

Serial Analysis of Gene Expression in Neurofibromatosis Type 2–Associated Vestibular Schwannoma

Stacey L. Halum, Paul Popper, Joseph A. Cioffi, and P. Ashley Wackym

Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin,
Milwaukee, Wisconsin, U.S.A.

Hypothesis: The genesis, morphology, and growth characteristics of vestibular schwannomas are determined by genetic alterations which vary gene transcript expression and this transcript expression can be qualitatively and quantitatively evaluated using the SAGE technique. By use of such technique, gene products with tumorigenic potential may be identified, providing insight and targets for future study.

Background: Serial analysis of gene expression (SAGE) is a powerful new technique that allows detailed qualitative and quantitative evaluation of cellular gene transcript expression. Tissue in limited quantity (5×10^4 to 2×10^6 cells) may be analyzed by a modified version of SAGE called microSAGE. Application of SAGE or microSAGE to study vestibular schwannoma gene expression has not been previously reported. **Methods:** Fresh, vestibular schwannoma specimen from an individual with the diagnosis of neurofibromatosis type 2 was attained intraoperatively and maintained in a sealed container at -80°C until the time of analysis. The tissue was processed

according to the microSAGE protocol, using 180 mg of vestibular schwannoma as starting material.

Results: The protocol resulted in the generation and sequencing of a tag library involving 458 tags representing 277 different gene products, including many transcripts known to be expressed in vestibular schwannomas. Several gene products with tumorigenic potential were identified.

Conclusions: These data demonstrate that microSAGE is a useful technique to study vestibular schwannoma gene expression. Future studies will include building more comprehensive libraries and comparing libraries from various vestibular schwannoma phenotypes to identify useful diagnostic or prognostic markers, and targets for therapeutic intervention. **Key Words:** Acoustic neuroma—Expression—Gene—Neurofibromatosis Type 2—NF2—Serial analysis of gene expression (SAGE)—Schwannoma—Vestibular.

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Vestibular schwannomas develop from an overproliferation of Schwann cells along the vestibular nerve, usually at the junction of peripheral and central myelin. Vestibular schwannomas are the most common tumor of the cerebellopontine angle and consist of 6% to 8% of all intracranial tumors (1). Although they often develop in a sporadic unilateral fashion, a bilateral vestibular schwannoma predilection has been demonstrated in individuals with the autosomal dominant disorder neurofibromatosis Type 2 (NF2).

Genetic studies have linked both sporadic and NF2-associated vestibular schwannomas to a single gene called the *NF2* gene, located on chromosome band 22q12 (2–4). In 1993, two groups independently identified a tumor suppressor protein that the *NF2* gene encoded. Rouleau et al. (5) named the protein “schwannomin” because of the association with vestibular schwannomas, whereas Trofatter et al. (6) 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. Inactivating germline mutations in the *NF2* gene have been detected in 33 to 60% of patients with NF2 (7). Significant differences in types of *NF2* gene mutations have also been found among vestibular schwannomas associated with NF2. Approximately 65% of NF2 associated *NF2* mutations are non-sense and frame-shift mutations, which are expected to cause truncated gene products. These mutations are often associated with severe (Wishart) NF2 phenotypes (8,9).

Previous studies have shown schwannomin/merlin requires at least two important intramolecular associations,

Address correspondence and reprint requests to P. Ashley Wackym, M.D., Department of Otolaryngology and Communication Sciences, Medical College of Wisconsin, 9200 West Wisconsin Avenue, Milwaukee, WI 53226, U.S.A.; Email: wackym@mcw.edu

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including one between its amino and carboxyl terminal domains, to function as a negative growth regulator (10,11). A truncated protein product cannot form these intramolecular associations, and, in fact, has been shown to result in lack of growth suppression (12). At least one recent study suggests intermolecular interactions also play a critical role in merlin/schwannomin tumor suppression; at a low cellular density, schwannomin/merlin is phosphorylated, growth permissive, and exists in 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 (13). It is likely that schwannomin/merlin has many other similar critical protein-protein interactions to regulate cell proliferation. Despite intense study of the *NF2* gene and its schwannomin/merlin protein, the regulatory mechanisms and biological pathways involved are still largely unknown.

