

Gene discovery using a human vestibular schwannoma cDNA library constructed from a patient with neurofibromatosis type 2 (NF2)

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BACKGROUND: Despite a strong association of schwannomin/merlin gene mutations with vestibular schwannoma formation, the regulatory mechanisms and biologic pathways involved are still largely unknown. The hypothesis of this study is that the genesis and growth characteristics of neurofibromatosis type 2 (NF2)-associated vestibular schwannomas are determined by genetic alterations that vary in gene transcript expression; this transcript expression includes oncogenic gene products that may be identified by construction and sequencing of a cDNA library from NF2-associated vestibular schwannoma.

METHODS: Approximately 3 mL of fresh tumor was obtained during resection of a 4-cm vestibular schwannoma from a patient with NF2. Poly(A)⁺ mRNA was isolated, synthesized into double-stranded cDNA, and unidirectionally inserted into Uni-Zap XR (Stratagene, La Jolla, CA) bacteriophage vectors. Bacteriophage vectors containing cDNA inserts were processed into phagemids according to Uni-Zap XR protocol, and inserted vectors were sequenced and analyzed using BLAST software (National Institutes of Health, Bethesda, MD) with GenBank, EMBL, DDBJ, and PDB databases.

RESULTS: The cDNA library contained 2.4 million primary plaques. Inserts averaged 1.8 kilobases (kb)

in length, with a range of 0.8 to 3.0 kb. BLAST multi-database comparison of the sequence data obtained from 50 randomly selected clones yielded identification of 13 sequences representing known human genes and 17 sequences representing cloned sequences with unknown function. Three clones represented sequences not previously described in vestibular schwannomas but strongly implicated in oncogenesis within other tissues.

CONCLUSIONS: These data have implications for understanding the molecular mechanisms of vestibular schwannoma tumor biology. Identified genes may provide future diagnostic/prognostic markers and therapeutic targets. (*Otolaryngol Head Neck Surg* 2003;128:364-71.)

Vestibular schwannomas (VSs) develop from overproliferation of Schwann cells along the vestibular nerve, usually at the junction of peripheral and central myelin.¹ VSs are the most common tumor of the cerebellopontine angle (CPA) and consist of 6% to 8% of all intracranial tumors.¹ They have no known sex or racial predilection. Studies from both United States and Denmark have estimated yearly incidence to be 1:100,000 individuals.²

VSs often develop in a sporadic unilateral fashion; however, a bilateral VS predilection has been demonstrated in individuals with the autosomal dominant disorder neurofibromatosis type 2 (NF2). Although the hallmark of NF2 is bilateral VSs, it is also associated with the formation of other cranial/peripheral nerve schwannomas, meningiomas, gliomas, neurofibromas, posterior lens-capsule opacifications, and cerebral calcifications.³ Clinically, patients with NF2 have been found to present at a younger age and have a higher likelihood of facial nerve sacrifice on resection than patients with spontaneous, unilateral VSs of the same size.⁴ NF2 has been divided into 2 categories based on disease severity. The severe phenotype (Wishart) is associated with early onset

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and multiple tumors, whereas the less severe phenotype (Gardner) has a later onset with slower clinical progression and few tumors.

Genetic studies have linked both sporadic and NF2-associated VSs to a single gene called the *NF2* gene, located on chromosome band 22q12.⁴ In 1993, 2 groups independently identified a tumor suppressor protein that the *NF2* gene encoded. Rouleau et al named the protein “schwannomin” because of the association with VSs, whereas Trofatter et al named the protein “merlin” for its similarity to a class of 4.1 superfamily of proteins (*moesin*, *ezrin*, and *radixin*) that mediate cytoskeleton linkage to plasma membrane (reviewed in Welling⁴). More recently, the *NF2* gene has been cloned and its mutations extensively analyzed. Patients with spontaneous, unilateral VSs carry only somatic *NF2* gene mutations within the tumor.⁵ Previous genetic analyses have found *NF2* gene-inactivating germline mutations in 33% to 60% of patients with NF2.⁴ NF2 is considered an autosomal dominant trait because one *NF2* gene mutation is inherited and the other occurs because of a high baseline spontaneous mutation rate in the homologous allele. Although both spontaneous and NF2-associated VSs have been associated with *NF2* gene mutations, significant differences in frequency distribution and type of mutation have been found.⁴ Furthermore, significant differences in types of *NF2* gene mutations have been found among VSs associated with NF2. Approximately 65% of NF2-associated *NF2* mutations are nonsense and frame shift mutations, which are expected to cause truncated gene products. These mutations are often associated with severe (Wishart) NF2 phenotypes.⁴

