UNIVERSITY OF CALIFORNIA, SAN DIEGO

Gene Expression of Growth Factors and Receptors in Vestibular Schwannoma

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by

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ABSTRACT OF THE THESIS

Gene Expression of Growth Factors and Receptors in Vestibular Schwannoma

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Vestibular schwannomas (VS) are known to carry mutations on the neurofibromatosis type 2 (NF2) tumor suppressor gene. However, the mechanism by which vestibular schwannomas occur is not well understood. The gene expression of several growth factors and receptors implicated in oncogenesis were evaluated in greater auricular nerve control tissue and vestibular schwannoma tissue taken from patients with sporadic VS and those with NF2-related VS using quantitative real-time polymerase

chain reaction and normalized with standardization to a single constitutively expressed control gene, human cyclophylin. Result demonstrated significant upregulation of fibroblast growth factor receptor 1(FGFR-1), glial cell line derived growth factor receptor alpha 1(GFRA-1), insulin-like growth factor 1(IGF-1), vascular endothelial growth factor (VEGF), transforming growth factor beta 1(TGFB-1), nerve growth factor receptor(NGFR), and downregulation of platelet derived growth factor beta (PDGFR-B) in sporadic VS. Significant differences in fibroblast growth factor receptor 1(FGFR-1), glial cell line derived growth factor receptor alpha 1(GFRA-1), and vascular endothelia growth factor(VEGF) gene expression was found between sporadic and NF2-related VS. Also the proportion of VS samples that exhibited upregulation of gene expression of FGRA-1 and VEGF were significantly greater in sporadic VS than in NF2-related VS

Introduction

Vestibular Schwannomas (VS), also known as acoustic neuroma, is a benign neoplasm arising from Schwann cells on the vestibulocochlear nerve (VIIIth cranial nerve). Schwann cells are glial cells responsible for supporting the nerve cell and forming myelin sheath around the axon of the nerve cells. In the case of VS, the Schwann cells surrounding the vestibular division of the vestibulocochlear nerve lose their growth control mechanism. This nerve is responsible for transmitting information regarding both balance and sound from the inner ear to the brain and the vestibular section of this nerve is the one responsible for transmitting balance information. When Schwann cells develop into a tumor, the tumor mass can compress the vestibular nerve and also affect the cochlear division that lies in close proximity and is responsible for transmitting sound to the brain. This is why patients commonly experience tinnitus, and hearing loss along with an impaired sense of balance, or even vertigo. Furthermore, if the tumor grows large enough, it may compress the brainstem and affect other adjacent nerves such as the trigeminal nerve (Vth cranial nerve) and, rarely, facial nerve (VIIth cranial nerve) causing symptoms such as the loss of sensation in face and mouth of the affected side and facial weakness, respectively. In rare cases and in patients with Neurofibromatosis type 2 (NF2), large and/or bilateral VS can lead to increased intracranial pressure, stroke, or even death.

VS in adults can take the form of a unilateral, sporadic tumor originating from the VIIIth cranial nerve that is non-inherited and arises from spontaneous somatic mutation of the *NF2* gene. However, VS can also occur as a result of Neurofibromatosis Type 2

(NF2), which is a familial disorder inherited in an autosomal dominant fashion, but may also occur through spontaneous germ line mutation in 50% of cases. Approximately 5 to 10% of VS cases are due to NF2. NF2 is characterized by the development bilateral tumors on the VIIIth CN, typically arising before the age of 21, and highly variable tumor growth rates even among patients of similar age from the same family[1]. NF2 occurs as result of mutation of a gene on chromosome 22 encoding the protein merlin also known as neurofibromin 2 or schwannomin. NF2 can also lead to Meningiomas, which are tumors that develop from cells in the arachnoid villi of the arachnoid, one of the three the layers of membranes covering the brain. Additionally, patients with NF2 develop spinal ependymomas, other cranial nerve schwannomas (most commonly VII, III, V, and IX, in order of decreasing frequency, and posterior subcapsular lens opacities, which can lead to blindness.

Functional merlin is a membrane-cytoskeleton scaffolding protein that seems to affect the movement and formation and cells and is thought to be a tumor suppressor. Although studies have shown that overexpression of merlin can block cell proliferation and tumor development, and induce cell cycle arrest, unlike other known tumor suppressors that activate or inhibit specific cell cycle related molecules or pathways, merlin does not appear to be directly involved in control of the cell cycle due to its localization to the plasma membrane and cytoskeletal junction. Instead, merlin is regarded as a protein with various functions that may contribute to its tumor suppressor properties such as contacted-mediated growth inhibition and membrane organization. Many studies have provided evidence that merlin regulates the localization of growth factor receptors to the cell surface and endocytosis of these receptors[2]. This suggests that

merlin plays a role in receiving and interpreting a variety of signaling pathways and that some of these pathways may contribute to VS proliferation when NF2 mutation results in the loss of functional merlin.

There are numerous cellular pathways, related receptors and mitogens that regulate Schwann cell proliferation during normal development and repair. Many of which have been implicated in various other forms of human cancer. The degree to which they are involved in VS and possible feedback to VS-related mutations are largely unclear, and results at times have been conflicting. Since merlin is related to the transduction of numerous signaling pathways, without functional merlin, it is possible for any number of these pathways to become involved in VS development and proliferation, depending on the individual. This may at least partially explain the diverse pattern of tumor growth observed in VS patients, seemingly without any regards to age, sex, or familial relations as noted above. It is been shown that Vestibular Schwann cells are a distinct subpopulation of peripheral glial cells with specific responsiveness to growth factors that differ significantly from even other types of peripheral glial cells[3], therefore the expression of various growth factors, receptors, and the response they elicit in VS are likely to be distinct as well. The expression of vascular endothelial growth factor (VEGF), insulin-like growth factor 1 (IGF1), transforming growth factor-beta 1 (TGFB1), Fibroblast growth factor receptor 1 (FGFR1), GDNF family receptor alpha 1 (GFRA-1), platelet derived growth factor receptor beta (PDGFR-B) and p75 neurotrophin receptor (p75-NTR) were studied in this project.

