHAPTER 2

SIGNIFICANCE OF RESEARCH

The significance of this research lies in two major areas. First, characterization of the regulatory region in the \textit{NF2} gene allows the opportunity to identify \textit{NF2} mutations which may not reside in the coding region in a given family member. As noted earlier, even when exhaustive searches for mutations have been carried out within the \textit{NF2} gene in vestibular schwannoma tissues, at best 84\% of the time is a mutation identified (Zuchman-Rossi et al. 1998). Linkage analyses do not implicate another gene in patients where a mutation is not identified in the \textit{NF2} gene. In NF2, identification of a familial mutation allows the performance of direct DNA diagnostic testing on other family members, leading to an early, even presymptomatic or prenatal diagnosis of affected patients. Early detection of mutation in the \textit{NF2} gene makes a distinct difference in the ability to successfully treat patients with vestibular schwannomas (Glasscock et al., 1989; Welling et al., 1999). If mutation within the \textit{NF2} gene is identified before the patient is symptomatic, efficient screening with magnetic resonance imaging can detect
tumors as small as 2 millimeters, which would in turn lead to decreased morbidity and mortality. NF2 patients often suffer profound bilateral deafness (Welling et al., 1990). Early detection however may allow hearing preservation by tumor removal before pressure from the tumor destroys all cochlear nerve function. Additionally, hearing restoration by cochlear implantation is only possible in NF2 patients diagnosed early enough to allow surgical sparing of the cochlear nerve. Cochlear implantation, if possible, is likely to achieve far superior hearing results when compared to auditory brainstem implants (Hoffman et al., 1992; Grant et al., 2000). An additional benefit is the peace of mind individuals who have not inherited a mutant \textit{NF2} gene will be granted, in addition to the savings in time and expense for repeated medical evaluations and imaging studies (Koizuka et al., 1991). Complete characterization of the \textit{NF2} gene including its 5’ and 3’ regions will allow a search for mutations within these regulatory regions.

Another potential benefit to be realized by identification of the \textit{NF2} promoter is the opportunity to develop a better tissue-specific targeting for a mouse model of NF2. Thus far, no mouse model has developed schwannomas on the vestibular nerve. Giovaninni et al. demonstrated schwannoma formation and Schwann cell hyperplasia in the mouse model by utilizing a Schwann cell-specific myelin basic protein specific P0 promoter (Giovaninni et al., 1999). Also, tissue-specific gene therapy may be aided by refined promoter definition.

Secondly, because there has not been a good genotype-phenotype correlation with mutation analysis, understanding other coregulated genes should
lead to understanding the pathways through which the \textit{NF2} gene product acts as a tumor suppressor. Understanding specific mechanism of action is essential to the development of specific new treatment modalities and to a better understanding of phenotypic response. Growth of vestibular schwannomas was not detected in all tumors when followed serially with magnetic resonance imaging. If the course of tumor growth could be predicted by molecular markers, treatment options base upon specific individual tumors characteristics rather than on broad generalizations could be utilized for better clinical decision making.

Finally, what may seem to be a minor point, but is actually an important benefit to this research, is that many patients are reassured to know that they are assisting in the ongoing process to find better diagnostic and treatment modalities by allowing us to study their tumors.
CHAPTER 3

CLINICAL STUDIES

As noted in the vita above, we have been and are currently active in clinical studies of vestibular schwannomas as well as more basic molecular studies. As the clinical studies are not the focus of this dissertation, only a brief overview of the recent findings will be presented here and the interested reader is directed to the published data if further information is desired.

3.1. Improvement in Diagnosis

High-resolution magnetic resonance imaging techniques improve early diagnosis of vestibular schwannomas and enhance treatment options by identifying the nerve of tumor origin and by detecting more subtle changes in tumor growth. Schmalbrach et al., compared a new technique of MRI imaging, the 3D T2-weighted prototype Segment-Interleaved Motion Compensated Acquisition in Steady state (SIMCAST), with the currently used gold standard Gadolinium-DTPA contrast enhanced spoiled gradient echo (SPGR) technique (Schmalbrach et al.,1999). Tumor volumes and the surrounding anatomy including the cranial nerves were
evaluated. The results showed that tumors were clearly identified with both techniques for sizes ranging from 0.06 to 3.0 cc. Tumor size measurements agreed on average within 14%. The information obtained from both techniques was complementary. The SIMCAST images usually delineated the CSF spaces better, whereas the SPGR images more clearly showed the tumor-brain boundary. This new technique better allows improved surgical planning by identifying the nerve of origin of the tumor. Schwannomas originating from the inferior vestibular nerve are more likely to share blood supply with the cochlear nerve, and therefore have a poorer prognosis for hearing preservation during surgical removal than those originating from the superior vestibular nerve. These results indicate that while both methods were suitable for tumor detection and volume measurements, the new SIMCAST excelled for visualization of the surrounding anatomy and depiction of the 7th and 8th cranial nerves (Schmalbrach et al., 1999).

Utilizing the new SIMCAST technique, risks factors associated with hearing loss in the patients who were being electively followed for their vestibular schwannomas were identified (Massick et al., 2000). An increase in tumor volume of greater than 15% occurred in 66% of the study population; however, the pattern of volumetric change was found to be extremely variable. Multiple regression analysis revealed significant correlations between the changes in the tumor volume and in pure tone average and speech discrimination score, which had not been previously demonstrated. Unexpectedly, the presence of NF2 had an independent protective effect against deterioration of the pure tone average when compared to those
without NF2. This finding suggests that observations made from the unilateral vestibular schwannoma patients pertaining to hearing loss during a period of observation can not necessarily be generalized to patients with bilateral tumors. Future studies should be designed to confirm this observation.
Figure 3.1. Right vestibular schwannoma viewed on magnetic resonance imaging with A) 3D T2-weighted Segment-interleaved Motion Compensated Acquisition in Steady State and B) Gd-DTPA contrast enhanced spoiled gradient echo.
3.2. Improvement in Treatment

a. Preoperative Studies

In an attempt to decrease postoperative facial nerve swelling and thus the incidence of facial nerve paralysis, we studied the effect of preoperative administration of steroids to patients undergoing vestibular schwannoma resection (Welling, et al., 1999). Preoperative antibiotics were also studied. The significant effect of this study was to decrease the time of hospitalization by one day on average. This study demonstrated no benefit to steroids or antibiotics administered 12 hours preoperatively when compared to the delivery of intravenous steroids and antibiotics at the time of anesthetic induction.

b. Intraoperative Studies

In an attempt to quantify the value of the experience of the surgical team in relationship to patient outcomes, including facial paralysis, hearing loss, cerebrospinal fluid leak, stroke and death, we reported our experience with the first 160 patients undergoing resection of vestibular schwannomas by our surgical team (Welling, et al., 1999). When evaluated in clusters of 20 patients, we detected an improvement in facial nerve outcome when controlling for tumor size and other confounding variables. Following the first 20 patients, outcomes similar to other
highly experienced groups were demonstrated. Interestingly, hearing preservation was not correlated with experience in this series, but the rate of cerebrospinal fluid leak improved with experience of the team. We also reviewed hearing preservation techniques during the excision of vestibular schwannomas, comparing the outcomes of various surgical approaches, stereotactic radiation and observation (Welling et al., 1999). The results showed a similar rate of hearing preservation with both surgical and radiation treatment options. Unfortunately only approximately 30% of patients were hearing preservation candidates at the time of tumor discovery.

c. Postoperative Studies

One specific complication of vestibular schwannoma removal which predisposes to meningitis is cerebrospinal fluid (CSF) leak. This occurs in approximately 8% of patients undergoing vestibular schwannoma removal. Rarely patients will have an unusual anatomic connection between the petrous apex and the medial portion of the eustachian tube. This air cell tract predisposes the postoperative patient to CSF leaks which are recalcitrant to normal occlusion procedures. Grant et al., (Grant et al., 1999) described this anatomic anomaly, a diagnostic paradigm for detection, and the surgical approach for closure which has been very useful in such difficult cases. The results of this study indicate that an awareness of this potential route of CSF leak, and closure of the tract of air cells between the posterior cranial fossa and the nasopharynx is successful at terminating long-standing CSF leaks.
As mentioned earlier, if early patient diagnosis of NF2 could be made, hearing preservation could be attempted with satisfactory results in many cases (Glasscock et al., 1989). In cases when the auditory portion of the 8th cranial nerve could be spared, even though hearing could not be preserved due to compromise of the cochlear artery or to neural compression from the tumor, hearing might be restored to the patient by using a cochlear implant. Grant et al., (Grant et al., 2000) described a patient who had received excellent hearing from a cochlear implant following the removal of bilateral vestibular schwannomas. The patient received an open set speech recognition score of 76% which far surpasses scores obtained with auditory brainstem implantation. Our findings again emphasized the importance of early detection in treatment.
CHAPTER 4

CHARACTERIZATION OF THE HUMAN NEUROFIBROMATOSIS TYPE 2 GENE

PROMOTER

4.1. INTRODUCTION

Characterization of the entire genomic sequence of the \( NF2 \)gene is important for understanding the underlying mechanisms of vestibular schwannoma formation. As noted earlier, even when exhaustive searches for mutations have been carried out within the coding region of the \( NF2 \) gene in vestibular schwannoma tissues, frequently mutations were identified in only a portion of the tumors studied (Sanson et al., 1993; Bourn et al., 1994; Irving et al., 1994; Jacoby et al., 1994; MacCollin et al., 1994; Twist et al., 1994; Merel et al., 1995; Kluwe et al., 1996; Welling et al., 1996; Zucman-Rossi et al., 1998)(Table 4.1). In NF2, identification of a familial mutation allows the performance of direct DNA diagnostic testing on other family members, leading to an early, even presymptomatic diagnosis of affected patients. Early detection makes a distinct difference in the ability to successfully treat patients with vestibular schwannomas (Glasscock et al., 1989; Welling et al., 1999; Grant et
al., 2000). Complete characterization of the \textit{NF2} gene in the 5' and 3' areas will allow a search for mutations within these regulatory regions.

The objective of this study was to isolate the human \textit{NF2} gene and characterize its transcriptional regulatory elements. A bacterial artificial chromosome (BAC) human genomic DNA library and a partial genomic DNA library in bacteriophages were used to isolate the human \textit{NF2} gene. \textit{NF2} promoter-luciferase constructs were generated and promoter activities assayed.