Despite intense study of the *NF2* gene and its schwannomin/merlin protein, the regulatory mechanisms and biologic pathways involved in VS genesis and growth are still largely unknown. The study of gene expression in VSs and other tissues of interest can be performed via a multitude of techniques. These analytic techniques can better be understood by classification into one of 2-systems: closed or open.⁶ Closed-system techniques investigate gene expression based on a known, predetermined set of genes; thus, inherent limitations include the current incomplete knowledge of the human genome. Examples of closed-

systems include oligonucleotide or cDNA array hybridization techniques. Open-system gene analysis has the advantage of not limiting findings to a set of known gene products; thus, even novel gene transcripts may be depicted. One of the most basic examples of an open-system technique is creation of a cDNA bacteriophage library for random sequencing. To create a cDNA bacteriophage library, mRNA is isolated from the tissue and cDNA copies are made using the enzyme reverse transcriptase. The cDNA copies are then integrated into an engineered bacteriophage vector. These vectors containing the cDNAs are placed into bacteria where, via replication, large quantities of the tissue-specific cDNA can be generated. These libraries can be maintained in perpetuity, providing a resource for studying genes unique to the tissue of origin (Fig 1). Such cDNA libraries have frequently been used for gene discovery in the field of otology. Multiple inner ear cDNA libraries have been constructed from microdissected tissues, thereby using a region-specific approach. These libraries have been used to identify inner ear-specific genes and have the potential for discovering auditory or vestibular hair cell or primary afferent neuron transcripts. One example is the unique nicotinic acetylcholine receptor $\alpha 9$ subunit found in guinea pig outer hair cells⁷ and in the rat vestibular end organs.⁸ With an understanding of what genes are unique to the inner ear and eighth nerve, there is tremendous potential for insight into the molecular pathology underlying many auditory and vestibular disorders.⁹ After the identification of a unique gene via such an open-system approach, closed-system approaches such as in situ hybridization histochemistry can be used to anatomically localize the specific cell(s) transcribing the gene. Additional tissue specimens can also be screened to determine the prevalence of the gene expression. Ultimately, determination of the mechanisms of the expressed gene may provide a target for novel treatment modalities such as gene transfer therapy.

In the current study, mutational analysis of the germline DNA was completed in an NF2 patient to identify and describe the associated *NF2* gene mutation. The patient's surgically resected VS tissue was then used to construct a cDNA library. Sequence data is reported from 50 randomly se-