VEGF is an angiogenic growth factor (promotes growth of blood vessels from pre-existing blood vessels), and it is considered, by many, to be the most potent and

pathologically important factor in tumor growth and progression. It induces the formation and proliferation endothelial cells via various receptors: VEGF-R1 and VEGF-R2. VEGF has been found in several types of brain tumors including astrocytic tumors, menigiomas, and neuronal tumors, as well as many normal tissue types. In a study, a sample of 34 VS tumor samples taken from patients of various age, sex, symptom duration, tumor size, were stained for VEGF, VEGFR-1 (VEGF receptor 1) and VEGF-R2 (VEGF receptor 2), only one was found to express VEGF and one to express VEGFR-1. The authors believe their findings to be in accordance with the non-aggressive, slow growing characteristics of VS and suggest VS may not be vascular tumors and their growth may not be angiogenesis-dependent[4]. However, in separate experiment performed by an independent group of researchers, VEGF and VEGF-R1 concentrations were determined by ELISA in a sample of 27 VS tumor cell homogenates from patients of varied symptom duration, tumor size and growth rate. These researchers reported that out of 27 samples, all contained both VEGF and VEGFR-1 and the concentrations of both VEGF and VEGFR-1 correlated positively with tumor growth rate, but not symptom duration or tumor size. They conclude that VEGF and VEGFR-1 appeared to be directly related to the growth pattern of VS[5].

IGF1 is a growth factor with high sequence similarity to the hormone insulin and binds various IGF receptors found on almost all cells in the human body. IGF-1 is one of the most potent natural activators of the AKT pathway, responsible for stimulating cell proliferation and inhibiting apoptosis. IGF can regulate cell growth and DNA synthesis in many cell types and its role is especially important in nerve cells. It has been shown IGF in combination with fibroblast growth factors can promote the survival of rat Schwann

Cell Precursors. These cells normally undergo rapid apoptosis when removed from axons, suggesting that these factors work together to promote Schwann cell development[6]. Furthermore, IGF-1 has been shown to significantly increase the cell density of vestibular schwann cell isolated from rats in culture when compared to control group after an exposure period of 7 days[3].

TGFβ1 is a cytokine that acts upon Transforming Growth Factor Beta Receptor Type I and II, and its functions include regulating cell growth, proliferation, differentiation, and death in many different cell types in the human body. It is synthesized and released by cells as an inactive complex and transported to the extracellular matrix where they are stored until needed. It has been suggested that TGFβ1 plays in a role in the interaction between axons and its surrounding Schwann cells and the proliferation of Schwann cells. This is based on the discovery that TGFβ1 expression is increased during Wallerian degeneration, the process in which an axon that has been separated from the nucleus of the neuron due to trauma breaks apart and is cleared away along with its myelin sheath. As part of the process, Schwann cells subsequently release growth factors and attract nerve fibers to grow towards the site eventually repairing the damage and replacing the axon[7]. TGFβ1 is frequently found to be upregulated in tumor cells. Interestingly, TGFβ1 can exert both positive and negative effects on cancer progression. On one hand, it may acts as a growth inhibitor in the early stages of cancer progression. However, most of the other activities of TGFβ1 contribute to tumor growth and invasion of other tissues. This is especially true if there are mutations to its receptors or other altercations to its signaling pathway[8]. TGFB1 expression has also been reported in VS[9], and cultured schwannoma showed increased proliferation when exposed to the growth

factor[10], providing evidence for its role in tumor growth. Furthermore, TGFB1 has been shown to act synergistically with Glial derived neurotrophic factor (GDNF) in neurons and found to be co-expressed in 21 out of 22 non-NF2 VS samples tested. However, this study reports the expression of these growths factors and their receptors had no correlation with Ki-67 (cell proliferation marker), tumor growth rate, and patient demographic, no significant correlation was found[11].

GFRα-1 or GDNF family receptor alpha 1 is the receptor for glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN), two structurally related neurotrophic factors that regulate neuron survival and differentiation. GFRα-1 is expressed in myelinated nerves[12] and has been shown to be part of a signaling complex along with two molecules of RET, which it recruits to lipid rafts following the binding of GDNF and results in activation of RET and RET/src assocation[13]. The complex is essential for GDNF signaling and the proper development the nervous system[14]. As mentioned above, GFRα-1 is also related to TFGβ1 which activates the transport of the receptor to the cell surface[15].

FGFR1 is one of the receptors for the fibroblast growth factor (FGF) family of proteins and binds to a specific subset of FGFs. FGFs affect many different cell types including neurons and the effects they exert include regulation of cell division, cell differentiation, angiogenesis, and Wallerian degeneration. As mentioned above, FGFs combined with IGFs promote the survival of rat Schwann cell precursors that would otherwise undergo apoptosis[6]. This corresponds to the finding that FGFR1 is present on newborn Schwann cells at a much higher level than adult cells and that newborn Schwann cells are responsive to FGFs while adult cells are not. These researchers also

discovered that Schwann cell response to various growth factors differ depending on the medium they are cultured in, and the length that have been cultured. They suggest this may explain many of the conflicting results that have been published over the years[16].