In 1995, Velculescu et al. (14) introduced serial analysis of gene expression (SAGE), an open system technique that allows rapid detailed qualitative and quantitative analysis of large numbers of cellular transcripts and requires no previous genetic information. Recently, SAGE has been used for strategic analysis in comparing cancerous and normal cell gene expression profiles in the National Cancer Institute-funded Cancer Genome Anatomy Project (CGAP) (15,16). The CGAP SAGEmap Web site (16) currently contains over 3 million tags from 88 different libraries. These libraries have demonstrated that between cancerous and normal tissue, only a small fraction of the total gene expression profiles are differentially expressed (17,18). Investigation into the differentially expressed genes identified by SAGE has resulted in discovery of novel regulatory genes (19) and identified pathways critical to tumorigenesis and growth (20). The aim of this investigation was to enhance understanding of the changes in gene expression involved in the genesis, growth, and differentiation of *NF2*-associated vestibular schwannomas by use of SAGE.

PATIENTS AND METHODS

The protocol was submitted to the institutional review board for exemption and approved based on the Federal (Basic DHHS) Policy for the Protection of Human Subjects Title 45, Code of Federal Regulations, part 46.101 b4. This states, in brief, that a study is exempt if it involves existing documents or pathologic 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.

Tissue acquisition

Fresh vestibular schwannoma tissue was obtained during resection of a 4.1-cm left-sided VS via a retrosigmoid approach by the senior author (P. A. W.) in a 29-year-old woman with the diagnosis of *NF2*. The patient had been diagnosed with *NF2* on the basis of a bilateral vestibular schwannoma (VS), but she was otherwise healthy, with no evidence of meningiomas or lens opacifications. After frozen section confirmation of the diagnosis of VS, approximately 500 mg of fresh tumor was

rapidly frozen on dry ice as it was removed. The tumor was stored at -80°C until mRNA extraction.

Micro-SAGE protocol

From the VS specimen, 180 mg of tissue was excised and polyA⁺ RNA was isolated using Dynabeads mRNA Direct kit (DynaL A.S., Oslo, Norway) according to the manufacturer's protocol (Fig. 1). Double-stranded cDNA was synthesized from the isolated, Dynabead-bound RNA using the Gibco BRL Superscript Choice System for cDNA synthesis (Life Technologies, Inc., Gaithersburg, MD, U.S.A.). This bound cDNA was then separated into two equal pools and cleaved with the *Nla* III restriction endonuclease (anchoring enzyme). This *Nla* III restriction endonuclease has a four base-pair (bp) recognition site and is therefore expected to cleave an average of every 256 bp. The bound cDNA from each pool was then ligated to a linker sequence containing a restriction site specific to the *Bsm*FI restriction endonuclease (tagging enzyme). The products were then cleaved with the *Bsm*FI tagging enzyme, which is a Type IIS restriction endonuclease that cleaves consistently 14 bp away from its recognition site, thus yielding a short segment of cDNA with its linker segment. Next, the ends were blunted with the addition of Klenow, and the tags from both pools were combined and ligated to each other to create ditags flanked by linkers (linker-ditags). The linkers contained both primer sequences for polymerase chain reaction (PCR) amplification and anchoring enzyme (*Nla* III) recognition sites. To determine the optimal linker-ditag dilution to use for large-scale amplification, a small aliquot of linker-ditags was amplified at various dilutions by PCR. These PCR products were analyzed by high-concentration NuSieve agarose gel electrophoresis (Fig. 2). The optimal linker-ditag concentration (1:50 dilution) was then used for large-scale PCR. The PCR product was isolated via polyacrylamide gel electrophoresis and the 102-bp bands, which represented the linker-ditags, were carefully extracted. The isolated linker-ditag sequences were then cleaved with the *Nla* III restriction endonuclease. The cleaved ditags were allowed to self-ligate at the restriction sites to yield a concatenate-