### 4.2. Isolation of human \textit{NF2} genomic DNA

A full-length human \textit{NF2} cDNA was isolated by reverse transcription-polymerase chain reaction (RT-PCR) using a specific \textit{NF2} primer set (Table 4.2) and the Titan one-tube RT-PCR kit (Boeringer-Mannheim, Indianapolis, IN) according to the manufacturers recommendations (Innis et al., 1990). The PCR product was 2.2-kb in length and was confirmed by sequence analysis to be consistent with the previously published \textit{NF2} cDNA (Trofatter et al., 1993; Rouleau et al., 1993). The full-length \textit{NF2} cDNA was then subcloned into the pCR2.1-TOPO plasmid (Invitrogen, Carlsbad, CA).
<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>NF2 Patients</th>
<th>Non-NF2 Patients</th>
<th>% Detected</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Welling et al.</td>
<td>1996</td>
<td>32</td>
<td>29</td>
<td>54</td>
<td>HA, DS</td>
</tr>
<tr>
<td>Zucman-Rossi et al.</td>
<td>1998</td>
<td>19</td>
<td>0</td>
<td>84</td>
<td>DGGE, DS</td>
</tr>
<tr>
<td>Rutledge et al.</td>
<td>1996</td>
<td>111</td>
<td>0</td>
<td>54</td>
<td>SSCP</td>
</tr>
<tr>
<td>Mautner et al.</td>
<td>1996</td>
<td>9</td>
<td>3</td>
<td>75</td>
<td>SSCP</td>
</tr>
<tr>
<td>Merel et al.</td>
<td>1995</td>
<td>91</td>
<td>0</td>
<td>35</td>
<td>DGGE</td>
</tr>
<tr>
<td>Jacoby et al.</td>
<td>1994</td>
<td>8</td>
<td>30</td>
<td>53</td>
<td>SSCP</td>
</tr>
<tr>
<td>MacCollin et al.</td>
<td>1994</td>
<td>33</td>
<td>0</td>
<td>64</td>
<td>SSCP</td>
</tr>
</tbody>
</table>

**Table 4.1 Mutation-detection summary.** HA, heteroduplex analysis; DS, direct sequencing; DGGE, denaturing gradient gel electrophoresis; SSCP, single-strand conformation polymorphism analysis.
<table>
<thead>
<tr>
<th>Primer Pairs</th>
<th>Primer Name</th>
<th>Sequence</th>
<th>Base Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length NF2 cDNA-F</td>
<td>NF2-105B-f</td>
<td>AGCCGTCAGGACCGTCCCCCAAC</td>
<td>24</td>
</tr>
<tr>
<td>Full-length NF2 cDNA-R</td>
<td>NFBM3-r</td>
<td>GTCGTTCAAGGCAAT</td>
<td>18</td>
</tr>
<tr>
<td>5’ NF2 cDNA-F</td>
<td>NF2-241-f</td>
<td>CATGAGCTTCAGCTCTCTCAAG</td>
<td>22</td>
</tr>
<tr>
<td>5’ NF2 cDNA-R</td>
<td>NF2-659-r</td>
<td>CTGGACGGCGTAAGAAGCCAG</td>
<td>22</td>
</tr>
<tr>
<td>MidNF2 cDNA-F</td>
<td>NFBM2-f</td>
<td>GAACAGACTGACCCCAAG</td>
<td>20</td>
</tr>
<tr>
<td>MidNF2 cDNA-R</td>
<td>NFBM2-R</td>
<td>TATGTCAGGGGCAACGGGT</td>
<td>19</td>
</tr>
<tr>
<td>3’ NF2 cDNA-F</td>
<td>NFBM3-f</td>
<td>AGCGAAAGCCAGACGA</td>
<td>18</td>
</tr>
<tr>
<td>3’ NF2 cDNA-R</td>
<td>NFBM3-r</td>
<td>GTCGTTCAAGGCAAT</td>
<td>18</td>
</tr>
<tr>
<td>5’ Flanking probe-F (8257)</td>
<td>NF2P8257-f</td>
<td>GTCCGCGCCTCAACCAGTCT</td>
<td>21</td>
</tr>
<tr>
<td>5’ Flanking probe-R (8257)</td>
<td>NF2P8257-r</td>
<td>GCTGCGCCGGGTCCCGAGCGGCAGCACGTG</td>
<td>40</td>
</tr>
</tbody>
</table>

These primers were constructed using NF2 sequences published in Trofatter er al., 1993; Touleau et al., 1993, and Zucman-Rossi et al., 1998.

**Table 4.2 Primers for RT-PCR.**
To isolate the NF2 genomic DNA, a human genomic BAC library was screened using the full-length human NF2 cDNA as the probe. A positive BAC clone designated BAC18345 was isolated. Sequential hybridization with the 5', mid, or 3' portion of the NF2 cDNA showed that nearly all of the NF2 gene was contained in BAC18345 (Figure 4.1). In collaboration with Dr. Robert Daniels, an otology fellow, further restriction digestion, subcloning and sequencing revealed that the genomic DNA insert in BAC18345 contained the NF2 gene from intron 1 to the 3' end. BAC 8257 genomic clone was restriction digested with Apa I, BamH I, EcoR I, Kpn I, Not I, Sal I, or the Sph I enzyme. Digested DNA fragments were separated by pulse-field gel electrophoresis, transferred to a GeneScreen-Plus membrane (NEN, Boston, MA), and then sequentially hybridized with $^{32}$P-labeled probes containing the 5', middle, or 3' portion of the NF2 cDNA. Figure 4.1 shows hybridization with each of the probes (5', mid and 3') to various elements in the pulsed-field gel electrophoresis indicating that the BAC 18345 contained elements from each of the regions of interest. As shown in Figure 4.2, the relative location of exons in the NF2 DNA are consistent with those reported by Zucman-Rossi et al (1998).

To complete isolation of the genomic sequence of the NF2 gene, an NF2 5' flanking DNA-enriched, partial genomic DNA library was subsequently constructed and screened with probe 8257 (Figure 4.3). A positive clone containing a 2.4-kb NF2 insert was obtained by in situ colony hybridization. Subcloning, restriction digestion, and sequencing confirmed that it contained exon 1 and the 5' upstream region of the NF2 gene (Figure 4.2). With the help of Elena M. Akhmametyeva, a
postdoctoral fellow, we cloned the 5’ end of the NF2 gene including its 5’ flanking sequences by the following approaches.

Human SK-N-AS neuroblastoma cell DNA was digested with Apa I, Pst I, or a combination of Xho I and Hind III enzymes. Digested DNA fragments were separated on a 0.7% agarose gel, transferred to a GeneScreen-Plus membrane and probed with a 600-bp 5’ NF2 DNA fragment (designated probe 8257) obtained by PCR (Table 4.2). As shown in Figure 4.3, a 2.3-kb hybridizing DNA was detected when the ApaI-digested genomic DNA fragments were probed with the 5’ NF2 DNA. DNA fragments from the corresponding hybridized region were then eluted, and used to construct a partial genomic DNA library in the pCR2.1-TOPO vector. The genomic DNA library should enrich with clones containing the 5’ flanking NF2 DNA. In situ colony hybridization was used to identify clones containing the 5’ flanking region of the NF2 gene using the same 8257 probe. A clone designated pNF2P was thus isolated (Figures 4.2 and 4.3).

The transcription start site of the NF2 gene was sought with primer extension analysis. Preliminary results showed the major transcription initiation site to be near the first nucleotide of the published cDNA sequence (Welling et al., 2000).

Further analysis with RNase protection assay has been carried out to confirm the NF2 transcription initiation site (Chang et al., 2002).
Figure 4.1A.
Figure 4.1B

Figure 4.1C
Figure 4.1. Southern hybridization of genomic BAC 18345 digest with a) 5'-human NF2 cDNA, b) mid-human NF2 cDNA, and c) 3'-human NF2 cDNA radiolabeled probe. Note hybridization with all three probes to various fragments of the pulse-field gel electrophoresed genomic DNA. (The hybridization to the 3'-NF2 probe appears weaker because the same filter had been washed and rehybridized after the 5' - and mid-hybridizations. Another possible explanation may be that the 3'-probe utilized may give rise to some non-specific hybridization to repetitive elements in the genomic DNA).
Figure 4.2. Diagrammatic representation of the human NF2 gene. The uppermost line depicts the genomic human NF2 DNA with its 17 exons marked by cross hatched lines. The corresponding 100-kb of BAC 18345 is shown in the next line including all but exon 1 and a portion of intron 1 of the genomic DNA. Additional 5' DNA was isolated to obtain sequence for exon 1 and 5' flanking regions (see text). The lower cartoons represent the promoter luciferase reporter cassette constructs for evaluation of the promoter activity.
Figure 4.3. Isolation of the 5’ flanking DNA of the human NF2 gene by screening partial genomic human DNA library with *in situ* colony hybridization (see text).
4.3 NF2-Luciferase Analysis of the NF2 Promoter

A 2.4-kb 5’ flanking DNA from the NF2 gene was ligated to a luciferase expression cassette from pGL2-Basic (Promega, Madison, WI) to generate the pNF2P2.4-LUC plasmid (Figure 4.2). The 2.4-kb promoter segment was isolated by ligating the 2.3-kb 5’ flanking DNA from pNF2P with an additional 100-bp of the 3’ end of the 600-bp promoter from clone 8257 which contained some additional 5’-untranslated region. Several 5’ and 3’ deletion derivatives of pNF2P2.4-LUC were generated by PCR or restriction digestion.

The NF2-luciferase constructs (20 µg each) plus 2 µg of pSV2-βgal were transfected into three human cell lines, SK-N-AS neuroblastoma, 293 embryonic kidney, and NT2/D1 teratocarcinoma, by calcium phosphate technique. A positive control, using a known promoter from the SV40 virus, and a negative control, using the pGL2-Basic plasmid which has no promoter, were also transfected. Forty-eight hours after transfection, cells were harvested and luciferase activities were measured using the Luciferase Assay System (Promega, Madison, WI) and the β-galactosidase activity was measured using Galacto-Light chemiluminescent reporter assay (Tropix, Bedford, MA) in a Lumat LB9401 luminometer (Berthold, Germany). At least two independent experiments were performed for all transfection assays.
The luciferase activity expressed from each promoter construct was normalized to the β-galactosidase activity.

When a construct containing the 2.4-kb *NF2* promoter DNA was fused to the luciferase reporter expression cassette (pNF2P2.4-LUC) and assayed in transient transfection, it expressed a high level of luciferase activity as compared to the pGL2-control plasmid which contains the SV40 early promoter and enhancer (Figure 4.4). In transfected 293 cells, the 2.4-kb *NF2* promoter activity is 5 times that expressed by the SV40 promoter, and in SK-N-AS neuroblastoma cells, it is about 60% of the SV40 promoter activity. The control pGL2 plasmid is utilized because it contains a strong promoter for expression in various cell types, and thus it is a useful standard by which to compare the relative strength of other promoters. The pGL2-Basic plasmid, with no enhancer or promoter, showed very little activity as expected (Figure 4.4).

To examine if there is any contribution from the 5' untranslated region of the *NF2* gene to promoter expression, the pNF2P2.3-LUC construct, with a 0.1-kb deletion which removed most of the 5' untranslated region DNA, was generated. Interestingly, the slightly smaller pNF2P2.3-LUC construct, when transfected, gave rise to a much higher promoter activity (2-3 fold higher) when compared to the pNF2P2.4-LUC in all three cell lines (293, SK-N-AS and NT2/D1). (Figure 4.4) These results indicate the presence of a negative regulatory element located within the 100-bp 5’ untranslated DNA of the *NF2* gene.
**Figure 4.4** Results of promoter fusion constructs with luciferase reporter cassettes. The upper three graphs show human 5' flanking DNA promoter-luciferase reporter constructs on the left and the corresponding luciferase activity on the right. Controls of the pGL2-Basic plasmid without a promoter and the pGL2-Control with the SV40 promoter and enhancer are shown below.
To localize other upstream regulatory elements, several 5’ unidirectional deletion derivatives of pNF2P2.4-LUC were generated. Preliminary transfection analysis showed that deletion to about a 600-bp promoter DNA (pNF2P0.6-LUC) reduced the promoter activity two to three fold in all three cell lines, indicating the presence of positive regulatory element between the 2.4- and 0.6-kb upstream regions (Figure 4.4). Note that the 600-bp NF2 promoter activity was still two-fold higher than the SV40 promoter in transfected 293 cells, and was about 30% of the SV40 promoter activity in SK-N-AS neuroblastoma cells. Taken together, these results suggest the presence of both positive and negative elements regulating the NF2 promoter activity.

4.4 Discussion

Characterization of the promoter regions of the human NF2 gene aids in understanding regulating factors which are likely to play an important role in the transcription of the NF2 gene. The results of this study demonstrate strong NF2 promoter activity in the 5’ flanking region of the NF2 gene when compared to a known potent SV40 promoter. DNA from schwannomas or from patients with NF2, wherein no previous mutations have been identified, can now be examined for potential mutations within the non-coding, 5’ regulatory region. Mutation within the promoter region may be one factor which could affect production of the tumor suppressor merlin.
In this study, human SK-N-AS neuroblastoma, 293 embryonic kidney, and NT2/D1 teratocarcinoma cells were used for transient transfection analysis of the NF2 promoter-luciferase constructs. It would have been ideal to utilize Schwann cells or NF2-derived schwannoma cell lines for evaluation; however, a human schwannoma cell line is not currently available. Several, including our group, are working diligently to obtain such a human schwannoma cell line.