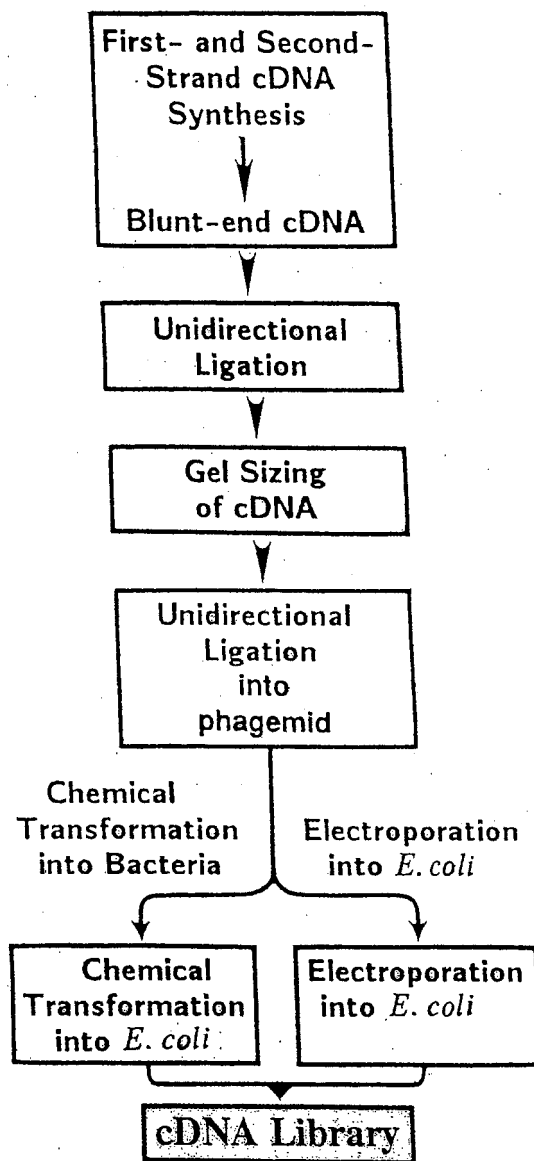


Fig 1. Algorithm for cDNA library construction. With a small amount of VS tissue, first-strand cDNA synthesis is accomplished using reverse transcription-PCR. A complementary second strand is synthesized using a single round of low-stringency PCR. Many species of DNA polymerase will have a nonspecific 3'-end nucleotide extension of PCR products, and a specific enzymatic process is used to remove these extensions to create blunt-end cDNA. The cDNA is then inserted in a specific orientation via a ligation reaction into a circular piece of DNA. This plasmid containing the inserted cDNA is then separated by size in electrophoresis gel. Selected cDNAs are then ligated into a bacteriophage (phagemid). This viral bacteriophage containing the cDNA of interest will infect and remain within specific bacteria after chemical or electrical insertion. These bacteria will continue to grow and thereby perpetuate the cDNA as a cDNA library.

lected clones from the cDNA library and the significance of the known and previously unreported genes in the biology and future treatment of VS are discussed.

MATERIALS AND METHODS

A 32-year-old woman with NF2 underwent resection of a 4-cm right-sided VS. Preoperative magnetic resonance imaging showed a VS on the contralateral side as well as 2 small intracranial meningiomas. There was no evidence of spinal tumors or lens opacifications. She was otherwise healthy. The surgical resection was accomplished via the retrosigmoid approach and performed by the senior author (P.A.W.). After frozen section confirmation of the diagnosis of VS, approximately 3 mL of fresh tumor was frozen on dry ice as it was removed. The tumor was stored at -70°C until mRNA extraction. Blood was obtained by phlebotomy for use in mutational analysis of her germline DNA. The protocol was submitted to the institutional review board (IRB) for exemption based on the Federal (Basic DHHS) Policy for the Protection of Human Subjects Title 45, Code of Federal Regulations, part 46.101 b4, which states, in brief, that a study is exempt if it involves existing documents, pathological or diagnostics specimens and the information is recorded by the investigator in such a manner that the subjects cannot be identified, directly or through identifiers linked to the subject.

Mutational Analysis

Approximately 20 mL of blood was drawn into separate Vacutainer tubes (Sherwood Medical, St Louis, MO) containing sodium citrate as an anticoagulant. Samples were immediately frozen on dry ice and stored at -20°C until use. A QIAamp Blood Mini Kit (QIAGEN Inc, Valencia, CA) was used to isolate and purify DNA from the whole blood. The standard QIAGEN DNA extraction protocol was followed.

A sample of the DNA solution was diluted 1:25 with distilled water and analyzed with a spectrophotometer (GeneQuant II DNA Calculator; Pharmacia Biotech, Piscataway, NJ). Absorbance at 260 nm was 0.118, with ratio of 1.871 and calculated concentration of 147 ng/ μL .