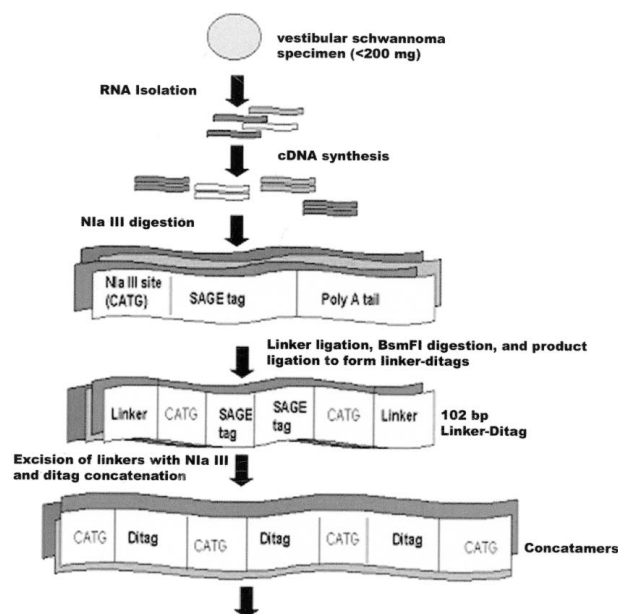


FIG. 1. MicroSAGE protocol applied to vestibular schwannoma tissue.

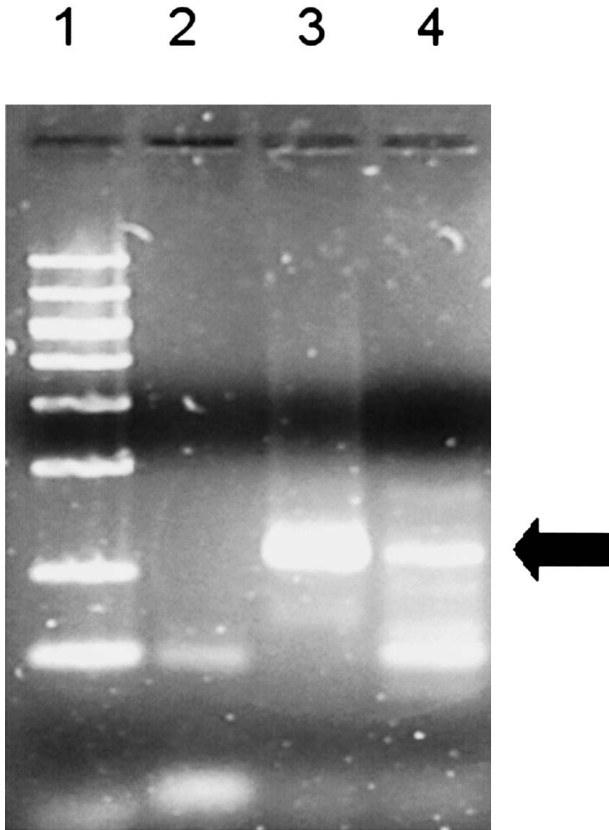


FIG. 2. The 102-bp linker-ditag PCR product (arrow) at 1:50 dilution (Lane 3) and 1:100 dilution (Lane 4). Lane 1 is a 50-bp ladder and Lane 2 is the negative control. The product is optimally amplified at the 1:50 dilution. The 40-bp band represents primer-dimers.

nated product containing ditags separated by the 4-bp anchoring enzyme restriction site sequence. The concatenated product was then separated via polyacrylamide gel electrophoresis. Products greater than 300 bp and less than 2,000 bp were excised, extracted, and cloned into the Sph1 site of pZERO (Invitrogen, Carlsbad, CA, U.S.A.). Colonies were then screened for inserts by PCR with the MI3 forward and MI3 reverse sequences located outside the cloning site as primers (Fig. 3). Amplified inserts were sequenced, which yielded a tag library. Sequence files were analyzed with the SAGE software by Velculescu et al. (14,16), which identifies the anchoring enzyme sites with the proper spacing, extracts the two intervening tags, and records them into a database.