Although this study specifically addresses the 5' regulatory region and the role which it may play in vestibular schwannoma formation, mechanisms whereby transcription of the NF2 gene might be down-regulated or terminated include methylation of essential promoter regions, mutations within introns, loss of heterozygosity because of deletions of large portions of the NF2 gene, or mosaicism. The latter two mechanisms have been previously reported as potentially important mechanisms (Zucman-Rossi et al., 1998; Evans et al., 1998). The isolation of the NF2 promoter region in this study will allow examination of methylation as a promoter regulator as has been proposed recently in NF2 and other gene regulation (Kino et al., 2001). The isolation of BAC 18345 containing NF2 intronic DNA regions together with that reported by Zucman-Rossi et al. (1998) will also allow a means for a more thorough evaluation of the potential regulatory sites not located within the coding region.

Patients with NF2 have a high predilection for tumor formation in specific tissues of the body such as the vestibular nerves and meninges, even though the NF2 mutation is carried systemically. Tissue-specific regulators of the NF2 gene are
not yet known and need further study. Promoters are known to be important for specific tissue targeting and may be important in future gene therapy (Chen et al., 1998). Transfection with a functional \textit{NF2} gene may restore the tumor suppressor activity and stop tumor growth. Alternatively, transmission of a suicide gene which, when activated, results in cell death to tumor cells may also be of benefit.

A current clinical benefit of knowing the mutation within NF2 families is the ability to screen at risk family members prior to the onset of symptoms. Debates on the ethical application of current molecular biological techniques are common in the lay and scientific press. Knowledge of a disease carrier state while still asymptomatic, when no effective therapy is eminently available, may lead to decreased insurability and to discouragement on the part of the patients. Fortunately, in NF2, early diagnosis is of great benefit to the treatment of patients with the disease (Glasscock et al., 1989). Hearing and facial nerve preservation are closely tied to tumor size, and presymptomatic treatment is currently the best hope to avoid severe morbidity and mortality.

4.5 Conclusion

The NF2 gene is approximately 100-kb long and contains both positive and negative transcriptional regulatory elements in its 5’ flanking region. The promoter activity of a 2.3-kb NF2 promoter fusion construct is particularly strong and is greater than that of the SV40 control promoter in the three cell lines tested in this study. The
future diagnosis and treatment of NF2 and vestibular schwannomas will be grounded in the understanding of the basic molecular mechanisms of this disease.
5.1. Introduction

The mechanism by which merlin acts as a tumor suppressor is presently not understood. The control of the growth of Schwann cells is lost by the inactivation of the \textit{NF2} gene, which strongly suggests that \textit{NF2} deficiency disrupts some aspect of intracellular signaling that leads to cellular proliferation (Gusella et al., 1999; Gutman et al., 2001). A number of proteins which associate with merlin have been identified as mentioned earlier. Although important information about the function of the \textit{NF2} gene and its protein product has been reported, mechanism-based treatment is not yet available.

The objective of this study was to evaluate the gene expression profiles in vestibular schwannomas when compared to normal vestibular nerve tissues, and to identify pathways which may be altered in schwannomas. Vestibular schwannomas are known to harbor mutations in the neurofibromatosis type 2 tumor suppressor gene (\textit{NF2}), but the mechanism of \textit{NF2} action is not well understood. Identification of genes differentially expressed in normal and diseased tissues using a large-scale
cDNA microarray approach may lead to increased understanding of pathways leading to tumor formation.

5.2 Vestibular Schwannoma Phenotypes

Various types of vestibular schwannomas are encountered which have distinct clinical features, including unilateral sporadic vestibular schwannomas, bilateral or NF2-associated schwannomas, and cystic schwannomas. The reported incidence of new vestibular schwannomas ranges from 10 to 25 per million per year (Nestor et al., 1988; Tos et al., 1992). Of these, unilateral sporadic tumors comprise more than 90% of the total number of tumors and bilateral or NF2-associated vestibular schwannomas and cystic schwannomas are both distinctly less common, each occurring at approximately 4% the rate of unilateral schwannomas respectively (Charabi et al., 1994). NF2-associated and cystic tumors are more likely to be multilobulated than unilateral sporadic tumors. Cystic vestibular schwannomas are associated with either intratumoral or extratumoral cysts which develop in the loosely organized Antoni B tissues. In addition, a higher degree of nuclear atypia is seen in cystic tumors.

Magnetic resonance imaging (MRI) distinguishes clearly between the subtypes of vestibular schwannomas. Cystic regions of the tumors are signal intense on T2 weighted images without contrast enhancement. The non-cystic component of the cystic tumor enhances with gadolinium, just as is seen in unilateral and NF2-associated schwannomas. Clinically, the distinction between the subtypes
of schwannomas is marked. Cystic vestibular schwannomas are much more aggressive resulting in increased morbidity because of invasion of surrounding cranial nerves and rapid growth rates. When cystic tumors are removed surgically, the rate of preservation of facial nerve function is only 35% (Kameyama et al., 1996) compared to 66% House grade I or II with NF2-associated vestibular schwannomas and 90% House grade I or II rate in sporadic unilateral vestibular schwannomas (Charabi et al., 1994; Welling, 1998). Stereotactic radiation failure rate is also much higher in cystic tumors (Pendl et al., 1996).

Although quite distinct clinically and radiographically, the underlying molecular differences among the subtypes of vestibular schwannomas are not understood. Mutations in the NF2 gene have been identified in NF2-associated and unilateral spontaneous schwannomas. These include point mutations, deletions, and occasional missense mutations (Welling et al., 1996). To date, the status of the NF2 gene in cystic schwannomas has not been reported. Given that most mutations in the NF2 gene in both unilateral and bilateral tumors cause a complete loss of merlin protein, other yet unknown regulatory factors may play a role in the clinical differences manifested in the subtypes of schwannomas.

5.3. cDNA Microarrays

Semi-quantitative cDNA microarrays allow examination of the gene expression profile or the so-called ‘transcriptome’ of a tissue, and gives a means of exploring a broad view of the basic biology of tumors. Data from the human genome
project in physical map development, cDNA library production, and genome sequencing make these expression profiles more useful. Microarray gene expression analysis has been successfully utilized in the evaluation of a number of solid tumors including colon, renal cell, breast, prostate and ovarian carcinoma, and Ewing's sarcoma. It is also applied to understanding of pharmacologic manipulation aiding drug development (Marton et al., 1998; Bubendorf et al., 1999). In addition, cDNA microarray analysis may be applied to compare normal and diseased tissues by giving a global assessment of genes expressed in both tissues helping to identify those genes which are deregulated in the diseased tissues.

Three complementary approaches to evaluate the transcriptome of vestibular schwannomas are considered as follows. The first is to identify pathways of tumorigenesis common to clinically similar tumors. Regions of recurrent abnormality in malignant tumors may include malfunction of components of damage surveillance, DNA repair or mitotic apparatus that leads to genetic instability (Lockhart and Winzeler, 2000; Gray and Collins, 2000).

The second approach has been to study differential gene expression profiles within tumors of the same type. Substantial genomic and expression differences have been shown to exist between tumors that are similar histologically. Factors, which lead to molecular classification rather than histological classification, may also better predict the response of specific tumor types to specific therapies (Klausner, 1999). Vestibular schwannoma characteristics including growth rates, adherence to the adjacent facial and cochlear nerves, and age of onset are highly variable and
cannot be explained by the current understanding of the mutation types alone (MacCollin, 1995; Kluwe et al., 1996; Mautner et al., 1996; Parry et al., 1996; Ruttledge et al., 1996; Scoles et al., 1996; Evans et al., 1998; Gutmann et al., 1998; Kluwe et al., 1998; Welling, 1998; Ruggieri, 1999; Hung et al., 2000).

A third way to organize data from gene expression microarrays is to utilize cluster analysis. Cluster analysis uses standard statistical algorithms to arrange genes according to similarity in pattern of gene expression (Eisen et al., 1998; Ross et al., 2000;). The value of such analysis is yet to be fully elucidated.

In the current study, cDNA microarray analysis has been used to generate and compare the expression profile of more than 25,000 genes or expressed sequence tags (ESTs) in vestibular schwannomas and a paired normal vestibular nerve. To confirm the up or down regulation of several genes of interest, real-time polymerase chain reaction (PCR) has been performed to quantitatively assess mRNA levels. Real time quantitative PCR measures PCR product accumulation through a dual-labeled fluorogenic probe (Heid et al., 1996). As the polymerase chain reaction occurs, the fluorescence is measured automatically and a very accurate and reproducible quantitation of gene copies can be performed. The real-time PCR method has a large dynamic range of starting target molecule determination and is less labor-intensive than other quantitative analysis techniques.

Additionally, to evaluate the protein product of these genes, immunostaining has been carried out.
5.4. Methods

5.4.1. Tissue procurement

The human subjects protocol for tissue procurement was approved by the institutional review board and informed patient consent obtained. Seven vestibular schwannomas including three sporadic schwannomas, one NF2-associated schwannoma, and three cystic schwannomas were procured. A vestibular nerve from one of the sporadic schwannoma patients was also removed at the time of surgery. Approximately one half of the tumor and the entire vestibular nerve tissues were snap frozen in liquid nitrogen. The remaining tumor tissues were sent for histopathology and immunostaining. Total RNA was prepared from frozen tissue using the TRIzol procedure (Life Technologies). The RNA concentration was measured by optical density and the quality of RNA was checked by electrophoresis on a 1% agarose gel (Sambrook, 1989).

5.4.2. Filter hybridization

For probe labeling, tissue RNA (1 to 10µg) was reverse-transcribed using oligo dT primer in the presence of [33P]dCTP. Five cDNA microarray filters with each filter containing 5,184 different known genes or ESTs (gene filters 200, 201, 202, 203, and 204) were purchased from Research Genetics, Huntsville, AL. Prehybridization of the microarray filters was carried out with 20 ml of hybridization solution containing 20 µg of cot-1 DNA (Life Technologies) and 10µg of poly(dA) (Research Genetics) as blocking reagents in a hybridization roller tube at 42°C for 2
hours. For hybridization, the purified cDNA probe was denatured for 3 minutes in a boiling water bath, quick chilled, and added to the gene filter membranes at 42°C for 12-18 hours. The filters were then washed twice at 50°C in 2X SSC and 1% SDS for 20 minutes, and once at 55°C in 0.5X SSC and 1% SDS for 15 minutes. The hybridization signals on the filters were captured by exposure to a phosphor-imaging screen and the phosphor image was acquired using a Storm 860 Phosphoimager (Molecular Dynamics). The paired vestibular schwannoma and vestibular nerve samples were run concomitantly on the same batch of gene filters to provide a similar filter background.