Primers based on the 17 exon-intron boundaries of the *NF2* gene were synthesized by Genset Oli-

gos Inc (La Jolla, CA).¹⁰ Stock primer was diluted to a concentration of 10 $\mu\text{mol/L}$ for use in polymerase chain reactions (PCRs). Each PCR contained a total volume of 50 μL , which included 2.5 mmol/L MgCl_2 , 0.5 unit *Taq* DNA polymerase, standard PCR buffer II (Perkin-Elmer, Foster City, CA), 200 $\mu\text{mol/L}$ dNTPs (deoxynucleoside triphosphates [deoxyadenosine, deoxyguanosine, deoxycytidine, deoxythymidine]), 2 μL of diluted primer, and 1 μL of extracted DNA. Initial denaturation was for 5 minutes at 94° C, after which 35 cycles were run under the following conditions: 94° C for 1 minute, 55° to 58° C for 1 minute, and 72° C for 1 minute.

After amplification, 10 μL of product was separated in a 1.6% agarose electrophoresis gel, stained with ethidium bromide, and photographed during exposure to UV light. Products showing 1 band were purified with a QIAquick PCR Purification Kit (QIAGEN Inc) and eluted in distilled water. Products with a single weak signal were reamplified under the same conditions or rerun with 2 to 4 μL of native DNA. These were then purified and prepared for sequencing.

DNA sequencing was performed in the core sequencing facility at the Utah State Biotechnology Center (Utah State University, Logan, UT) using a Model 373 Stretch Sequencing System (Perkin-Elmer Cetus, Norwalk, CT). BLAST software (National Institutes of Health, Bethesda, MD) was used for computer analysis of the sequence data.

cDNA Library Construction and Gene Discovery

Poly(A)⁺ RNA was isolated from total cellular RNA extracted from the tumor. Oligo(dT) primers were used to synthesize the cDNAs using reverse transcriptase, and these were unidirectionally inserted in Uni-Zap XR (Stratagene, La Jolla, CA) bacteriophage vectors.

One microliter of the amplified and titered cDNA library (concentration of lambda phage, 1.4×10^5 plaque-forming units [pfu]/mL) was preincubated (37° C for 15 minutes) with 200 μL of host culture (*Escherichia coli*, *XL1-Blue MRF'*) (Fig 2). NYZ (NZ amine casein enzymatic hydrolyzate) agar (3 mL, T = 48° C), 0.5 mol/L IPTG (isopropyl- β -D-thiogalactopyranoside) (15 μL),

and 50 μL of X-gal (250 mg/mL of DMF [*N,N*-dimethyl formamide]) was then added, and the mixture was plated onto NZY agar. After overnight incubation at 37° C, 50 recombinant (white) plaques were selected and transferred to sterile tubes, each containing 500 μL of SM buffer (Stratagene) and 20 μL of chloroform. For the single-clone excision procedure, 250 μL of phage stock (containing 1×10^7 phage particles) was combined with 200 μL of XL-1 Blue MRF' (OD₆₀₀ of 1.0 [optical density = 1 at λ = 600 nm]) and 1 μL of the ExAssist helper phage ($>1 \times 10^6$ pfu/ μL) (Stratagene) in Falcon 2059 tubes (Becton Dickinson Labware, Lincoln Park, NJ) and incubated at 37° C for 15 minutes. Then 3 mL of LB (Luria-Bertani) broth was added, and overnight incubation at 37° C with shaking was completed. The tubes were heated to 70° C for 20 minutes and then centrifuged at $1000 \times g$ for 15 minutes. Next, 10 μL of phage supernatant containing the excised pBluescript phagemid (Stratagene) (diluted 1:200) was added to 200 μL freshly grown SOLR cell (Stratagene); after preincubation at 37° C for 15 minutes, the mixture was plated onto LB-ampicillin agar plates (50 $\mu\text{g/mL}$). After overnight incubation at 37° C, colonies containing pBluescript double-stranded phagemid with cloned DNA inserts were streaked onto the new LB ampicillin agar plates (Fig 2). Then, 3 mL of LB medium containing 50 $\mu\text{g/mL}$ ampicillin was inoculated with individual colonies using sterile toothpicks. The culture was incubated at 37° C for 16 hours with vigorous agitation. Plasmid DNAs were isolated using QIAprep Spin Plasmid Miniprep Kit (QIAGEN) and analyzed by digestion with *EcoRI* and *XhoI* restriction enzymes. DNA sequencing was performed in the core sequencing facility at the Medical College of Wisconsin (Milwaukee, WI) using a Model 310 Gene Sequencing System (Perkin-Elmer Cetus). BLAST software (National Institutes of Health) was used for computer analysis of the sequence data.