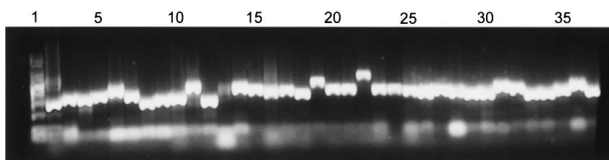


FIG. 3. PCR product of 36 colonies run on 1.5% agarose to determine insert size. Lane 1 represents a 100-bp ladder. Inserts ranged from 300 to 700 bp. Those 500 bp or longer (Lanes 11, 19, and 22) were selected for sequencing.

RESULTS

A SAGE library was developed from the NF2-associated vestibular schwannoma specimen (Tables 1 and 2). Of the 458 total tags in the library, there were 277 different tags, with 155 tags expressed once and 122 tags expressed more than once. Two tags corresponded with apoptotic related transcripts, and several tags represented proliferation or tumor-associated transcripts (Table 2). Four tags had multiple gene product matches, which were all associated with housekeeping function.

The two apoptotic related transcripts included Wilms tumor-related protein and FAST kinase. Wilms tumor-related protein is a zinc finger protein found in Wilms tumor of the kidney and is thought to act as a tumor suppressor by regulating the expression of several growth-related genes (21). FAST kinase is a serine/threonine kinase activated by Fas ligation to phosphorylate TIA-1, which is a nuclear RNA-binding protein thought to be an effector of apoptosis (22).

There were nine proliferation or tumor-associated transcripts identified in the library, and the majority were represented by more than one tag (Table 2). Thymosin β -10 was found to be the most highly expressed transcript in the library. It is expressed in other SAGE libraries from both normal and malignant tissues; however, it is overexpressed in colon cancer, ovarian cancer, and uterine cancer when compared with the respective normal tissues (23). In addition, chemotherapeutic agent-induced growth inhibition is associated with decreased levels of thymosin β -10 in breast cancer cell lines (24).

Another tumor associated product was the LLRep 3, also known as human ribosomal protein S2. This S2 protein demonstrates elevated expression in several cancers including human head and neck squamous cell carcinoma and premalignant leukoplakia in comparison with adjacent normal tissue (25).

The next most abundant tumor associated transcripts were nucleophosmin (B23) and the 90-kDa heat shock protein. Nucleophosmin (B23) is a nucleolar phosphoprotein whose levels correlate well with cell proliferation in human neuroblastoma and colon cancer cell lines (26). Colorectal neoplasms have also demonstrated elevated levels of B23 when compared with normal colorectal mucosa (27). Although the precise role of the 90-kDa heat shock protein is unknown, it is strongly associated with a wide range of tumors (28). These neoplasms include acute leukemia (29), melanomas (30), gastrointes-

TABLE 1. Neurofibromatosis type 2 vestibular schwannoma serial analysis of gene expression library analysis

	No.
Total no. of sequenced tags	458
Tags with SAGE database matches	291
No. of different transcripts matched	150
Tags with unknown function	22
Tags with known function	269
No. of tags with no database match	167

TABLE 2. Summary of neurofibromatosis type 2 vestibular schwannoma serial analysis of gene expression library