5.4.3 Microarray gene filter analysis

Analysis of gene expression profiles was carried out using the Pathways™ software (Research Genetics). First, the gene expression array profiles from the paired vestibular schwannoma and vestibular nerve samples from the same patient were compared. After filter normalization, a three-fold or greater difference in the intensity of gene expression between the vestibular nerve and schwannoma tumor tissues was sought. Genes over-expressed or under-expressed were catalogued and utilized for comparison with other schwannomas. Next, a panel of genes known to be involved in cell growth and signaling pathways were selected and evaluated. Lastly, the three tumor types (NF2, sporadic, and cystic schwannomas) were evaluated for inter-tumor differences in gene expression profiles.
5.4.4 Real-time PCR

Real-time PCR technique (Heid et al., 1996) was used to confirm quantitatively the mRNA expression of selected genes of interest. The primers and probes were designed using Primer Express™ software (Applied Biosystems) and are shown in Table 5.1. The RNA samples from the normal vestibular nerve and various vestibular schwannoma samples were reverse transcribed and standard PCR experiments were performed to verify that the designed primer pair amplified the correct-sized product. Then, real-time PCR analysis of the cDNA samples was carried out using an ABI Prism® 7700 Sequence Detection System. Approximately 125 ng of each cDNA sample were added to Taqman Universal Master Mix (Applied Biosystems) containing 9 µM of each primer and 2 µM of the probe, which were labeled with fluorescent reporters Fam and Vic. Amplification of human ribosomal RNA (Applied Biosystems) was included as the endogenous control. The ABI Prism Sequence Detection System software was utilized to analyze the data. Quantitative expression data of the specific target were obtained for each cDNA sample. The comparative threshold of cycle \( (C_{T}) \) method was then used to determine any difference in target expression between the tumor and the control vestibular nerve.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>TaqMan Probe®</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteonectin</td>
<td>TTTGCCACAAAGTGCACCC</td>
<td>AGAAGGGGCCACAA</td>
<td>GTATTTGCAAGGC</td>
</tr>
<tr>
<td>or SPARC</td>
<td></td>
<td>GCTCCACCTGG</td>
<td>CCGATGTA</td>
</tr>
<tr>
<td>LUCA-15</td>
<td>GGCTGTGCTTCCTCAGTCG</td>
<td>AGAGGGGAGTTGATTTTGAT</td>
<td>TGCTCCAGAGTCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TCAGCTGT</td>
</tr>
</tbody>
</table>

Table 5.1. Primer sequences for real time-polymerase chain reaction analysis.
5.4.5 Immunohistochemistry

Selected genes of interest were examined for protein expression in the tumor and normal nerve tissues using immunohistochemical staining. Paraffin-embedded tissue sections were mounted on slides. For deparaffinization, slides were heated for 1 hour at 60°C, cooled and treated with xylene and graded ethanol solutions. For antigen retrieval, rehydrated tissue sections were treated with 3% hydrogen peroxide in methanol for 5 minutes and then placed in a citric-acid target retrieval solution (Dako) at 94°C for 30 minutes. For antibody staining, various dilutions of antibodies to Rho-B, merlin, osteonectin (Santa Cruz) and endoglin (Dako) were added to tissue sections for 1 hour at 37°C. After extensive washing, the slides were treated with biotinylated-linking antibody for 20 minutes, conjugated streptavidin for 20 minutes, and substrate chromogen for 5 minutes. A hematoxylin counterstain was then applied and light microscopy used to visualize the stained tissues. Additionally, three schwannomas were stained with a neurofilament antibody (Santa Cruz) to assess the distribution of axons within the tumors.
5.5. Results

5.5.1. Clinical

Tumors from seven patients were studied including three patients with sporadic vestibular schwannomas, one with an NF2-associated schwannoma, and three with cystic schwannomas. A range of clinical manifestations of vestibular schwannomas was represented as shown in Table 5.2. Pre and post-operative pure tone average (PTA) and speech discrimination scores are included. Pure tone averages are obtained by averaging pure tone thresholds at 500, 1000, and 2000 hz. Speech discrimination scores are determined by scoring the number of words correctly identified from a standardized list (NU-25) at the most comfortable listening level for the patient. All patients underwent translabyrinthine tumor excision. The three patients with unilateral non-cystic vestibular schwannomas (tumors a0715, b0728, t0721) had successful surgical removal and normal facial nerve function (House grade I). Conversely, the patient with NF2 (tumor s0721) suffered bilateral profound hearing loss, facial paralysis, moderate dysequilibrium, unilateral blindness from corneal ulceration, and a recurrent multi-lobulated unilateral vestibular schwannoma with brainstem compression. The three patients with cystic schwannomas (tumors f0928, y0918, mcryH) were all associated with unilateral hearing loss and worse postoperative facial nerve function (average House grade
III). The facial nerve was splayed throughout the cystic tumors making removal difficult.

**5.6.2. Microarray analysis of schwannomas**

Gene expression profiles of seven vestibular schwannomas were evaluated with cDNA microarrays analysis (Figure 5.1). Three sporadic vestibular schwannomas, one NF2-associated schwannoma, and three cystic schwannomas were compared to a paired normal vestibular nerve from one of the patients with sporadic schwannoma. Of 25,920 genes or ESTs screened, 42 genes (0.2%) were up regulated 3-fold or more in at least 5 of the 7 tumors. Because there were more genes whose expressions were down regulated than up regulated, 109 genes known to be involved in cell growth and signaling were selected for evaluation, and eight were found to be significantly down regulated. A summary of the results is shown in Tables 5.3 – 5.5.

Of the genes that were significantly up or down regulated, several were noted that are important in cell growth and signaling. Osteonectin, also known as SPARC (secreted protein acidic and rich in cysteine) or BM40, was significantly up regulated in 5 of the 7 tumors studied. Osteonectin is a secreted glycoprotein that interacts with extracellular matrix proteins to decrease adhesion of cells from the matrix, thereby inducing a biological state conducive to cell migration. It is also an angiogenesis mediator (Vajkoczy et al., 1999, 2000).
<table>
<thead>
<tr>
<th>Tumor ID</th>
<th>Age</th>
<th>Sex</th>
<th>Size (cm)</th>
<th>Type</th>
<th>PTA Preop</th>
<th>PTA Postop</th>
<th>% Discrim Preop</th>
<th>% Discrim Postop</th>
<th>Facial Nerve Final</th>
<th>Surg Appr</th>
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</thead>
<tbody>
<tr>
<td>a0715</td>
<td>53</td>
<td>F</td>
<td>2.3 x 1.6</td>
<td>unilateral</td>
<td>54</td>
<td>110</td>
<td>64</td>
<td>0</td>
<td>1/6</td>
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</tr>
<tr>
<td>b0728</td>
<td>45</td>
<td>F</td>
<td>2.0 x 1.6</td>
<td>unilateral</td>
<td>38</td>
<td>110</td>
<td>88</td>
<td>0</td>
<td>1/6</td>
<td>TL</td>
</tr>
<tr>
<td>t0721</td>
<td>39</td>
<td>M</td>
<td>2.5 x 2.3</td>
<td>unilateral</td>
<td>42</td>
<td>110</td>
<td>28</td>
<td>0</td>
<td>1/6</td>
<td>TL</td>
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<td>10918</td>
<td>75</td>
<td>F</td>
<td>2.0 x 2.5</td>
<td>cystic</td>
<td>25</td>
<td>110</td>
<td>80</td>
<td>0</td>
<td>3/6</td>
<td>TL</td>
</tr>
<tr>
<td>y0918</td>
<td>67</td>
<td>M</td>
<td>1.5 x 1.0</td>
<td>cystic</td>
<td>110</td>
<td>110</td>
<td>0</td>
<td>0</td>
<td>1/6</td>
<td>TL</td>
</tr>
<tr>
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<td>M</td>
<td>3.0 x 3.2</td>
<td>cystic</td>
<td>CNE</td>
<td>110</td>
<td>DNT</td>
<td>0</td>
<td>4/6</td>
<td>TL</td>
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<tr>
<td>s0721</td>
<td>31</td>
<td>M</td>
<td>3.2 x 3.0</td>
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<td>90-</td>
<td>110</td>
<td>2</td>
<td>0</td>
<td>4/6</td>
<td>TL</td>
</tr>
</tbody>
</table>

Table 5.2. Clinical Manifestations of Vestibular Schwannomas. PTA = pure tone average, %discrim = speech discrimination, TL = translabyrinthine surgical approach. CNE = could not evaluate. DNT = did not test.
**Figure 5.1.** cDNA microarray comparison of gene expression from patient 0715 vestibular schwannoma and adjacent normal vestibular nerve (gene filter 200, Research Genetics, Huntsville, Al). The phosphor image shows red genes over expressed in the tumor and green genes over expressed in the vestibular nerve. Yellow images are gene which are nearly equally expressed in both tissues.
Endoglin (a transforming growth factor-β receptor) is also known to be an endothelial marker for angiogenesis in solid tumors (Fonsatti et al., 2001). Endoglin was found to be up regulated an average 3.3 fold in all of the solid tumors in this study but not in any of the cystic tumors.

RhoB GTPase, which is important in cell signaling, was up regulated in all of the tumors, with 5 of 7 greater than 3-fold. The RhoB and the ERM proteins are involved in reorganization of the actin cytoskeleton. (Kjoller and Hall, 1999; Bishop and Hall, 2000; Prokopenko et al., 2000). Perturbation of this signaling pathway may be in part the consequence of inactivation of merlin during schwannoma tumorigenesis.

The similarity between the protein 4.1 superfamily (including the ERM proteins) and merlin suggests that merlin may physically associate with both the cell membrane and the cytoskeletal structures (Gronholm et al., 1999; Gutmann et al., 1999). The comparative expression profiles for members of the protein 4.1 superfamily including merlin are shown in Table 5.5. Ezrin was significantly down regulated in 5 of the 7 tumors. Intriguingly, the only tumor, which showed significant reduced *NF2* RNA expression, was the NF2-associated schwannoma (s0721).

Furthermore, the recently described apoptotic-related gene LUCA-15, a lung cancer tumor suppressor located on 3p21.3 (Edamatsu et al., 2000; Sutherland et al., 2000, 2001), was down regulated considerably in 6 of the 7 tumors with a mean reduction of 27-fold.
<table>
<thead>
<tr>
<th>Schwannoma Type</th>
<th>spontan a0715</th>
<th>spontan b0720</th>
<th>spontan t0721</th>
<th>cystic f0918</th>
<th>cystic y0918</th>
<th>cystic m0630</th>
<th>nF2 sc0721</th>
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<tr>
<td>Vimentin</td>
<td>-10.9</td>
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<td>-7.3</td>
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<td>-2.8</td>
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<td>Human cytoplasmic beta-actin gene, complete cds</td>
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<td>-2.7</td>
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<td>-3.0</td>
<td>-4.3</td>
<td>-4.8</td>
<td>-6.0</td>
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<td>Transforming protein RhoB</td>
<td>-3.4</td>
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<td>-2.3</td>
<td>-2.7</td>
<td>-4.0</td>
<td>-3.4</td>
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<tr>
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<td>-2.4</td>
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<td>-2.4</td>
<td>-5.4</td>
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<tr>
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<td>-5.0</td>
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<td>-9.1</td>
<td>-7.1</td>
<td>-6.7</td>
</tr>
<tr>
<td>Human cytoplasmic beta-actin gene, complete cds</td>
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<td>-2.5</td>
<td>-3.9</td>
<td>-2.2</td>
<td>-4.1</td>
<td>-3.5</td>
<td>-6.1</td>
</tr>
<tr>
<td>Regulator of G-protein signalling 1</td>
<td>-5.6</td>
<td>-3.1</td>
<td>-1.9</td>
<td>-2.9</td>
<td>-16.5</td>
<td>-7.8</td>
<td>-6.6</td>
</tr>
<tr>
<td>Actin, alpha 2, smooth muscle, aorta</td>
<td>-10.0</td>
<td>-5.4</td>
<td>-8.4</td>
<td>-3.9</td>
<td>-13.9</td>
<td>-27.6</td>
<td>-11.7</td>
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<tr>
<td>SPARC/osteonectin</td>
<td>-3.3</td>
<td>-4.0</td>
<td>-3.4</td>
<td>-2.9</td>
<td>-2.2</td>
<td>-5.0</td>
<td>-4.9</td>
</tr>
<tr>
<td>Human thymosin beta-4 mRNA, complete cds</td>
<td>-3.3</td>
<td>-2.6</td>
<td>-3.1</td>
<td>-10.6</td>
<td>-22.8</td>
<td>-22.6</td>
<td>-6.7</td>
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<tr>
<td>S100 calcium-binding protein A10 (annexin II ligand)</td>
<td>-4.7</td>
<td>-2.3</td>
<td>-3.8</td>
<td>-13.1</td>
<td>-13.5</td>
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<td>-5.4</td>
<td>-15.4</td>
<td>-5.0</td>
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<td>Beta-2-microglobulin precursor</td>
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<td>-2.2</td>
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<td>-5.0</td>
<td>-16.4</td>
<td>-7.0</td>
<td>-6.7</td>
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<td>Interferon-inducible protein I-BD</td>
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<td>-3.7</td>
<td>-3.2</td>
<td>-6.0</td>
<td>-13.3</td>
<td>-5.1</td>
<td>-3.7</td>
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<tr>
<td>Fibronectin 1</td>
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<td>-2.8</td>
<td>-3.2</td>
<td>-2.1</td>
<td>-5.4</td>
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<td>Fibronectin 1</td>
<td>-3.2</td>
<td>-2.3</td>
<td>-2.5</td>
<td>-1.3</td>
<td>-4.</td>
<td>-3.3</td>
<td>-2.6</td>
</tr>
<tr>
<td>ESTs, Highly similar to PNG gene [Hsapers]</td>
<td>-3.8</td>
<td>-10.9</td>
<td>-9.9</td>
<td>-3.9</td>
<td>-14.1</td>
<td>-1.1</td>
<td>-2.2</td>
</tr>
<tr>
<td>ESTs, Weakly similar to neuronal olfactomedin-related ER localized protein [Hsapers]</td>
<td>-3.2</td>
<td>-3.0</td>
<td>-2.6</td>
<td>-3.1</td>
<td>-3.1</td>
<td>-3.0</td>
<td>-3.0</td>
</tr>
<tr>
<td>ESTs, Similar to WW-domain binding protein 1</td>
<td>-3.9</td>
<td>-2.9</td>
<td>-8.9</td>
<td>-3.9</td>
<td>-17.2</td>
<td>-2.2</td>
<td>-5.7</td>
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</table>