PDGFR-β is a receptor for the platelet derived growth factor (PDGF) family of molecules. PDGFR-β dimerizes with either another monomer of itself or its isomer PDGFR-α forming either PDGFRαβ or PDGFRββ depending on which PDGF it binds. There are four different PDGF monomers: A, B, C, and D all of which are inactive until they form homodimers or heterodimers with another monomer. Each dimer has different affinities towards the different receptor dimers[17]. PDGFs play a large variety of roles in various cell types and in various stages life including cell proliferation, differentiation, growth, and development. Various mutations in PDGFs and PDGFRs have long been linked to many different types of cancer[18]. One study showed PDGFR-β to be highly expressed in two neurofibromin deficient cell lines derived from Neurofibromatosis type 1 (NF1) patients, however, it is expressed at very low levels in a cell line derived from a malignant Schwannoma from a non-NF1 patient. They also found that PDGF BB (an isoform with high affinity to PDGFR-ββ) was highly mitogenic in the two NF1 derived cell lines, but not for the non-NF1 cell line. The researchers believe the abnormal expression of PDGFRs may be in important step in leading to NF1-associated VS proliferation[19]. Other studies have also shown that PDGFR-β may be involved in NF2 VS as well. One group of researchers found PDGFR-β to be overexpressed by almost 2 fold in NF2-derived Schwannoma cells when compared to Schwann cells. The degradation and inactivation of PDGFR-β were also slower in the Schwannoma cells than the Schwann cells. Their data shows that PDGFR-β activation leads to a strong and

sustained activation of ERK1/2 (extracellular signal-regulated kinase 1/2) which has been show to lead to survival, growth, and proliferation in neuronal cells. Their data also show that PDGFR-β activation leads to the activation of AKT the known to apoptosis inhibitor[20]. In an independent study, researchers studied an established NF2 mutated human VS cell line (HEI 193) by overexpression of merlin through viral transfection and found that it lead to inhibition of cell proliferation in serum-free conditions and that PDGFR-β stimulated ERK1/2 and AKT activation were also greatly reduced. Furthermore, they also showed that overexpression of merlin also led to an acceleration of PDGFR degradation when compared to the control HEI 193 cells[21]. The mechanism of PDGFR-β regulation is thought to be merlin-mediated PDGFR-β internalization and targeting the receptor for degradation, thereby silencing the receptor signaling.

Nerve growth factor receptor (NGFR or p75-NTR) is a receptor for neurotrophins which are factors that promote the survival, differentiation, and myelination of neurons. P75-NTR is also sometimes known as the Low Affinity Nerve Growth Factor Receptor and is one of the two types of receptor types for neurotrophins, the other one being the high affinity receptor family also known as Trk receptor tyrosine kinases which normally promotes neuron growth and differentiation. The role of p75-NTR is neurotrophin signaling seems to be complicated and has been controversial. It is widely expressed in the nervous system during development, down regulated during adulthood, and up regulated after injury. Research has shown that p75-NTR may play several roles in nerve cells including as a co-receptor for Trk receptors that may refine or modify their response to neurotrophins. In the absence of Trk receptors p75-NTR can also act alone to be a receptor of neurotrophins, however, in this case it can activate different pathways that

promote cell death instead[22]. Other research suggest that it may have tumor suppressor properties in certain cancer cell types[23].

The development of polymerase chain reaction (PCR) in 1983 by Kary Mullis made it possible to copy a given segment of DNA millions of times within a few hours. This technique revolutionized biological research by providing researchers with a way to relatively easily generate enough genetic material to study and perform experiments. Then in 1993, Russell Higuchi and his team discovered that the relationship between the amount of target DNA and the copies of the DNA produced by PCR is linear after a specific number of cycles. This discovery allowed for the development of real-time quantitative PCR (qPCR)which uses complimentary DNA reverse transcribed from mRNA to detect and quantify the expression of specific genes in a given tissue sample. This technique has several advantages when compared other techniques such as Southern blot and fluorescence in situ hybridization in detection of gene transcription, namely reduced hands-on time and risk for contamination, minimal cDNA input, shorter time interval required to perform experiments and the large range of precise quantification. In qPCR, fluorescence is generated relative to the amount of product generated and the fluorescence in the sample is measured after each cycle of PCR using either dye-based or probe-based detection to determine the amount of mRNA present in the original sample. Dye-based detection utilizes non-specific double stranded DNA binding dye that only emit low amounts of fluorescence until it binds to dsDNA, therefore, as more dsDNA is produced after each cycle of PCR the amount of fluorescence in the sample also increases and can be measured. Probe based detection on the other hand, utilizes DNA probes labeled with a fluorescent reporter and a quencher that prevents fluorescence when the

two are in close proximity. These probes are sequence specific and are designed to bind to the gene of interest. When the DNA polymerase generates a copy of the gene by going over the sequence the probe is displaced and the fluorescent reporter and quencher are separated resulting in fluorescence which is then measured at the end of each cycle. Constitutively expressed reference genes are used to normalize the data to eliminate variations due to variations in the amount of cDNA in each sample.

In this study, the gene expression levels of VEGF, IGF1, TGFB1, FGFR1, GFRA-1, PDGFR-B, and p75-NTR in a total of 28 VS patients were studied using qPCR. Of those samples 23 had complete clinical data and were included in this study. This pool contained patients with sporadic (n = 11) and NF2 (n = 12) tumors. The expression levels of each gene was compared to that of a constitutively active human housekeeping gene, human cyclophyllin, as an internal control. Expression levels of these genes were analyzed in respect to patient age, gender, NF2 status, and tumor size.