Housekeeping function	Tags
Ribosomal	60
Mitochondrion	12
Other	134
Structural or plasma membrane-associated	
Vimentin	2
α -Tubulin	2
Ras-related C3 botulinum toxin substrate (rac)	4
Keratin type II	3
β -Actin	2
E-cadherin	1
Cell adhesion molecule CD44	1
Other	13
Tag matches with unknown function	
Clone hRPK.268 F 2	3
Clone KAT02707	2
Clone DKFZp434N212	2
Clone HEP02567	2
Clone DKFZp434K1210	2
KIA0242 protein	2
KIAA0795 protein	2
Other	7
Tags with no database match	
GGCTGGTCTG	6
CAAGCATCCC	4
CACTAAAAAA	4
ATAATGAATA	3
ATAGTTATTT	3
ATGGCAAGGG	3
GAGACAAACT	3
GATGATGCTA	3
GCCCCAGGTA	3
GTCTGAAAAT	3
Other	132
Apoptotic potential	
FAST kinase	3
Wilms tumor-related protein (QM)	2
Proliferation-related or tumor-associated	
Thymosin β -10	12
LLRep 3	6
Nucleophosmin (B23)	3
90-kDa heat shock protein	3
Translationally controlled tumor protein (TPT)	3
Leptin	3
Platelet-derived endothelial cell growth factor	2
Prepromegakaryocyte potentiating factor	1
Growth factor inducible 2A9	1

tinal cancers (31), ovarian cancer (32), and breast cancer (33). It is hypothesized to play a role in reorganization of chromatin structure and to provide tumor cells with a survival advantage (28).

Two tumor-associated transcripts, platelet-derived (PD) endothelial cell growth factor (ECGF) (also known as thymidine phosphorylase [TP]), and leptin, are implicated in tumor angiogenesis. Leptin is a hormone secreted by white adipocytes that has proliferative effects in some cells, including mammary epithelial cells (34). PD-ECGF is also highly expressed in tumors and functions in angiogenesis by stimulation of endothelial cell migration (35). It has also been shown to suppress Fas-induced apoptosis. TP inhibitors have been developed as chemotherapeutic agents to inhibit this TP-mediated tumor angiogenesis (36).

Another tumor-associated product was the prepro-megakaryocyte potentiating factor. This is a precursor protein that is proteolytically processed into megakaryocyte potentiating factor and mesothelin. SAGE libraries have demonstrated mesothelin to be consistently present in mesothelioma, ovarian cancer, and pancreatic cancer but not in normal pancreas cells, and such findings have been confirmed with reverse-transcriptase PCR and in situ hybridization (37). A member of the mesothelin/MPF family has recently been identified as a circulating soluble tumor antigen that is detectable in the majority of cases of ovarian carcinoma, but it is not detectable in healthy controls or in those with benign ovarian tumors (38).

The final proliferation or tumor-associated products in the library were translationally controlled tumor protein and growth factor-inducible A29. Translationally controlled tumor protein is a growth-related protein that is regulated at the translational level (39), and its mRNA has been shown to bind the dsRNA-activated protein kinase PKR that mediates diverse processes such as cell growth, differentiation, stress response, and apoptosis (40). Expression of growth factor-inducible 2A9 mRNA is elevated in many human acute myeloid leukemias, and its expression is inducible by PD-ECGF or TP (41).

DISCUSSION

The SAGE library presented demonstrates that less than 200 mg of NF2 VS tissue can be used to successfully create a SAGE tag library. The SAGE technique (Velculescu et al. (14)) is based on two principles. First, a 9-bp nucleotide sequence can uniquely identify a transcript, provided it is isolated at a defined distance within the transcript. A 9-bp sequence can distinguish up to 4^9 or 262,144 transcripts, provided there is random nucleotide distribution at the tag site. This should be sufficient to cover all of the transcripts encoded by the human genome, which is estimated to contain 35,000 to 40,000 genes. Second, concatenation of short-sequence tags allows analysis in a serial manner by sequencing of multiple tags within a clone. The expression pattern of any population of transcripts can be quantitatively evaluated by determining the abundance of individual tags and identifying the genes corresponding to each tag. The process of concatenation of tags minimizes the sequencing time and expense involved in gene expression library creation.