Table 5.3: Genes Overexpressed in Vestibular Schwannomas. Genes overexpressed in the schwannomas are shown as negative numbers.
<table>
<thead>
<tr>
<th>Schwanionma Type</th>
<th>Spontan</th>
<th>Spontan</th>
<th>Spontan</th>
<th>Cystic</th>
<th>Cystic</th>
<th>Cystic</th>
<th>Cystic</th>
<th>nf2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor ID</td>
<td>a0715</td>
<td>b072B</td>
<td>t0721</td>
<td>1091B</td>
<td>y091B</td>
<td>m0630</td>
<td>s0721</td>
<td></td>
</tr>
<tr>
<td>Insulin-like growth factor 2 receptor</td>
<td>2.5</td>
<td>3.6</td>
<td>4.1</td>
<td>2.5</td>
<td>2.4</td>
<td>-2.2</td>
<td>2.51</td>
<td></td>
</tr>
<tr>
<td>Insulin-like growth factor 2 (somatomedin A)</td>
<td>5.8</td>
<td>18.9</td>
<td>5.2</td>
<td>5.9</td>
<td>7.1</td>
<td>-1.9</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Homing sapers mRNA for NB thymosin beta, complete cds</td>
<td>2.7</td>
<td>2.7</td>
<td>6.2</td>
<td>4.7</td>
<td>3.2</td>
<td>-1.5</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>Neuroblastoma RAS viral (v-ras) oncogene homolog</td>
<td>2.4</td>
<td>4.4</td>
<td>4.1</td>
<td>3.9</td>
<td>3.0</td>
<td>-4.1</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>Thymosin beta-10</td>
<td>7.0</td>
<td>21.5</td>
<td>3.4</td>
<td>5.8</td>
<td>7.7</td>
<td>-2.1</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>Human guanine nucleotide exchange factor mss4 mRNA</td>
<td>2.3</td>
<td>1.5</td>
<td>8.1</td>
<td>3.9</td>
<td>3.6</td>
<td>-3.5</td>
<td>4.1</td>
<td></td>
</tr>
<tr>
<td>Human protein tyrosine kinase t-Ror1 (Ror1) mRNA</td>
<td>4.6</td>
<td>2.6</td>
<td>5.7</td>
<td>5.7</td>
<td>3.1</td>
<td>-4.7</td>
<td>6.1</td>
<td></td>
</tr>
<tr>
<td>Eph-related receptor tyrosine kinase ligand 5</td>
<td>4.3</td>
<td>4.6</td>
<td>7.1</td>
<td>8.9</td>
<td>5.3</td>
<td>-1.3</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>Integrin, alpha 6</td>
<td>2.0</td>
<td>2.9</td>
<td>3.5</td>
<td>2.7</td>
<td>3.0</td>
<td>-1.7</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>ESTs, Highly similar to insulin-degrading enzyme [Rattus norvegicus]</td>
<td>2.8</td>
<td>2.6</td>
<td>9.5</td>
<td>6.3</td>
<td>3.0</td>
<td>-2.5</td>
<td>10.3</td>
<td></td>
</tr>
<tr>
<td>Human putative tumor suppressor (LUCA15) mRNA, complete cds</td>
<td>7.9</td>
<td>62.9</td>
<td>16.3</td>
<td>21.9</td>
<td>72.4</td>
<td>-1.3</td>
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Table 5.4 Genes Underexpressed in Vestibular Schwannoma. Genes underexpressed in the schwannomas are shown as positive numbers.
<table>
<thead>
<tr>
<th>Schwannoma Type</th>
<th>spontan a0715</th>
<th>spontan b0728</th>
<th>spontan t0721</th>
<th>cystic f0918</th>
<th>cystic y0918</th>
<th>cystic m0630</th>
<th>nf2 s0721</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ezrin (villin2)</td>
<td>1.9</td>
<td>16.2</td>
<td>3.7</td>
<td>4.9</td>
<td>14.0</td>
<td>1.4</td>
<td>4.5</td>
</tr>
<tr>
<td>Radixin</td>
<td>-2.6</td>
<td>-5.0</td>
<td>-3.1</td>
<td>1.1</td>
<td>-1.5</td>
<td>-2.0</td>
<td>-2.4</td>
</tr>
<tr>
<td>Moesin</td>
<td>-2.0</td>
<td>-2.7</td>
<td>-1.7</td>
<td>-1.2</td>
<td>-2.1</td>
<td>-2.6</td>
<td>-2.1</td>
</tr>
<tr>
<td>NF2</td>
<td>1.2</td>
<td>-1.5</td>
<td>-2.8</td>
<td>-1.8</td>
<td>-2.8</td>
<td>-3.4</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Table 5.5 ERM-associated Genes in Vestibular Schwannoma. Genes overexpressed in the schwannomas are shown as negative numbers. Genes underexpressed in the schwannomas are shown as positive numbers.
5.5.3. Real-time PCR

We conducted real-time PCR to quantitatively evaluate mRNA expression on LUCA-15 which was down regulated, and osteonectin which was up regulated in our cDNA microarray analysis. Strong correlation was found in both genes.

For LUCA-15, decrease of its RNA expression was detected in the same six tumors that showed down regulation by cDNA microarray analysis (Table 5.6). For osteonectin, increase of its RNA expression was also detected in all of the tumors that showed up regulation by cDNA microarray analysis, albeit the levels of increased RNA expression were higher in quantitative real-time analysis (Compare Table 5.6 with Table 5.5).
<table>
<thead>
<tr>
<th>Pt. ID</th>
<th>Osteonectin Microarray</th>
<th>Osteonectin RT-PCR</th>
<th>LUCA-15 Microarray</th>
<th>LUCA-15 RT-PCR</th>
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</thead>
<tbody>
<tr>
<td>F918</td>
<td>2.89</td>
<td>133</td>
<td>-21.87</td>
<td>-4.8</td>
</tr>
<tr>
<td>y918</td>
<td>2.18</td>
<td>867</td>
<td>-72.46</td>
<td>-8.8</td>
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<tr>
<td>a715</td>
<td>3.34</td>
<td>1075</td>
<td>-7.92</td>
<td>-17.5</td>
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<td>4.03</td>
<td>750</td>
<td>-62.65</td>
<td>-10.7</td>
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<tr>
<td>T721</td>
<td>3.43</td>
<td>5220</td>
<td>-16.31</td>
<td>-10.7</td>
</tr>
<tr>
<td>s721</td>
<td>4.94</td>
<td>600</td>
<td>-14.12</td>
<td>-9.0</td>
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</table>

**Table 5.6 Comparison of cDNA Microarray and Real-time PCR Results.**

Numbers represent ratio increase or decrease of gene in tumor compared to vestibular nerve. Genes over-expressed in the VS tissue have a positive value. Tumor mCRY had insufficient remaining RNA for RT-PCR evaluation and thus was not included.
5.5.4 Immunohistochemical staining

Immunohistochemistry analysis of tissue sections from the tumors that showed increased osteonectin RNA expression using an anti-osteonectin antibody demonstrated strong staining in the extracellular matrix and cytoplasmic compartment of schwannoma cells compared to adjacent vestibular nerve tissue which showed minimal staining (Figure 5.2).

Immunohistochemistry analysis on RhoB was performed with tumor dependent results as shown in Figure 5.3. Note that strong RhoB staining was detected in the tumor (Figure 5.3a) correlating with increased RNA expression (Table 5.3). Little or no staining was seen in other schwannomas (Figure 5.3b).

To further compare the expression of the NF2 protein, we carried out an immunohistochemistry analysis on several schwannomas. Interestingly, even though the NF2 RNA was detected, the anti-merlin antibody gave rise to little or no staining in the tumor tissue (Figure 5.4a). As a comparison, the cranial nerve control showed merlin staining readily (Figure 5.4b).

Furthermore, the location of axons scattered within the body of the tumor was demonstrated with antineurofilament antibody staining (Figure 5.5a). In contradistinction, the adjacent uninvolved vestibular nerve demonstrates highly concentrated axons visualized with Bielshowsky axonal stain (Figure 5.5b).
Figure 5.2. Immunohistochemical staining of schwannoma with an anti-osteonectin antibody. Note the prominent uptake in the cytoplasm of the tumor (T) but the relative lack of staining in the adjacent vestibular nerve (VN) (original magnification x 100).
Figure 5.3. Immunohistochemical staining of schwannoma with an anti-RhoB antibody. A shows positive brown staining was detected in the cytoplasm of a schwannoma, in contrast to another schwannoma without anti-RhoB tumor stain seen in B. The arrow in A points to an area of Antoni A cells, and the arrow in B depicts the loosely arranged Antoni B cells (original magnification, X 200).
Figure 5.4. Immunohistochemical staining of schwannoma with an anti-merlin antibody.  a) anti-merlin antibody staining (brown) shows no staining in the vestibular schwannoma tissues but b) readily detects cytoplasmic staining in normal cranial nerve.  The arrows point out Schmidt-Lantermann clefts which correspond to nodes of Ranvier or discontinuity in the myelin sheath (original magnification, X 200).
**Figure 5.5.** Immunohistochemical staining of schwannoma with an anti-neurofilament antibody.  

**a)** Anti-neurofilament antibody staining slender, linear axons coursing through a vestibular schwannoma shown by arrows. (x200).  

**b)** Bielshowsky axonal stain detects strong axonal staining in the vestibular nerve (VN) but with light staining in the adjacent schwannoma (T) (original magnification, X 100).
5.5.5. Mutation analysis

Mutation analysis detected the mutation in the $NF2$ coding region in five schwannomas (Table 5.7). Interestingly, the NF2 tumor showed a missense mutation at nucleotide 1460, resulting in an amino acid change from methionine to isoleucine which was associated with a severely affected phenotype in all family members in this NF2 kindred.