Materials and Methods

To detect endogenous expression levels of the genes in VS tumors, quantitative reverse transcriptase PCR (qPCR) was used using cDNA samples previously isolated from VS tissue donated by the House Ear Institute (IRB approval No. 97-157). Tumor and nerve tissue obtained following this protocol are snap frozen in liquid nitrogen and stored at -70˚C. The diagnosis was verified by conventional histopathologic examination of the tumor. Total RNA was then extracted from VS and control nerve samples using the Trizol reagent (Gibco Invitrogen, Carlsbad, CA) following the manufacturer's instructions. mRNA purification was performed by running total RNA over oligo-dT columns (Invitrogen, Carlsbad, CA), and mRNA (5 μg per 50 μL sample) was reverse transcribed using the Gene AMP kit (Perkin-Elmer; Waltham, MA) in a DNA Engine Peltier Thermal Cycler (PTC-200; MJ Research, Watertown, MA) at 42˚C for 1 minute, followed by 72˚C for 2 hours, then held at 4˚C to construct cDNA samples. Each qPCR reaction contained 7.5 μL Taqman Universal PCR Master Mix (2x buffer containing polymerase; Applied Biosystems, Foster City, CA), 0.75 μL 20x solution of primers and fluorophor-labeled probe (Taqman Gene Expression Assays; Applied Biosystems, Foster City, CA)(Table I) for the transcript encoding the receptor or mitogen of interest, and an internal control (human cyclophyllin, a mitochondrial structure protein, and 2 μL of cDNA (reverse-transcribed mRNA 0.1 μg/μL). All expression assays were designed to span exon boundaries, to eliminate the risk of contamination from DNA or unprocessed RNA.

A total 23 VS samples were analyzed, out of those 12 were NF2 related and 11 were sporadic. As control for normal Schwann cell gene expression levels, qPCR analysis

was performed using two independent greater auricular nerve (GAN) specimens as control for baseline mRNA expression levels.

An ABI Prism 7,000 Sequence Detection System was used for all reactions with the following parameters: 50˚C for 2 minutes, 95˚C for 10 minutes, then 50 cycles of (95˚C for 10 seconds, 60˚C for 1 minute) for amplification and detection. Reactions were performed in duplicate, and an internal control of human cyclophyllin was amplified within the same sample well using a different fluorophor reporter for detection (VIC; Applied Biosystems, Foster City, CA) for simultaneous detection. Results were analyzed using a Microsoft Excel spreadsheet designed for qPCR quantification based on internal control mRNA levels, to control for differences in the mRNA integrity between samples and amount of sample loaded due to human error. The spreadsheet also calculates mRNA transcript levels as fold-induction of baseline tissue to provide comparative data.

Statistical Analysis

Genes were considered upregulated if their expression levels showed at least 2-fold increase when compared to the controls. Clinical information and qPCR data were combined and analyzed statistically using the Fisher's Exact test (for age, tumor size in centimeters, NF2 status, and expression data), the Mann-Whitney U test (for NF2 vs. sporadic VS data), and the Spearman R for correlations.

Table 1: Gene Expression Assays Used in Quantitative Real Time PCR Experiments

Transcript	Taqman Gene Expression Assay No.	Amplicon Length (nt)	Reporter Sequence	Chromosomal Location
VEGF	Hs00900054_m1	60	CACGAAGTGGTGAAGTTCATGGATG	6p12
IFG1	Hs00153126_m1	70	GTGATTTCTTGAAGGTGAAGATGCA	12q22-q23
TGFB1	Hs99999918_m1	125	GGACATCAACGGGTTCACTACCGGC	19q13.2;19q13.1
FGFR1	Hs00241111_m1	81	GGCTACAAGGTCCGTTATGCCACCT	8p11.2-p11.1
GFRα-1	Hs00237133_m1	69	ATCTGCAGATCTCGCCTTGCGGATT	10q26
PDGFR-β	Hs00182163_m1	86	GCAGCAAGGACACCATGCGGCTTCC	5q31-q32
NGFR	Hs00609976_m1	59	CCCTGCCTGGACAGCGTGACGTTCT	17q21-q22

Results

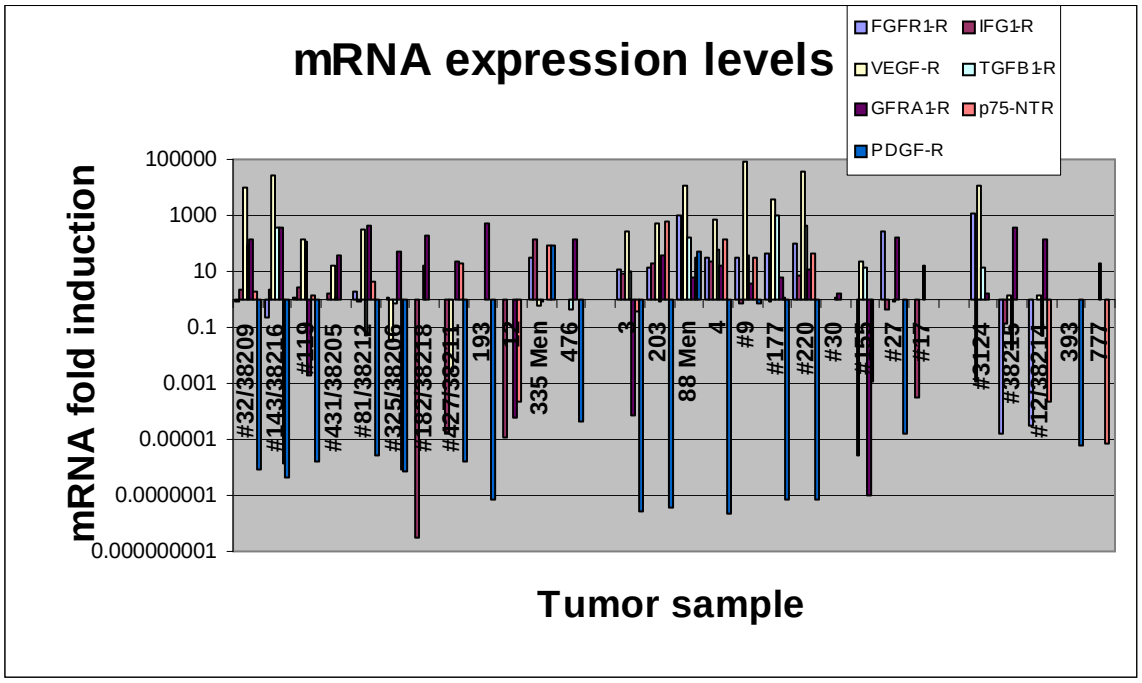

Figure 1: Gene expression in individual VS samples shown as fold induction.