Velculescu and colleagues (14) used the SAGE technique in pancreatic cells and found the 10 most abundant transcripts were derived from genes of known pancreatic function such as procarboxypeptidase A1, trypsinogen 2, and chymotrypsinogen. The quantitative nature of SAGE was confirmed via hybridization analysis with an oligo(dT)-primed pancreatic cDNA. SAGE was also shown to identify a previously uncharacterized pancreatic transcript, demonstrating that SAGE could be used to identify and characterize expression of novel genes.

Recently, SAGE has been used for strategic analysis in comparing cancerous and normal cell gene expression

profiles in the National Cancer Institute-funded CGAP. The CGAP SAGEmap Web site (16,42,43) currently contains over 3 million tags from 88 different libraries. These libraries have demonstrated that between cancerous and normal tissue, only a small fraction of the total gene expression profiles are differentially expressed (42,43). Investigation into the differentially expressed genes identified by SAGE has resulted in discovery of novel regulatory genes (44) and identified pathways critical to tumorigenesis and growth (44–46). For example, SAGE recently identified 14 transcripts, including many novel gene products that are significantly up-regulated by the tumor suppressor gene *p53* (45). Up further study, many of these transcripts were found to be critical effector proteins involved in common regulatory pathways of *p53*-mediated tumor suppression (45–47). The regulatory pathways of tumor suppressor gene APC have been illuminated in a similar fashion with the help of SAGE (48).

Despite the ability of SAGE to accurately generate gene expression profiles and identify novel gene products, the literature to date has no reports on the use of SAGE in neurotology or elsewhere in the field of otolaryngology. In contrast, the study of gene expression using microarrays has become increasingly popular in neurotology in recent years. For example, Welling and colleagues (49) recently studied the expression of over 25,000 genes and expressed sequence tags (ESTs) in VS specimens and normal vestibular nerve. Although such a study can provide an abundance of information about global and differential gene expression, the findings are limited to investigation of a predetermined set of genes, sequences, or ESTs. Although the ESTs in microarrays may represent novel sequences or sequences with unknown function, the proportion of ESTs representing novel sequences has been shown to be decreasing significantly over time (50). This is thought to be due to near complete identification of transcripts in the human genome. On the basis of this idea, the benefit of an open system technique such as SAGE, which is not limited to known genes, sequences, or ESTs, has been debated. However, recent studies have demonstrated that nearly 70% of unmatched SAGE tags are derived from novel transcripts (51), and many SAGE tags represent novel genes not yet identified in the human genome (52). The sequences corresponding with the identified novel or unmatched tags can be determined by use of reverse SAGE (45,53,54), which uses the novel tag of interest to design a primer for amplification and eventual sequencing of the transcript of interest. In the current study, several highly expressed tags had no SAGE database match (Table 2). The SAGE database can be used to further investigate these tags by demonstrating their relative levels of expression in other SAGE libraries. Candidates for reverse SAGE may include those demonstrating differential gene expression in neoplastic SAGE libraries when compared with normal tissue or those that are rare to nonexistent in other SAGE libraries. The tag ATGGCAAGGG is one example of a tag demonstrated to be highly expressed in

medulloblastoma but found in lower levels in other libraries (Fig. 4). Further investigation with reverse SAGE may be warranted.

In the current study, several genes with apoptotic or tumorigenic potential were identified. Clearly, most of these products are expressed in both normal and malignant tissues, so their presence in itself is not as informative as if the products had demonstrated differential expression when compared with a normal control (vestibular nerve). However, with the relatively small size of the current library, the fact that several proliferation or tumor-associated factors were present in multiple copies suggests that they could be highly expressed in the library. It could also be hypothesized that several of the transcripts found in the library are interrelated. Growth factor inducible 2A9 is known to be inducible by TP, which is coexpressed in the current library. TP may therefore actually be responsible for the presence of 2A9. TP is also an inhibitor of Fas-induced apoptosis, so conceivably FAST kinase was detectable in the library in response to a TP-mediated inhibition downstream in the apoptotic pathway.