Mutation analysis of cystic schwannomas has not been previously reported. Mutations were identified within the $NF2$ gene in cystic tumor samples y0918 and f0918; however, insufficient RNA was available for evaluation of cystic tumor mcryH.
<table>
<thead>
<tr>
<th>Tumor ID</th>
<th>Tumor Type</th>
<th>Mutation†</th>
<th>Nucleotide Position‡</th>
<th>Exon</th>
<th>Result</th>
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</thead>
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<tr>
<td>a0715</td>
<td>Sporadic</td>
<td>None</td>
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<td></td>
<td></td>
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<tr>
<td>t0721</td>
<td>Sporadic</td>
<td>CGA→TGA (Arg→Stop)</td>
<td>592</td>
<td>2</td>
<td>Stop</td>
</tr>
<tr>
<td>B0728</td>
<td>Sporadic</td>
<td>4-nt insertion (GTCA)</td>
<td>505-506</td>
<td>1</td>
<td>Frame-shift</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CGA→TGA (Arg→Stop)</td>
<td>592</td>
<td>2</td>
<td>Stop</td>
</tr>
<tr>
<td>f0918</td>
<td>Cystic</td>
<td>TTG→TAG (Leu→Stop)</td>
<td>1458</td>
<td>11</td>
<td>Stop</td>
</tr>
<tr>
<td>y0918</td>
<td>Cystic</td>
<td>TAC GAC C→TAC ACC 1-nt deletion</td>
<td>883</td>
<td>5</td>
<td>Frame-shift</td>
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<tr>
<td>mcryH</td>
<td>Cystic</td>
<td>DNT†</td>
<td>DNT</td>
<td>DNT</td>
<td>DNT</td>
</tr>
<tr>
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<td>NF2</td>
<td>ATG→ATA (Met→Ile)</td>
<td>1458</td>
<td>11</td>
<td>Missense</td>
</tr>
</tbody>
</table>

Table 5.7. Mutation Analysis of the *NF2* gene in schwannomas. †The *NF2* coding region was searched for any mutations by RT-PCR and DNA sequencing. The mutated nucleotide detected is underlined. ‡Relative to the major transcription initiation designated as +1 [61]. ¶DNT, data not tested
5.6. Discussion

Microarray analysis of vestibular schwannomas has opened many new potential avenues of investigation. A more complete understanding of why NF2 mutation leads to tumor formation is dependent upon understanding other interacting genes whose expression are deregulated during tumorigenesis. Data generated by microarray analysis requires new methodological techniques for comprehensive understanding. In this study alone, over 1.75 million raw data points have been accumulated. Early evaluation of selected deregulated genes has focused on confirmation of the cDNA microarray data with real-time polymerase chain reaction. Furthermore, evaluation of protein expression via immunohistochemistry has been useful. Future large-scale proteonomics analysis (Futterman and Lemberg, 2001; Gorin et al., 2001) will assist in these efforts as well as cluster analysis (Eisen et al., 1998) of larger numbers of tumors in each category may prove useful.

The choice of normal adjacent vestibular nerve as a control tissue for comparisons of deregulated gene expression in this study needs to be carefully considered. The normal vestibular nerve contains predominantly axons surrounded by Schwann cells, but the tumor is predominantly schwannoma cells with fewer axons scattered throughout as demonstrated in Figure 5.5. Differing proportions of expressed mRNA might be expected from different tissue proportions. Normal
cranial nerves without associated schwannomas were therefore utilized for immunohistochemical comparisons as well. Another consideration was that the vestibular nerve adjacent to the schwannoma might have some change in its expression array. Pure primary cultures of Schwann cells and perhaps schwannoma cells could also be used. These studies are currently being carried out, although it is known that marked changes in expression profiles do occur when cells are taken from fresh tissues and cultured (Lipshutz et al., 1999). So the results from using cultured cells must also be interpreted carefully. Nevertheless, the use of normal vestibular nerve as a control yielded correlated findings on RNA and protein analysis. In vivo analysis of the genes discovered will likely be useful for better understanding the role they play, if any, in schwannoma formation. Hypotheses can be formed and tested based upon these data.

As an example, RhoB may potentially associate with merlin. RhoB is a member of a family of molecular switches called G proteins that regulate many essential cellular processes, including the organization of the actin cytoskeleton, gene transcription, cell adhesion, cell-cycle progression, and differentiation cytoskeleton (Takahashi et al., 1997; Yonemura et al., 1998; Bretscher, 1999; Kjoller and Hall, 1999; Bishop and Hall, 2000; Prendergast and Oliff, 2000; Prokopenko et al., 2000; Ramirez et al., 2001). Evidence suggests that merlin exists as both an open and a closed conformation as do other members of the ERM family. We hypothesize that wild-type merlin association with RhoB directly or indirectly may allow the open conformation to interact with actin in the cytoskeleton binding it to the
cell membrane allowing regulation through cell membrane signaling molecules. (Figure 5.6) Further testing of such an hypothesis is necessary.

It is interesting to note that vestibular schwannomas expressed NF2 RNA but showed little or no $NF2$ protein staining. The fact that mutations in the NF2 coding region have been identified in the tumors suggest that the mutant $NF2$ protein is likely unstable. These results are in agreement with previous studies which showed significantly absent or reduced merlin expression in vestibular schwannomas (Gutmann et al., 1997). Thus, the $NF2$ mRNA may be present, but the protein may not be detectable.

Comparisons of RNA detection by microarray and real-time PCR confirmed up or down regulation of osteonectin and LUCA-15, respectively, as noted in Table 5.4. Differences in amplitude of expression between the methods may reflect differences in the selection of the cycle thresholds for comparison in real-time PCR which are logarithmic in nature. Also, differing internal controls were utilized for the osteonectin and LUCA-15 real-time PCR (ribosomal RNA and $\beta$-actin, respectively) which may have yielded a differing amplitude of response. Note that internal controls were consistent throughout the cDNA microarray experiments and the data was reliably reproduced (for an example, see a comparison of two different cDNAs tested for fibronectin 1 in Table 5.3). Nevertheless, the pattern of up or down regulation of the mRNA expression evaluated by both techniques was consistent. Immunohistochemical analysis also confirmed these findings.
Mutation analysis of cystic schwannomas has not been previously reported. The detection of \( NF2 \) mutation in cystic tumor samples y0918 and f0918 confirm that \( NF2 \) mutation is also responsible for this type of schwannoma with unique clinical properties. Any correlation between phenotype and \( NF2 \) mutation in these tumors remains to be further investigated. The missense mutation identified in the patient with NF2 is associated with early onset of tumors, multiple intracranial tumors and early death in his kindred. Initially we found that missense mutations associated with milder phenotypes (Pendl et al., 1996), however, other studies have also identified severe phenotypes associated with missense mutations (Kluwe and Mautner, 1996; Scoles et al., 1996). It is possible that the location and type of mutation may alter a critical function of the NF2 protein.

The marked decrease of LUCA-15 cDNA in the vestibular schwannomas was interesting, given the association of this tumor suppressor gene with lung carcinoma (Edamatsu et al., 2000). Recent evidence suggests that LUCA-15 is important in the control of apoptosis (Sutherland et al., 2000; Sutherland et al., 2001). Thus, the significant down regulation of LUCA-15 in schwannomas may be beneficial for tumor cell survival.

It is noteworthy that endoglin was up regulated in all of the solid tumors, but not in any of the cystic tumors in this study. These results suggest that there may be a difference in the mechanisms of tumor formation between these tumor types. Endogolin has been found in the proliferating endothelial cells of vessels associated with solid tumors (Fonsatti et al., 2001).
The blood vessels associated with schwannomas clinically resemble the thin-walled vessels of hereditary hemorrhagic telangiectasia associated with Osler-Weber-Rendu syndrome (Pau et al., 2001). It bears mention that angiogenesis inhibitors are currently under trial for malignant tumors such as gliomas (Vajkoczy et al., 1999; Fine et al., 2000; Laird et al., 2000; Geng et al., 2001).
Figure 5.6. A schematic diagram of a potential association between RhoB and the ERM proteins. Merlin displays a closed and open state like other of the ERMs. Membrane bound signaling molecules such as CD44 and intercellular adhesion molecules (ICAM) have been shown to bind a specific domain of the ERMs.
Secreted protein acidic and rich in cysteine (SPARC) or osteonectin, which is a cellular marker of glioma invasiveness, was elevated in 5 of 7 tumors in this study. This finding may be of particular clinical interest because osteonectin responds to the angiogenesis inhibitor SU5416, which is a selective inhibitor of the vascular endothelial growth factor signal-transduction pathway (Vajkoczy et al., 1999; Vajkoczy et al., 2000). Further investigation of angiogenesis inhibitors in the treatment of vestibular schwannomas may be warranted.

5.8. Conclusions

In summary, this study presents the expression profiles of seven vestibular schwannomas compared to that of normal vestibular nerve tissues. Novel pathways potentially affecting cellular proliferation in vestibular schwannomas demonstrate significant deregulation. Further confirmational studies are warranted and many other genes are yet to be investigated.
CHAPTER 6

RETINOBLASTOMA PROTEIN-CDK PATHWAY DEREGULATION IN VESTIBULAR SCHWANNOMAS

6.1. Introduction

The mammalian cell cycle is divided into four phases: G1, S (DNA replication), G2, and mitosis (M) (Ford and Pardee, 1999). It has been shown that G1-to-S progression is tightly regulated by the retinoblastoma protein (pRb)-cyclin dependent kinase (CDK) pathway (reviewed in Figure 1). Hypophosphorylated pRb and its family members p107 and p130 bind to the E2F transcription factor whose binding sites are present in several genes necessary for G1-to-S progression and DNA replication. Hypophosphorylated pRb/E2F complexes repress these genes through the E2F-binding sites in their promoters, thereby down regulating their expression and consequently blocking cell cycle progression at G1.
Figure 6.1. Schematic diagram of the pRb-CDK pathway during G1-to-S progression. Upon addition of growth factors, quiescent cells are stimulated to enter the cell cycle by the induction of cyclin-CDK associated kinase activities which phosphorylate pRb (indicated by the addition of “P” to the protein). Hyperphosphorylation of pRb results in the release of the E2F transcription factor from its complexes. Free E2F can then transcriptionally activate the genes necessary for cycle progression.
The progression of the cell cycle is regulated by a group of protein kinases termed cyclin-dependent kinases. The CDK-associated kinase activities are dependent on the synthesis of and association with specific regulatory subunits known as cyclins. When quiescent cells are stimulated to enter the cell cycle, expression of both cyclin D and cyclin E are induced. The cyclin D-associated CDK4/6 kinase activity reaches a maximum at mid G1 phase, while the cyclin E-associated CDK2 kinase activity reaches a maximum at late G1 to early S phase. The cyclin-CDK complexes hyperphosphorylate pRb, and release the E2F transcription factor from its complexes. The E2F transcription factor is a heterodimer, consisting of one of the six E2F members (E2F-1~6) and one of the two associated DP proteins (DP-1~2). Free E2F can then transcriptionally activate the genes necessary for cell cycle progression.

The CDK activities are also regulated by a group of CDK inhibitors (CKIs). There are two families of CKIs. The Cip/Kip family includes p21, p27, and p57, and influences cyclin-CDK complexes during G1-S transition. The INK family includes p15, p16, p18, and p19, and inhibits cyclin-CDK4/6 complexes.

Alterations in growth regulatory genes have been frequently observed in human tumors (Ford and Pardee, 1999). Several genes in the pRb-CDK pathway have been implicated in human tumorigenesis; however, alteration in these genes in vestibular schwannomas (VS) has not been examined.

The cDNA microarray technology is a tool for analyzing gene expression in a large-scale fashion (Young, 2000). The expression profiles of thousands of genes in
a tumor can be compared to the normal tissue of origin. Genes that are up or down regulated in several human carcinomas have been identified and evaluated for their relation to growth regulation and specific signaling pathways (Reismann et al., 1989; Hensel et al., 1990; Phillips et al., 1994; Shimizu et al., 1994; Paggi et al., 1995; Alon et al., 1999; Moch et al., 1999; Wang et al., 1999; Chaib et al., 2001).

Following the findings of down-regulated cyclin dependent kinase 2 (CDK2) in the vestibular schwannomas (see Chapter 5), investigation of the retinoblastoma protein-CDK pathway, in relation to its potential role in vestibular schwannoma formation was selected for study.