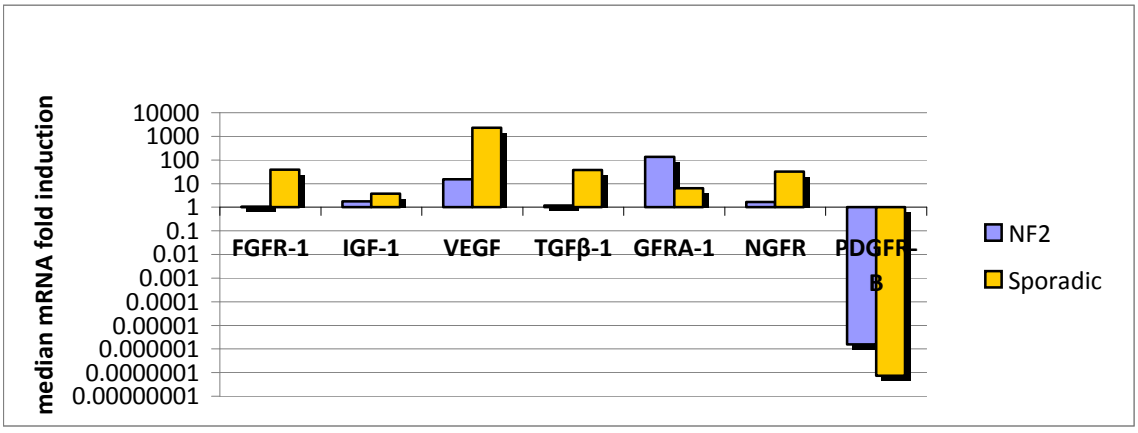

Figure 2: Growth Factors and Growth Factor Receptors mRNA median fold induction in NF2-related and sporadic VS. Median expression was calculated and shown here due the high variability of expression levels in VS samples. NF2-related VS are shown in light blue and sporadic VS in yellow.

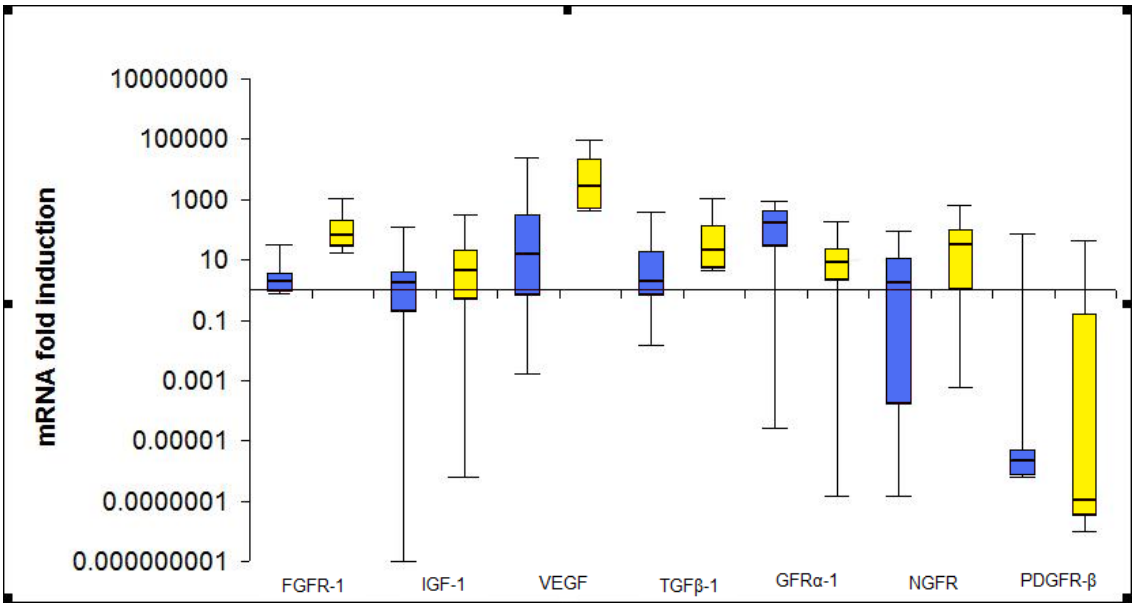

Figure 3: Median fold induction of genes for NF2-related and sporadic VS and the proportion of samples with upregulated gene expression. Although several genes appeared more upregulated in sporadic VS compared to NF2-related VS, this was only statistically significant in FGFR-1, and VEGF. Only GFRα-1 was expressed at a significantly higher level in NF2 VS compared to sporadic VS. FRGR-1 and VEGF were upregulated in a significantly larger proportion of sporadic VS samples than in NF2-related samples.

The average patient age at presentation for NF2 related tumors was 25.8 years of age, and average patient age at presentation for sporadic tumors was 50.8 years of age. The average size measured in the largest dimension was 1.95 cm for NF2 related tumors and 2.13 cm for sporadic tumors. The male to female ratio of patients was 1:1 for NF2 VS and 1:1.2 for sporadic VS.

A total of 1 of 7 (14%) NF2 VS samples upregulated of FGFR-1, and all 8 sporadic VS samples upregulated FGFR-1. This difference between the relative proportions of NF2 and sporadic VS samples with upregulated FGFR-1 was found to be statistically significant ($p<0.0014$). No statistically significant relationship was found between the relative proportions of samples that upregulated FGFR-1 to the proportions of male and female samples. FGFR-1 mRNA expression level was found to be more upregulated in sporadic VS samples compared to NF2 VS samples. A positive correlation

was found between patient age and FGFR-1 mRNA expression level ($p<0.036$). No statistically significant correlation was found between tumor size and mRNA expression.