RESULTS **Mutational Analysis**

Mutational analysis of the 17 exon-intron boundaries of the *NF2* gene revealed that there was a single base pair insertion in exon 2 causing a reading frame shift at codon 59 and resulting in

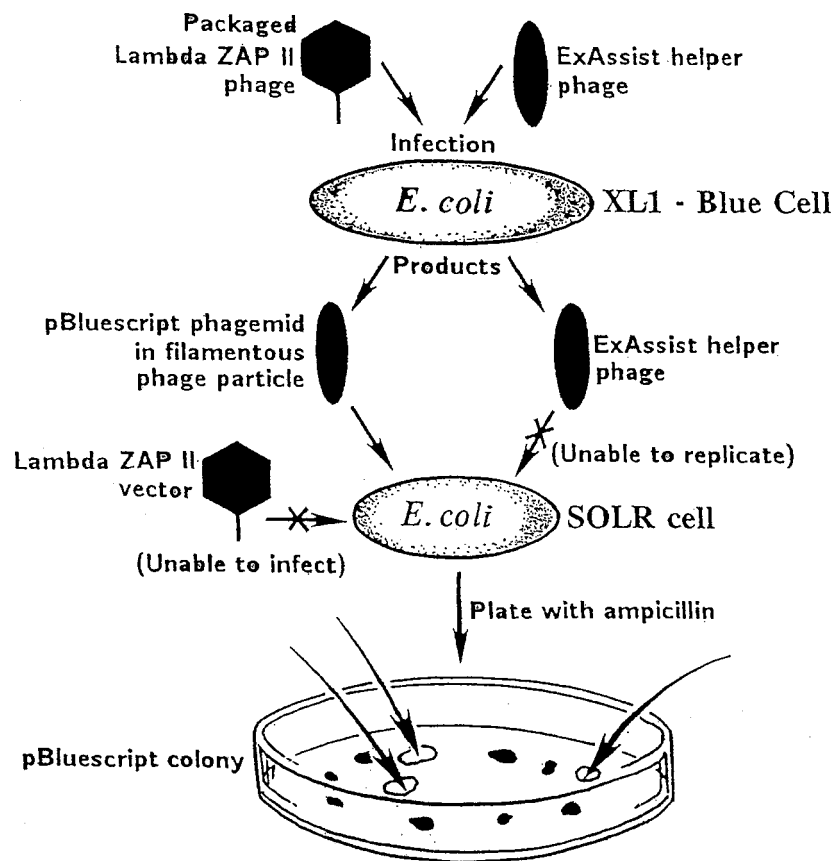


Fig 2. Summary of the excision procedure. The phage vector containing the cloned DNA insert is recognized by the helper phage (ExAssist helper phage), and a new DNA strand is synthesized within XL1-Blue cells (*E coli*), displacing the existing strand. The displaced strand is circularized and packaged as filamentous phage by the helper phage proteins and then packaged and secreted from the cell. pBluescript plasmids are recovered by infecting an *E coli F'* strain (SOLR cell) and growing in the presence of ampicillin (ampicillin-resistance gene within the pBluescript phagemid allows survival of bacteria containing the plasmids).