6.2. Methods

With the institutional review board approval of the human subject protocol, eight patients with vestibular schwannomas were included in this study. Four patients had sporadic unilateral schwannomas, three had sporadic cystic schwannomas, and one had a schwannoma associated with NF2. The tumors ranged in size from 1.5 cm to 3.0 cm. All eight patients had non-serviceable sensorineural hearing loss with poor discrimination scores on the side of the tumor, and had a translabyrinthine resection (see also Chapter 5).

Tissue procurement entailed meticulous dissection with the operating microscope during surgery to separate vestibular schwannoma from the normal vestibular nerve tissue. A paired normal vestibular nerve was also harvested from the division opposite the origin of a 1.5 cm vestibular schwannomas to eliminate any
tumor tissue contamination. Additionally, any surrounding tissue was dissected away from the nerve. The vestibular schwannoma samples were taken from the central portion of the tumor. Arachnoidal tissues surrounding the tumor were specifically avoided and removed. Histopathological examination confirmed each tumor sample was indeed a vestibular schwannoma.

Upon surgical removal, all specimens were immediately frozen in liquid nitrogen to prevent degradation of tissue RNAs. Total RNAs were isolated from the vestibular schwannoma and normal vestibular nerve using TRIzol® reagent as described in Chapter 5. Purified RNAs were reverse-transcribed into $^{33}$P-labeled cDNA and used in hybridization of the cDNA microarray gene filters as described before. The Pathways™ software (Research Genetics) was utilized to analyze the gene expression data obtained from microarray analysis.

For real time-polymerase chain reaction (PCR), the primers and probes for CDK2 were designed using the Primer Express™ software (Applied Biosystems) and shown in Table 6.1. The real time-PCR reaction was carried out as described previously (Chapter 5). Amplification of human β-actin cDNA was included as the endogenous control to normalize any difference in the amount of cDNA sample used as well as any inhibition of the reaction caused by the sample.
<table>
<thead>
<tr>
<th>CDK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Primer</td>
</tr>
<tr>
<td>Probe</td>
</tr>
<tr>
<td>Reverse Primer</td>
</tr>
</tbody>
</table>

**Table 6.1. Primers and Probes for Real-time PCR Analysis.** FAM is the reporter dye attached to the probe for Real-time PCR.
Immunohistochemical analysis was employed to examine the expression of the CDK2 protein within a surgical specimen containing both schwannoma and vestibular nerve tissues. Tissue sections were prepared as described (Chapter 5). An anti-CDK2 antibody (Santa Cruz Biotechnology) at 1:300 dilution was used in the analysis. Light microscopy was used to visualize the samples. Tissue expressing the CDK2 protein stained brown, while the hematoxylin counterstain appeared blue.

6.3. Results

6.3.1. Clinical

Eight patients with vestibular schwannomas were included in this study. There were six males and two females, and their ages ranged from 19 ~ 67 years. Four patients had spontaneous unilateral schwannomas and three had cystic tumors noted both on preoperative imaging and during surgery. One patient had NF2 and bilateral schwannomas. Additionally, the uninvolved superior vestibular nerve was harvested from the patient with a small vestibular schwannoma of the inferior vestibular nerve.
6.3.2. cDNA Microarray Analysis

The gene expression profiles from the paired normal vestibular nerve and the vestibular schwannoma tumor from the same patient were first compared. After normalization with the internal controls, genes that showed a three-fold or greater difference in the level of RNA expression between the paired normal vestibular nerve and schwannoma were considered for further evaluation. Approximately 0.5% of the 25,920 genes/ESTs surveyed displayed a three-fold or more difference in expression levels between the vestibular nerve and tumor. Several were noted to be members of the pRb-CDK pathway. A detailed search for the pRb-CDK pathway genes among the 25,920 genes or ESTs examined, identified 14 members of this pathway (Table 6.2). These include the retinoblastoma susceptibility gene (Rb-1), cyclins D1, D2, A, and E, the CKIs p18, p19, and p27, CDK2, CDK6, E2F-4, E2F-5, and DP-1. Also, the expression profile of the NF2 gene was included in the analysis.

Table 6.3 summarizes the results for the expression profiles of the 14 genes in the pRb-CDK pathway in vestibular schwannoma compared to the normal vestibular nerve. The Rb-1 gene was under-expressed in two tumors (VS5 and VS8). Cyclin D1 was over expressed in two tumors, and under expressed in one other. Cyclin D2 was under expressed in one VS. CDK6 was upregulated in VS-8. No significant change in cyclin A expression was found in all eight tumors analyzed.
Intriguingly, CDK2 was highly under expressed in seven of the eight tumors studied. The only tumor which did not show CDK2 down regulation is VS8; however, this cystic tumor displayed under expression of cyclin E and over expression of CDK6.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb-1</td>
<td>pRb protein</td>
</tr>
<tr>
<td>CDK2</td>
<td>cyclin-dependant kinase 2</td>
</tr>
<tr>
<td>CDK6</td>
<td>cyclin-dependent kinase 6</td>
</tr>
<tr>
<td>cyclin D1</td>
<td>forms CDK4/6 complex</td>
</tr>
<tr>
<td>cyclin D2</td>
<td>forms CDK4/6 complex</td>
</tr>
<tr>
<td>cyclin E</td>
<td>forms CDK2 complex</td>
</tr>
<tr>
<td>cyclin A</td>
<td>forms CDK2 complex</td>
</tr>
<tr>
<td>p18</td>
<td>CDK inhibitor (CDK4/6)</td>
</tr>
<tr>
<td>p19</td>
<td>CDK inhibitor (CDK4/6)</td>
</tr>
<tr>
<td>p27</td>
<td>CDK inhibitor (CDK2 and CDK4/6)</td>
</tr>
<tr>
<td>E2F-4</td>
<td>E2F-4 transcription factor</td>
</tr>
<tr>
<td>E2F-5</td>
<td>E2F-5 transcription factor</td>
</tr>
<tr>
<td>DP-1</td>
<td>forms a heterodimer with E2F</td>
</tr>
<tr>
<td>NF2</td>
<td>merlin</td>
</tr>
</tbody>
</table>

*Table 6.2. The pRb-CDK Pathway Gene Panel.*
<table>
<thead>
<tr>
<th>Gene</th>
<th>VS 1-C</th>
<th>VS 2-C</th>
<th>VS 3-S</th>
<th>VS 4-S</th>
<th>VS 5-S</th>
<th>VS 6-NF2</th>
<th>VS 7-S</th>
<th>VS 8-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb-1</td>
<td>-1.2</td>
<td>-1.1</td>
<td>1.1</td>
<td>-1.2</td>
<td>3.4</td>
<td>2.6</td>
<td>2.1</td>
<td>5.8</td>
</tr>
<tr>
<td>CDK6</td>
<td>1.9</td>
<td>1.5</td>
<td>-1.5</td>
<td>1.1</td>
<td>-1.6</td>
<td>-1.1</td>
<td>-2.6</td>
<td>-3.9</td>
</tr>
<tr>
<td>CDK2</td>
<td>7.1</td>
<td>25.4</td>
<td>14.4</td>
<td>31.1</td>
<td>11.4</td>
<td>15.8</td>
<td>3.5</td>
<td>1.8</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>-2.9</td>
<td>-6.5</td>
<td>-2.3</td>
<td>-2.2</td>
<td>-2.1</td>
<td>-6.1</td>
<td>8.4</td>
<td>2.8</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>3.1</td>
<td>2.2</td>
<td>-1.4</td>
<td>-1.9</td>
<td>1.8</td>
<td>1.86</td>
<td>-1.5</td>
<td>-1.5</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>1.1</td>
<td>1.2</td>
<td>1.2</td>
<td>-1.4</td>
<td>2.4</td>
<td>2.8</td>
<td>1.5</td>
<td>4.7</td>
</tr>
<tr>
<td>Cyclin A</td>
<td>1.5</td>
<td>-1.1</td>
<td>-1.0</td>
<td>-2.5</td>
<td>2.1</td>
<td>1.6</td>
<td>1.4</td>
<td>2.0</td>
</tr>
<tr>
<td>P18</td>
<td>-1.4</td>
<td>1.0</td>
<td>1.0</td>
<td>-1.9</td>
<td>2.8</td>
<td>2.2</td>
<td>1.3</td>
<td>2.3</td>
</tr>
<tr>
<td>P19</td>
<td>2.2</td>
<td>1.6</td>
<td>1.1</td>
<td>1.1</td>
<td>2.1</td>
<td>1.6</td>
<td>-1.1</td>
<td>-1.6</td>
</tr>
<tr>
<td>P27</td>
<td>1.2</td>
<td>1.2</td>
<td>2.0</td>
<td>1.8</td>
<td>3.7</td>
<td>1.8</td>
<td>4.6</td>
<td>7.8</td>
</tr>
<tr>
<td>DP-1</td>
<td>4.4</td>
<td>16.8</td>
<td>4.9</td>
<td>7.4</td>
<td>5.2</td>
<td>8.2</td>
<td>-1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>E2F-4</td>
<td>1.7</td>
<td>1.4</td>
<td>1.7</td>
<td>1.0</td>
<td>3.2</td>
<td>3.3</td>
<td>2.7</td>
<td>1.8</td>
</tr>
<tr>
<td>E2F-5</td>
<td>-1.3</td>
<td>-2.2</td>
<td>-1.4</td>
<td>-1.8</td>
<td>3.4</td>
<td>2.7</td>
<td>1.2</td>
<td>-1.1</td>
</tr>
<tr>
<td>NF2</td>
<td>-1.8</td>
<td>-2.8</td>
<td>1.2</td>
<td>-1.5</td>
<td>3.8</td>
<td>3.5</td>
<td>3.8</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Table 6.3. Differential Gene Expression of the pRb-CDK Pathway in Vestibular Schwannomas Compared to the Normal Vestibular Nerve. Significantly under- or overexpressed genes are shown in bold. A 3-fold or greater difference is considered significant. Genes under expressed in the VS tissues are shown as positive values while those over expressed have a negative value. S-sporadic tumor, C-cystic tumor, and NF2-neurofibromatosis type 2.
Note that the VS8 tumor from a 19-year old male was the largest schwannoma (about 3x3 cm in size) from the group of tumors studied.

Of the three CKIs analyzed, only p27 was found to be under expressed in three vestibular schwannoma tumors including the VS8. It should be mentioned that all these three tumors were from young patients with large tumors (VS5, VS7, and VS8) and their ages were 39, 28, and 19 years old, respectively. Each tumor had a diameter greater than 2.5 cm. These results suggest that these vestibular schwannomas may have had a faster growth rate compared to the other tumors when taking the size and patient age into consideration.

The transcription factor E2F-4 was under expressed in two tumors including the VS5 that also had Rb-1 down regulation. No significant change in E2F-5 expression was seen for all eight vestibular schwannomas studied. Interestingly, the DP-1 protein, which associates with E2F, was under under-expressed in six of the eight tumors. As a comparison, the NF2 gene was under-expressed in three tumors and the others showed no significant difference in expression.

### 6.3.3. Real-time PCR Analysis

The real-time PCR was utilized to confirm the expression data from the cDNA microarray analysis. CDK2, which was under-expressed in six of the seven vestibular schwannomas examined, was further analyzed. Table 6.4 summarizes the real-time PCR data. Consistent with the cDNA microarray results, the real-time PCR analysis also showed CDK2 under expression in the same seven tumors compared
with the normal vestibular nerve (compare Table 6.3 to Table 6.4). However, the overall reduction in CDK2 expression was greater from real-time PCR than cDNA microarray analysis. Although CDK2 expression in VS8 appeared to be similar to that in normal vestibular nerve, a slightly greater CDK2 expression was detected using real-time PCR. VS7 had insufficient RNA to perform real-time PCR.