A total of 5 of 9 (44%) NF2 VS samples upregulated of IGF-1, and 6 of 11 (55%) sporadic VS samples upregulated IGF-1. No statistically significant relationship was found between the relative proportions of samples that upregulated IGF-1 to the proportions of NF2 and sporadic samples or to the proportion of male and female samples. There was no statistically significant variability in the expression of IGF-1 mRNA between NF2 and sporadic VS samples. Using logistic regression with clinical parameters no statistically significant correlation was found between the patient age at presentation and tumor size with IGF-1 mRNA expression.

A total of 5 of 9 (56%) NF2 VS samples upregulated VEGF, and all of the sporadic VS samples upregulated VEGF. This difference between the relative proportions of NF2 and sporadic VS samples with upregulated VEGF was found to be statistically significant ($p < 0.036$). No statistically significant relationship was found between the relative proportions of samples that upregulated VEGF to the proportions of male and female samples. VEGF mRNA expression level was also found to be significantly higher in sporadic VS samples compared to NF2 VS samples. A positive correlation was found between patient age and VEGF mRNA expression level ($p < 0.029$). No statistically significant correlation was found between tumor size and mRNA expression.

A total of 3 of 9 (33%) NF2 VS samples upregulated TGFβ-1, and 8 of 11 (73%) sporadic VS samples upregulated TGFβ-1. No statistically significant relationship was found between the relative proportions of samples that upregulated TGFβ-1 to the proportions of NF2 and sporadic samples or to the proportion of male and female

samples. There was no statistically significant variability in the expression of TGFβ-1 mRNA between NF2 and sporadic VS samples. No statistically significant correlation was found between the patient age at presentation and tumor size with TGFβ-1 mRNA expression.

A total of 9 of 11 (82%) NF2 VS sample upregulated GFRα-1, and 7 of 10 (70%) sporadic VS samples upregulated GFRα-1. No statistically significant relationship was found between the relative proportions of samples that upregulated GFRα-1 to the proportions of NF2 and sporadic samples or to the proportion of male and female samples. GFRα-1 expression in NF2 tumors were significantly higher than in sporadic tumors. Using logistic regression with clinical parameters no statistically significant correlation was found between the patient age at presentation and tumor size with GFRα-1 mRNA expression.

A total of 4 of 8 (50%) NF2 VS samples upregulated, NGFR and a total of 5 of 8 (63%) of sporadic VS samples upregulated NGFR. No statistically significant relationship was found between the relative proportions of samples that upregulated NGFR to the proportions of NF2 and sporadic samples or to the proportion of male and female samples. There was no statistically significant variability in the expression of NGFR mRNA between NF2 and sporadic VS samples. Using logistic regression with clinical parameters no statistically significant correlation was found between the patient age at presentation and tumor size with NGFR mRNA expression.

A total of 1 of 9 (11%) NF2 VS samples upregulated PDGFR-β, and a total of 1 of 8 (13%) if sporadic VS samples upregulated PDGFR-β. No statistically significant relationship was found between the relative proportions of samples that upregulated

PDGFR-β to the proportions of NF2 and sporadic samples or to the proportion of male and female samples. There was no statistically significant variability in the expression of PDGFR-β mRNA between NF2 and sporadic VS samples. Using logistic regression with clinical parameters no statistically significant correlation was found between the patient age at presentation and tumor size with PDGFR-β mRNA expression.

Table 2. Summary of results. Light blue highlighted genes where the proportion of NF2 and sporadic samples that showed regulation were significantly different. Yellow highlighted genes that showed a positive correlation between gene expression level and patient age. Purple highlighted samples that showed upregulation according to their median fold induction. Arrows pointed to genes were the gene expression level between NF2 and sporadic samples showed significant variability.

Gene	Proportion of VS samples showed upregulation		Correlations between expression level and -		Median Fold Induction		
	NF2	Sporadic	Age	Tumor size	NF2	Sporadic	
FGFR-1	**14%**	**100%**	**+**	N	1.053361	**39.02157**	**<**
IGF-1	44%	55%	N	N	1.465186	**3.781703**	
VEGF	**56%**	**100%**	**+**	N	**15.24221**	**2311.537**	**<**
TGFB-1	33%	73%	N	N	1.172835	**16.33619**	
GFRA-1	82%	70%	N	N	**136.2394**	**6.298804**	**<**
NGFR	50%	63%	N	N	1.656689	**31.82191**	
PDGFR-B	11%	13%	N	N	1.58E-06	7.36E-08	

Discussion

Several of the receptors and ligands were found to be upregulated in a larger proportion of sporadic VS samples than NF2 VS samples. Furthermore, the median expression levels of FGFR-1 and VEGF were also significantly higher in sporadic VS than NF2 VS, while that of GFRα-1 was higher in NF2 VS than sporadic VS. The differences in gene expression between NF2 and sporadic VS suggest the mechanisms affecting their growth are different as well. This may partially explain the different growth patterns observed clinically, with NF2 VS generally having a faster growth rate and being more invasive[25]. It is possible that since NF2 VS result from mutations NF2 (merlin), which loses its functions as the regulator of contact mediated growth inhibition and reception and interpretation of growth factors, the expression of growth factors and receptors are not necessarily affected. Sporadic VS on the other hand have been shown to have alterations in the expression of several growth factors and receptors implicated in oncogenesis of other types of cancer, it is possible Sporadic VS may occur as a result of alterations in one of more of these other pathways.

Furthermore, this may explain why a positive correlation was found between mRNA expression levels of several genes and the patient age at presentation since the average NF2 VS patient was in their mid-twenties while the average sporadic VS patient was over fifty years of age and sporadic VS samples usually showed higher fold induction than NF2 VS samples. The results of this study suggest that several of these growth factors or their pathways can be potential pharmacological targets for treatment of sporadic VS because they were found to be upregulated in most VS samples.