a stop codon at the start of exon 3. This stop codon would prematurely truncate the schwannomin protein and the resulting 84-amino acid protein would be 511 amino acids shorter than the wild type and would have 25 erroneous amino acids at the carboxyl-terminal end.

cDNA Library Construction and Gene Discovery

The cDNA library contained 2.4 million primary plaques. Inserts averaged 1.8 kb in length, and the range was 0.8 to 3.0 kb. Comparison of the sequence data obtained from the 50 randomly selected clones to all sequences in the GenBank, EMBL, DDBJ, and PDB databases yielded identification of 13 sequences representing known hu-

man genes, and 17 sequences representing cloned sequences with unknown function (Table 1). One sequence, the LIM domain of rhombotin, represents a site of frequent chromosomal translocations associated with T-cell tumorigenesis, but the presence of the translocation could not be established in this study due to limited sequence length. Two sequences represented cytoskeletal proteins: one (vimentin), which can act as a marker in VS histochemical staining, and the other (zyxin), which is abundant in many human tissues. Three sequences were consistent with gene products known to be involved in apoptosis. One sequence matched the highly conserved FERM domain found in the cytoskeleton-associated proteins of the band 4.1 superfamily including protein 4.1,

Table 1. Sequence data from 50 randomly selected clones

	No. of clones (n = 50)
House-keeping/structural function	
Ribosome	3
Mitochondrion	4
Major histocompatibility complex (MHC) antigens	6
Chaperonin	1
Vimentin	1
Zyxin	1
Translocating chain-associating membrane protein	1
FERM, RhoGEF, and pleckstrin domain protein 1	1
Other structural function	7
Apototic potential	
G protein-coupled receptor interacting protein	1
Complement cq-1 tumor necrosis factor-related	
N-myc downstream regulated (NDRG1) mRNA	1
Mcl-1 (MCL-1) and Mcl-delta S/TM	1
Sequences with unknown function	
Homo sapiens 12q BAC RP11-2H8	1
Homo sapiens KIAA0281 mRNA	1
Human DNA sequence from clone RP11-356B19	1
Homo sapiens chromosome 1 clone RP11-97014	1
Homo sapiens 12 q BAC RP11-473M14	1
Homo sapiens chromosome 17, clone HCIT524C5	1
Human DNA sequence from clone RP 11-96L14	1
Homo sapiens hypothetical protein FLJ10916	1
Homo sapiens hypothetical protein LOC55565	1
Homo sapiens 12q BAC RP11-421H10	1
Citrus sinensis mRNA for ACC synthase	2
Human DNA sequence from clone RP11-262B12	1
Homo sapiens chromosome 8, clone RP11-26K8	1
Homo sapiens chromosome 8, clone RP 11-250 P18	1
Homo sapiens, similar to protective protein for β -galactosidase	1
Homo sapiens BAC clone RP11-67L23	1
Tumor-associated translocation site	
LIM domain only 2 (rhombotin-like 1)	1
Tumor associated	
Annexin 2 mRNA	1
Prothymosin α	1
<i>Trk</i> oncogene mRNA	1

ezzrin, radixin, moesin, protein tyrosine phosphatases, and the tumor suppressor protein merlin (schwannomin). Three sequences were consistent with gene products known to be associated with tumorigenesis; these included the well-characterized human *trk* oncogene.

DISCUSSION

In this study, we used a PCR approach to identify mutations in the *NF2* gene of a patient with NF2. Each of 17 identified exons of the *NF2* gene was amplified and sequenced. This is in contrast to

other mutational analyses of the *NF2* gene, which have first screened the gene by single-strand conformational polymorphism (SSCP) analysis and then sequenced only those exons with abnormal migration.^{10,11} This approach identified an insertion at position 175 in exon 2 that caused a ready frame shift that resulted in a stop codon in exon 3. Using a similar approach, Irving et al¹² demonstrated a preponderance of mutations in exon 2 resulting in protein truncation in a large series of VS tumors. Not all NF2 patients screened by PCR-SSCP have identified *NF2* gene mutations. Ap-

proximately 42% to 71% of affected individuals have an abnormal SSCP analysis.^{10,11} Defects in the *NF2* gene have also been reported in other neoplasms, including meningioma, malignant mesothelioma, melanoma, and breast carcinoma.¹³