6.3.4. Immunohistochemistry

To examine the CDK2 protein expression, immunohistochemical analysis was performed on vestibular schwannoma tissue sections using an anti-CDK2 antibody. A representative CDK2 staining pattern is shown in Figure 6.2. The anti-CDK2 immunoreactivity could be seen primarily localized in the vestibular nerve along the surface of the tumor. Schwann cells, axons, and ganglion cells had high anti-CDK2 activity. In contrast, the palisading spindle-shaped schwannoma tumor cells showed only weak CDK2 staining. Normal cranial nerve was also stained with anti-CDK2 antibody and showed staining similar to the vestibular nerve alongside the tumor.
Table 6.4. Analysis of CDK2 Expression in Vestibular Schwannomas by Real-time PCR.
Genes under expressed in the VS tissues are shown as positive values. N/A = Insufficient quantity of RNA available to perform real-time PCR analysis. C\textsubscript{T} = comparative cycle threshold.

<table>
<thead>
<tr>
<th>Patient</th>
<th>CDK2 (C\textsubscript{T})</th>
</tr>
</thead>
<tbody>
<tr>
<td>VS1</td>
<td>22.6</td>
</tr>
<tr>
<td>VS2</td>
<td>171.3</td>
</tr>
<tr>
<td>VS3</td>
<td>243.9</td>
</tr>
<tr>
<td>VS4</td>
<td>163.1</td>
</tr>
<tr>
<td>VS5</td>
<td>156.5</td>
</tr>
<tr>
<td>VS6</td>
<td>98.4</td>
</tr>
<tr>
<td>VS7</td>
<td>N/A</td>
</tr>
<tr>
<td>VS8</td>
<td>-4.3</td>
</tr>
</tbody>
</table>
Figure 6.2. Immunohistochemical staining of schwannoma with an anti-CDK2 antibody. Note the prominent uptake in the vestibular nerve (VN) along the surface of the tumor (T). The arrows point to axons and Schwann cells that showed high anti-CDK2 activity. (Original magnification X 200).
6.4. Discussion

In addition to mutations in oncogenes and tumor suppressor genes, deregulated expression of growth regulatory genes frequently occurs in human tumors. Growth regulatory gene products are responsible for cellular proliferation. Alteration of expression of these genes will result in abnormal cell growth, leading to tumor formation. Consistent with this notion, our study shows that the pRb-CDK pathway is frequently deregulated in vestibular schwannomas.

Mutations in the NF2 gene are frequently found in vestibular schwannomas. We also identified mutations in the coding region of the NF2 gene in six of the eight tumors studied (data not shown). In addition, NF2 was significantly under-expressed in three of the eight schwannomas studied, suggesting that while NF2 RNA could be detected, mutations in the NF2 gene may cause instability in the NF2 protein as reported previously (Gutmann et al., 1997). However, how the NF2 protein is linked to the growth regulatory pathway is presently not understood. The fact that expression of the pRb-CDK pathway genes is altered in all vestibular schwannomas studied suggest that this pathway is a potential downstream target.

Alterations in Rb-1 expression may result in loss of pRb tumor suppressor function, and have been reported in lung cancer (Hensel et al., 1990; Shimizu et al., 1994), osteogenic sarcomas (Reismann et al., 1989), leukemias (Paggi et al., 1995),
prostate (Phillips et al., 1994), and bladder cancer (Wolff et al., 1994). Furthermore, loss of both Rb alleles leads to retinoblastoma formation (Friend et al., 1987). Curiously, we also found two of the eight vestibular schwannomas studied showed down regulation of the Rb-1 gene.

Cyclin D1 over-expression has been reported in breast, colon, lung, liver, gastric, head and neck, and bladder cancer (Ford and Pardee, 1999). Over expression of cyclin D2 and CDK 4 has been identified in testicular germ cell tumors (Schmidt et al., 2001). Also, CDK6 up regulation has been reported in some gliomas (Costello et al., 1997), while its down regulation has been observed in the majority of testicular seminomas (Schmidt et al., 2001). Intriguingly, we observed CDK6 overexpression and cyclin E under expression in a large cystic tumor when compared to the normal vestibular nerve. Most importantly, both the CDK2 RNA and protein are under expressed in the rest of seven vestibular schwannomas analyzed. Decreased CDK2 and cyclin E expression have been previously reported in testicular germ cell tumors (Schmidt et al., 2001). In contrast, CDK2 and cyclin E expression have been reported to be high in human cutaneous melanomas and their metastases when compared to other cyclin-dependent kinases and cyclins (Georgieva et al., 2001). Furthermore, cyclin E over expression has been used to predict the presence of metastasis with prognostic value in non-small cell lung en reported in lung cancer (Muller-Tidow et al., 2001). Also, over expression of CDK2 has been observed for oral and laryngeal squamous cell carcinoma (Dong et al., 2001; Mihara et al., 2001). In addition, proliferating Schwann cells established in
culture from rat sciatic nerves showed higher CDK2 protein expression than nonproliferating cells (Tikoo et al., 2000). Taken together our results suggest that decreased expression of CDK2 in vestibular schwannomas may relate to the slow growth rate of the tumor.

The cyclin-dependent kinase inhibitor p27 has been assigned to chromosome 12p12, a region often mutated in childhood acute lymphoblastic leukemia (ALL) (Komuro et al., 1999). Consistently, we found that three vestibular schwannomas showed p27 down regulation. In contrast, over-expression of p27 in non-Hodgkin’s lymphoma has been shown to be of prognostic value (Moller, 2000). Others have also reported that high expression of p27 and inhibition of cyclin E/CDK2 may favor survival of small cell lung cancer cells by preventing apoptosis in an environment that is not favorable for cell proliferation (Masuda et al., 2001).

The Rb-related protein p130 can be phosphorylated in a CDK2 dependent fashion resulting in the activation of E2F-4 and E2F-5 transcription factors (Cheng et al., 2000). Deregulation of E2F transcription factors would likely interrupt normal cell cycle progression. DP-1 associates with E2F transcription factors. The functional significance of under-expression of E2F-4, E2F-5, and their associated protein DP-1 in some vestibular schwannomas is currently unknown.

One important consideration should be pointed out in our analysis is the use of the paired normal vestibular nerve as the control tissue. Experiments using cultured Schwann cells may yield data for direct comparison with schwannoma tissue. However, the drawback of using such a cell culture is that culture conditions
could alter gene expression patterns. Studies with colon cancer have revealed that normal colon tissue, colon carcinoma tissue, and colon carcinoma cell culture yield distinctly different expression patterns (Alon et al., 1999). Additionally, limited division as well as growth arrest has been commonly observed with cultured human vestibular schwannoma cells, and a stable human Schwann cell line has not been established. Nevertheless, a direct comparison of protein expression (CDK2) in tissue section together with quantitative real-time PCR analysis yield results consistent with cDNA microarray data when schwannoma tumors were compared with the normal vestibular nerve.

6.5. Conclusions

Approximately 0.5% of genes expressed in vestibular schwannomas showed deregulation compared to those in the normal vestibular nerve. Among deregulated genes, several members of the pRb-CDK pathway, including Rb-1, cyclin D1, D2, and E, CDK2, CDK6, p27, E2F, and DP-1 were found. In particular, CDK2 expression was down regulated in the seven of the eight vestibular schwannomas studied. Further collaboration with other centers and investigation in vitro and in vivo into the regulatory mechanisms governing CDK2 expression may lead to a better understanding of vestibular schwannoma tumorigenesis.
CHAPTER 7

SUMMARY

7.1. Discussion

Although understanding of the molecular dysfunction in the \textit{NF2} gene has increased dramatically in the past decade, there are still critical gaps in our knowledge which prevent translation from the bench to the bedside. Several more pieces of the greater puzzle have been put into place as a result of this work, including characterization of the regulatory regions of the \textit{NF2} gene, cDNA microarray analysis of vestibular schwannomas, and evaluation of the role of the retinoblastoma-CDK pathway in tumor development. From our own work alone, many more questions have been generated which need attention. The effect of mutation or methylation on the regulatory regions can now be examined. The role of the \textit{NF2} promoter in directing tissue specificity for tumor formation is being evaluated in a transgenic mouse in an attempt to obtain a vestibular schwannoma model. This would be very useful for testing new therapies suggested by our microarray data. A mouse xenograft vestibular schwannoma model was recently
tested, however, the xenograft model did not reliably show an increase in tumor size even without treatment (data not shown).

The confirmed gene expression changes on microarray analysis will need to be pursued in the context of other biochemical pathways to derive hypotheses about tumor-specific regulatory alterations. With the identification of commonly altered genes, we hope to improve our understanding of \( NF2 \) signaling, subclassification of cystic, NF2-related, and unilateral sporadic tumors, tumor formation and progression, and finally, identification of potential therapeutic targets.

### 7.2. Future studies

The preceding three molecular biology studies, as is the case with most research, raise more questions and possible routes of investigation. In order to bring science from the bench to the bedside for patients who suffer as a result of vestibular schwannomas, much more must be accomplished. I’m certain it will occur only with the collaborative effort of many laboratories working in this field and other related fields of tumor biology and treatment delivery.

The following is a brief summary of future studies in progress or under consideration to further this work.

#### 7.2.1. Analysis of Promoter Mutations

With identification of regulatory elements of the human \( NF2 \) gene, these regions can now be studied in vestibular schwannoma tissues for mutations,
particularly in patients where no mutation is identified within the \textit{NF2} coding region and after evaluating for loss of heterozygosity. Also, methylation of the promoter regions is of interest in tumor and control tissues.

\textbf{7.2.2. Phenotypic Determinants}

As mentioned earlier, vestibular schwannomas can be divided into three general categories including unilateral spontaneous vestibular schwannomas, neurofibromatosis type 2 (NF2) associated vestibular schwannomas, and cystic type schwannomas. Analysis of mutations in the \textit{NF2} gene alone has not directly correlated with phenotypic expression. Factors which affect phenotypic expression are not yet known. A long-term objective of future studies is to identify factors which potentially play a role in determining the severity of clinical manifestations of vestibular schwannomas. Specifically, if the clinical behavior of various types of schwannomas was understood biologically, the treatment options currently available could be more judiciously utilized and future treatment options, based upon tumor phenotype, devised.

\textbf{7.2.3 Clinical Outcomes Studies}

Current treatment options for vestibular schwannomas include 1) observation with serial follow-up magnetic resonance imaging (MRI), 2) microsurgical removal or 3) stereotactic radiation. Microsurgical excision has been the historically established treatment for vestibular schwannomas, however, new data suggests that a period of
observation of tumor growth may be prudent in select cases as between 18 and 70% of tumors show no growth when followed conservatively (Fucci et al., 1999 Charabi et al., 1994). Those tumors which do grow, typically grow at a slow rate of 2 millimeters per year. Avoiding surgical intervention may avoid some of the associated morbidity.

Likewise, stereotactic radiation therapy has recently gained many advocates as it may also reduce treatment morbidity. Unfortunately, all current data pertaining to all three treatments are based on retrospective case series studies which are subject to strong inherent biases. The variation in these reports is great. Critical issues of tumor growth patterns and tumor control rate, facial nerve preservation and hearing preservation have not been adequately studied in a uniform, controlled setting. Standardization of outcome criteria is necessary for comparison of treatment modalities.

In order to evaluate new mechanistic based treatment options, as they become available, accurate growth rates without treatment and solid data regarding the outcomes of currently utilized interventions (surgical removal and radiation therapy) must be known for comparison. A multicenter outcomes study is needed to evaluate the effect of currently available treatment options on clinically measurable parameters (tumor size, facial nerve function, hearing) and patients perceived quality of life outcomes following treatment or observation. If carried out, not only will baselines be established, but future interventional studies will be facilitated.


Bell C. The nervous system of the human body. Appendix of cases, pp 112-114, 1830.


Dandy WE. Ventriculography following the injection of air into the cerebral ventricles. Ann Surg, 68:5, 1918.


Murthy A, Gonzalez-Agosti C, Cordero E, Pinney D, Candia C, Soloman F, Gusella J and Ramesh V. NHE-RF, a regulatory cofactor for Na(+)-H+ exchange, is a