FGFR-1 was found to be significantly upregulated in sporadic VS. As mentioned above it is involved in the signaling pathway of basic fibroblast growth factor (FGF-2), which promotes the survival and growth of many different types of neurons *in vitro* and regeneration *in vivo*. Furthermore, FGFRs has been found to be upregulated in many different types of human breast cancer and is often associated with poor patient prognosis. A low molecular weight compound PD173074 has been shown to be a selective and potent inhibitor of the tyrosine kinase activity of FGFR-1. In studies, PD173074 was able to successfully arrest the growth of several types of breast cancer cells as well as block the neurotrophic and neurotropic action of FGF-2 in granule neurons. PD173074, however, does not inhibit the actions of IGF-1 in promoting growth of various types of neurons[26, 27].

IGF-1 was found to be significantly upregulated in only sporadic VS. Since IGF acts together with FGF-2 to promote schwann cell growth and development and was also found to be upregulated in sporadic VS, its would have to inhibited also for a therapy involving the inhibition of FGFR-1 was to be effective. A compound currently being studied capable of effectively inhibiting the autophosphorylation and kinase activity of insulin-like growth factor-1 receptor (IGF-1R) by IGF-1 and other substrates by competitive binding to the receptor is AG538[28].

VEGF was found to be upregulated in some NF2 VS and highly upregulated in all sporadic VS samples. Although in the past there has been conflicting results regarding the expression of VEGF in VS, our data supports the previously published report that VEGF is indeed expressed in VS and that there are no correlation between VS expression and

patient age and tumor size. They did report, however, that a positive correlation was found between VEGF expression and tumor growth rate. Therefore, since VEGF has also been shown to play an important role in the growth and progression of many different types of tumors, it is also a potential target for VS treatment in the future. A VEGF inhibitor manufactured by Genentech/Roche known as Bevacizumab (Avastin) is currently FDA approved for the treatment of colorectal, lung, and breast cancer and being clinically tested for the treatment of several other types of cancer.

TGFβ-1 was found significantly upregulated in sporadic VS samples, but no correlation was found between mRNA expression and patient age, sex, or tumor size. This supports previous studies where it was reported to be upregulated in VS but lacked signficant correlation with the above mentioned clinical parameters, tumor growth rate, and cell proliferation[9,11]. This contradicts the study reporting TGFβ-1 increases cell proliferation in cultured schwannoma, but this discrepancy may suggest altered growth factor responsiveness and expression in cultured schwannoma cells compared to cells *in vivo*. Furthermore, as mentioned earlier TGFβ-1 and the TGFβ signaling pathway regulate many cellular processes in different cell types, and mutations in the pathway and their association with various types of cancer including VS at different stages are complex and not well understood. VS, like other types of tumors may also respond to TGFβ-1 differently at different stages of progression. Furthermore, TGFβ-1 is known to go through numerous post-transcriptional modifications and regulations and this study only provides a very limited picture of its possible role in VS.

GFRα-1was upregulated in a large proportion (76%) of VS samples tested in this study. This is in agreement with a previous study which reported that 100% of VS

samples were positive for GFRα-1 using immunostaining, and no correlation was found between expression level and patient demographic, tumor growth rate, and cell proliferation[11]. The study also mentions that GFRα-1was co-expressed with TGFβ-1 in VS. It is believed that TGFβ-1 activates and brings GFRα-1 to the cell surface. However, the effect of this activity on VS is still unknown.

About half of VS samples studied upregulated NGFR and 31% down regulated NGFR. Interestingly, in another study researchers found that NGFR was down regulated at the mRNA level.[24] In this experiment GAN was used as the baseline for NGFR expression in this experiment and unspecified peripheral nerve was used in the other study and may have led to this contradiction. NGFR is also known to be widely expressed in development, down regulated in adulthood, and up regulated after an injury. This may have also had something to do with the diverse pattern of gene expression.

PDGFR-β was found to down regulated in most NF2 and sporadic VS samples. In most VS samples PDGFR-β cDNA was undetectable after 50 cycles of qPCR reactions. As mentioned earlier, inactivation and degradation of PDGFR-β was found to be slower in schwannoma cells than schwann cells. Studies suggest this is because merlin regulates the internalization of PDGFR-β and targets it for degradation and in NF2 mutants this function is lost. Therefore, the increased PDGFR-β activity and its reported role in VS proliferation may not be necessarily result from increased expression. The slower internalization and degradation PDGFR-β at the protein level may account for why the expression of the gene is significantly reduced at the gene level. It is also possible the results were affected by experimental error. Lastly, it was also very possible that there were problems with the reagents such as the primer since Q-PCR reactions with the same

PDGFR-β primer were repeated and PDGFR-β cDNA was again virtually undetected in almost all of the VS samples. No other PDGFR-β primers were available at the time to verify this possibility. Interestingly, the only two samples that PDGFR-β was detected and found to be significantly upregulated in were the only two VS samples that came from patients who had also developed meningiomas.

In this study, a very limited number of VS samples were tested and variation was often very high. A much larger pool of VS samples should be examined to confirm the results. Also, many studies have reported correlations between the expression of the genes studied in this experiment and tumor growth rate. Patient age, sex, and tumor size were studied but the data for tumor growth not available for the VS samples used in this study. Although exact tumor growth rate of VS would be extremely difficult to measure, future studies should make it a point to included data on tumor growth rate whenever possible. This will allow for a better insight into the responsiveness of tumors to various growth factors and their role in tumor progression. These conditions may be difficult to meet because of the limited amount of tumors available. Furthermore, although PCR is considered to be one of the most sensitive assays, as shown in this study the low amount of RNA available led high variation. Although not as sensitive as PCR, protein expression assays should also be performed on the growth factors and receptors used in this study to confirm the results. This would also give insight to the numerous post transcriptional modifications and regulations that occur on these genes and possible mutations that may occur on these genes that this study does not take into account. Also, it should be noted that different control tissues whether it be any one of the peripheral

nerves often used or cultured schwann cells often express genes at different levels and can greatly affect the way data is interpreted depending on which one is used.