Two tumor-associated factors, leptin and TP, are involved in tumor angiogenesis. Both factors were well represented in the library. Such factors are especially interesting in light of potential therapeutic implications. The factors could conceivably provide targets for che-

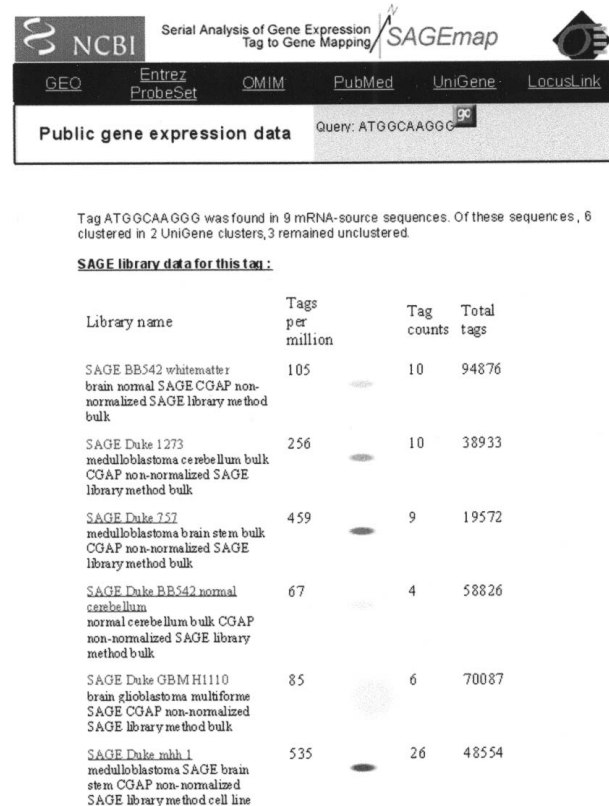


FIG. 4. Selected libraries from the NCBI SAGEmap Web site (www.ncbi.nlm.nih.gov/SAGE) demonstrating the levels of expression of the tag of interest.

motherapeutic intervention, such as use of TP inhibitors that are currently being developed (35). Ultimately, a chemotherapeutic role for VS management may be established.

Despite the usefulness of SAGE for the study of gene expression, there are several limitations to this study and limitations inherent in the SAGE technique. In 1995, at the time SAGE was developed, the rate of sequencing error for single-pass sequencing was estimated to be 1% for each nucleic base. Because SAGE relies on sequencing of a 9- to 10-bp tag to identify each library product, the rate of sequencing error for each tag was estimated to be 10%. To circumvent this source of error, omission of the tags counted only once was recommended, despite the fact that this may eliminate rarely expressed tags that do exist. Because of advances in sequencing technology over recent years, the rate of sequencing error has now been demonstrated to be only 1.67% for each tag (51). Thus, in the current study, some single tags were included in the final summary (Table 2), but emphasis was placed on those tags expressed more than once. Another limitation of SAGE is that in any given library, some of the tags will have multiple gene or gene product matches. This occurred in four of the singly expressed tags, and all were categorized as having a housekeeping function. In larger libraries, if this were to occur more frequently, it could make interpretation difficult. Such ambiguities need to be clarified by confirming the presence of the sequences or transcripts of interest with Northern blot, reverse-transcriptase PCR, or in situ hybridization. These studies are currently underway in our laboratory.

CONCLUSIONS

Although the current study results are preliminary in nature, progress is underway to develop more comprehensive SAGE libraries from different types of VS tissue and normal vestibular nerve. The gene transcripts identified as novel and differentially expressed can be targeted for further study via closed system approaches, possibly depicting the regulatory pathways of the *NF2* gene, schwannomin/merlin, and other critical genes and gene products. Ultimately, these genes and gene products may provide important diagnostic or prognostic markers and targets for therapeutic intervention.

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