The *NF2* gene has homology to the *moesin*, *ezrin*, and *radixin* family of genes. Translation of these genes is thought to produce membrane organizing proteins linking cytoskeletal proteins to the plasma membrane.¹⁴ Previous studies have shown schwannomin/merlin requires at least 2 important intramolecular associations, including one between its amino- and carboxyl-terminal domains, to function as a negative growth regulator. A truncated protein product, such as the one demonstrated in the current study, cannot form these intramolecular associations and has been shown to result in lack of growth suppression.¹⁵ At least one recent study also suggests that merlin/schwannomin growth suppression function may be dependent on its state of phosphorylation. In rat schwann cell culture at a low cellular density, schwannomin/merlin is phosphorylated, rendering it growth permissive due to formation of a complex with transmembrane hyaluronate receptor CD44, ezrin, and moesin. At high cell density, schwannomin/merlin becomes hypophosphorylated and inhibits cell growth again via an interaction with CD44.¹⁶ It is likely that schwannomin/merlin has many other similar critical protein-protein interactions to regulate cell proliferation.

In the search for genes or gene products that may contribute to VS formation and growth, the current study identified 4 sequences that matched genes or gene products associated with human tumor formation in nonotologic tissues (Table 1). In addition to identification of sequences with oncogenic potential, the current study also identified 3 clones representing gene products that may play a role in tumor apoptosis or cytotoxicity. These antitumorigenic factors may be upregulated in response to VS formation but inadequate to reverse the effects of coexistent tumorigenic factors.

The current study sought to identify genes and gene products that may be potential contributors to VS formation in addition to or in association with the identified *NF2* gene mutation. To meet this objective, open-system gene analysis was used.

Open-system analysis of gene expression can vary from the simple creation of a cDNA bacteriophage library for random sequencing or differential display to the complex protocols such as serial analysis of gene expression (SAGE) and total gene expression analysis (TOGA).^{17,18} These complex protocols have the primary advantage of analyzing large-scale gene expression (thousands of genes simultaneously) in a qualitative and quantitative nature, thus permitting analysis of differential gene expression among given specimens. The disadvantages of these protocols include their reliance on restriction digest, PCR amplification, and sequencing, which may incorporate significant error as a result of PCR contamination and inherent sequencing error, which can distort gene product results by up to 10%.¹⁹ Creation of a bacteriophage cDNA library has the advantage of being a well-established technique that can be performed with standard microbiology equipment and expertise. Libraries can be screened for inserts of cDNA over 100 bp, which, when sequenced, result in an error rates of less than 1%. The primary disadvantage of random sequencing of a cDNA library is that each detected sequence must be individually cloned and sequenced, which can result in significant time consumption and expense for a large number of sequences. Another potential disadvantage is that cloning the cDNA into bacteriophage vector is dependent on the presence of a given restriction enzyme site, thus cloning efficiency may vary for different cDNA fragments. This disadvantage is also present, however, in more complex protocols such as SAGE and TOGA.

Once the bacteriophage cDNA library has been used to identify gene transcripts as hypothetical contributors to VS formation, these genes and gene products can be targeted for further study via closed-system approaches. In the current study, it must be emphasized that this represents the partial sequence analysis of 50 clones taken from a single VS cDNA library. Follow-up studies with closed-system approaches will be necessary to establish the role of the identified sequences or gene products. Studies are currently under way to sequence an additional 50 clones. These closed-system approaches will include repeat detection and full-length sequencing of the gene products of interest from the original VS tissue to ensure complete

accuracy of the current clone representations. In situ hybridization will also be used to screen large numbers of VS specimens and determine the overall level and prevalence of expression of the identified genes. Additional studies to further characterize molecular pathways involved may ultimately provide important genetic targets for VS prevention, diagnosis, or treatment.

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