Appendix

Table 3: Fold induction of genes in samples along with clinical data. "X" denotes data not available.

tumor number	NF2	patient age	tumor size	gender	FGFR-1	IGF-1
#32/38209	1	13	1	M	0.907519	2.361985
#143/38216	1	50	4	M	0.244855	2.219139
#119	1	12	1.5	M	1.144724	2.770219
#431/38205	1	7	0.3	F	1.024557	1.777685
#81/38212	1	38	3	F	1.796265	0.784584
#325/38206	1	9	1.2	F	1.053361	1.152686
#182/38218	1	18	x	M	x	3.04E-09
#427/38211	1	15	3	M	x	1.54E-05
193	1	56	1	F	x	x
12	1	31	1.5	M	x	1.09E-05
335 Men	1	33	3	F	31.01727	132.5139
476	1	28	x	F	x	x
	Sporadic VS					
3	0	72	x	M	11.75335	8.693879
203	0	39	2	F	13.68952	19.83532
88 Men	0	57	4	M	926.0845	337.794
4	0	45	x	M	33.94201	22.16175
#9	0	47	0.8	F	34.17809	0.752623
#177	0	44	2	F	43.86505	0.882703
#220	0	44	2.4	F	102.8929	6.680703
#30	0	39	3	F	x	x
#155	0	66	x	F	x	2.72E-06
#27	0	61	0.7	M	279.17	0.450625
#17	0	45	x	M	x	0.000331
Average fold induction					152.1719	23.56194
Standard deviation					352.5743	73.83227
Average Fold induction NF2					5.312651	15.95336
Stdev Fold induction NF2					11.34373	43.72161
Average fold induction sporadic					180.6969	39.7252
Stdev fold induction sporadic					313.8503	105.0557
NF2 median					1.053361	1.777685
			Min	0	0.244855	3.04E-09
			q1	1	0.966038	0.196158
			Median	2	1.053361	1.465186
			q3	3	1.470494	2.326274
			Max	4	31.01727	132.5139
Sporadic median					39.02157	3.781703
			Min	0	11.75335	2.72E-06
			q1	1	28.87889	0.526125
			Median	2	39.02157	3.781703
			q3	3	146.9622	17.04996
			Max	4	926.0845	337.794

Table 3: Fold induction of genes in individual samples continued.

tumor number	VEGF	TGFβ-1	GFRA-1	NGFR	PDGFR-B
#32/38209	9508.486	88.03468	139.1021	1.952064	9.15E-07
#143/38216	25977.95	404.5012	372.217	1.42E-06	4.36E-07
#119	130.6896	115.3601	0.00194	1.361314	1.58E-06
#431/38205	15.24221	1.172835	37.92297	x	X
#81/38212	308.6868	0.05059	413.0006	4.242751	2.78E-06
#325/38206	0.032464	0.714497	53.44563	8.1E-07	6.74E-07
#182/38218	1.049717	16.33619	182.2784	x	X
#427/38211	0.002577	x	21.25897	20.60611	1.63E-06
193	x	x	531.8959	x	7.24E-08
12	x	x	6.59E-05	0.000237	x
335 Men	0.650671	0.907519	x	91.45562	78.24898
476	x	0.406126	136.2394	x	4.39E-05
3	284.0498	9.285631	7.18E-05	0.37501	2.55E-08
203	522.7582	0.795536	37.27147	636.934	3.66E-08
88 Men	11665.82	172.4459	6.364292	33.47472	49.35075
4	680.2871	61.3929	17.38776	137.6633	2.37E-08
#9	80405.13	37.27147	3.706352	30.16911	0.668964
#177	3942.787	1016.927	6.233317	1.252664	7.49E-08
#220	38833.12	463.0422	11.43195	44.17016	7.24E-08
#30	x	1.148698	1.693491	x	x
#155	20.96629	13.08643	1.07E-07	0.001169	x
#27	x	0.913831	176.6806	x	1.53E-05
#17	x	16.33619	x	x	x
Average fold induction	9192.275	102.3098	110.881	55.7588	7.126042
Standard deviation	19647.1	230.1555	156.5764	149.7977	21.20599
Average Fold induction NF2	3993.643	69.72041	171.5784	14.95226	8.694337
Stdev Fold induction NF2	8816.427	132.8536	185.4939	31.67659	29.57533
Average fold induction sporadic	17044.37	197.2662	28.7862	110.505	6.252466
Stdev fold induction sporadic	28788.31	341.5915	56.64971	217.4293	17.41591
NF2 median	15.24221	1.172835	136.2394	1.656689	1.58E-06
Min	0.002577	0.05059	6.59E-05	8.1E-07	7.24E-08
q1	0.650671	0.714497	29.59097	0.000178	6.74E-07
Median	15.24221	1.172835	136.2394	1.656689	1.58E-06
q3	308.6868	88.03468	277.2477	8.33359	2.78E-06
Max	25977.95	404.5012	531.8959	91.45562	78.24898
Sporadic median	2311.537	37.27147	6.364292	31.82191	7.36E-08
Min	20.96629	0.795536	1.07E-07	0.001169	2.37E-08
q1	463.0811	5.217165	2.196706	1.033251	3.38E-08
Median	2311.537	16.33619	6.298804	31.82191	7.36E-08
q3	18457.64	116.9194	15.89881	67.54344	0.167252
Max	80405.13	1016.927	176.6806	636.934	49.35075

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