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MicroRNA-21 Over-Expression Contributes to Vestibular Schwannoma Cell Proliferation and Survival

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Abstract

Hypothesis—Elevated levels of hsa-microRNA-21 (miR-21) in vestibular schwannomas (VSs) may contribute to tumor growth by down-regulating the tumor suppressor gene phosphatase and tensin homolog (PTEN) and consequent hyperactivation of protein kinase B (AKT), a key signaling protein in the cellular pathways that lead to tumor growth.

Background—VSs are benign tumors that arise from the vestibular nerve. Left untreated, VSs can result in hearing loss, tinnitus, vestibular dysfunction, trigeminal nerve dysfunction and can even become life threatening. Despite efforts to characterize the VS transcriptome, the molecular pathways that lead to tumorigenesis are not completely understood. MicroRNAs are small RNA molecules that regulate gene expression post-transcriptionally by blocking the production of specific target proteins.

Methods—We examined miR-21 expression in VSs. To determine the functional significance of miR-21 expression in VS cells, we transfected primary human VS cultures with anti-miR-21 or control, scrambled oligonucleotides.

Results—We found consistent over-expression of miR-21 when compared to normal vestibular nerve tissue. Furthermore, elevated levels of miR-21 correlated with decreased levels of PTEN; a known molecular target of miR-21. Anti-miR-21 decreased VS cell proliferation in response to platelet derived growth factor stimulation and increased apoptosis suggesting that increased miR-21 levels contributes to VS growth.

Conclusion—Since PTEN regulates signaling through the growth-promoting phosphoinositide 3-kinase (PI3K)/AKT pathway, our findings suggest that miR-21 may be a suitable molecular target for therapies aimed specifically at reducing VS growth.

Keywords

Acoustic Neuroma; MicroRNA; Neurofibromatosis Type 2; Vestibular Schwannoma

Vestibular schwannomas (VSs) are benign Schwann cell-derived tumors associated with the vestibular nerve and are a hallmark of the autosomal dominant genetic disorder neurofibromatosis type 2 (NF2). NF2 has been linked to mutations in the *NF2* gene which encodes the tumor suppressor protein “merlin” (aka schwannomin) (1,2). Although the exact mechanisms whereby merlin prevents tumor formation are not completely understood, recent efforts to define the associated genes and molecular pathways involved in tumorigenesis and

expansion have met with some success (3). VSs frequently go undiagnosed until clinical symptoms develop such as hearing loss, tinnitus, and balance impairment as the tumors grow larger. Left untreated, vestibular schwannomas can become life threatening. At present, the most common methods of treatment are observation with serial imaging studies, microsurgical removal and stereotactic radiosurgery.

MicroRNAs are evolutionarily conserved, small (~22 nt), non-coding RNA molecules that regulate gene expression post-transcriptionally. Mature microRNAs bind to specific mRNA targets in regions that are significantly complementary to the microRNA and, by a mechanism that is not completely understood, results in translational repression or mRNA degradation (4,5). The human genome encodes more than 1000 microRNAs with tissue- and cell-type specific expression (6). MicroRNAs have been shown to play important roles in such diverse cellular processes as differentiation, development, metabolism, apoptosis and cancer (7). Studies have shown that tumors generally exhibit aberrant microRNA expression profiles, and identified multiple microRNAs with reputed tumor suppressor or oncogenic properties (8-10).

In preliminary studies investigating microRNA expression profiles in four human VSs using a microarray platform, we found that hsa-miR-21 (herein referred to as miR-21) was consistently over-expressed when compared to normal vestibular nerve. Over-expression of miR-21 has been observed in many cancers including breast, liver, and glioblastoma (11-13). Phosphatase and tensin homolog (PTEN) tumor suppressor gene has been identified as a target of miR-21 (12). PTEN acts as a tumor suppressor via its inhibitory effect on the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) pathway, which promotes cell survival, cell proliferation, and tumor formation (14). The PI3K/AKT pathway has recently been shown to be active in VSs (15). Therefore, elevated levels of miR-21 in VS may contribute to tumor growth by down-regulating PTEN and consequent hyperactivation of AKT signaling.

The microRNA-21 gene has an upstream enhancer region containing two strictly conserved signal transducer and activator of transcription 3 (STAT3) binding sites and activation of STAT3 has been shown to induce the expression of miR-21 (16). STAT3 is a critical regulator of gene expression in response to many growth factors and cytokines (17). For example, the neurotrophic cytokines ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and interleukin 6 (IL-6) bind to specific ligand-binding receptor subunits and share the signal transduction subunit gp130 which signals through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (18). Interestingly, merlin has been shown to play a role in suppressing STAT3 activation through its interaction with hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) in a human schwannoma cell line (19). Furthermore, these authors showed that a naturally occurring *NF2* missense mutation interferes with hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) binding and abolishes the ability of merlin to inhibit STAT activation. This raises the possibility that over-expression of miR-21 in VSs may be a consequence of deregulated activation of STAT3 by an autocrine or paracrine mechanism involving neurotrophic cytokines or other growth factors.

In the present study, we sought to confirm our earlier microarray results showing over-expression of miR-21 in the four original VSs using quantitative real-time polymerase chain reaction assays, measure the expression of PTEN mRNA and protein levels, and determine whether CNTF, LIF, IL-6, the receptor subunits neurotrophic cytokines ciliary neurotrophic factor receptor alpha (CNTFR α), leukemia inhibitory factor receptor (LIFR), interleukin 6 receptor alpha (IL-6R α) and signal transduction subunit gp130, and STAT3 are expressed in these tumors. We also sought to examine miR-21 expression levels in additional VSs, greater auricular and normal vestibular nerve samples to increase the power of our statistical analyses. Finally, we sought to demonstrate that transfection of human VS cultures with anti-miR-21

oligonucleotides reduce their proliferative potential and promotes apoptosis which would suggest that over-expression of miR-21 contributes to VS growth.

Materials and Methods

Procurement of Vestibular Schwannoma, Vestibular Nerve and Greater Auricular Nerve Specimens

The human subject protocol for tissue procurement was approved by the Institutional Review Board and informed consent was obtained from all participating patients. During surgical resection of unilateral sporadic VSs via a retrosigmoid or translabyrinthine approach, fresh tumor specimens (n=8) were sterilely collected and immediately frozen in dry ice. Normal vestibular nerves (n=9) removed during vestibular neurectomy or adjacent to resected small VSs were collected in a sterile manner and immediately frozen in dry ice. Segments of greater auricular nerve were collected in a sterile manner during radical neck dissections performed in patients (n=5) with no clinical evidence of metastatic cancer and immediately frozen in dry ice. The tumor, vestibular nerve and greater auricular nerve specimens were stored at -80°C until they were used in the cell and molecular biology studies described below.

In an additional four fresh VS specimens acquired during microsurgical resection and that were not used for RT-PCR or western blot analysis, the samples were not frozen but were processed to produce primary VS cultures as described below.

Quantitative Real-Time RT/PCR

Real-Time reverse transcriptase/polymerase chain reaction (RT/PCR) was used to confirm previous microarray data showing elevated expression of hsa-miR-21 in VSs. Real-Time RT/PCR reagents for U6B small nuclear RNA (RNU6B) (control) and hsa-miR-21 were purchased from Applied Biosystems (Foster City, CA) and the reactions were performed as recommended by the manufacturer. Briefly, RT reactions containing total RNA, stem-looped primers, 1X RT buffer, reverse transcriptase, and RNase inhibitor were incubated for 30 min. each, at 16°C and at 42°C. Stem-looped primers were annealed to miRNA targets and extended by reverse-transcriptase. Reactions containing miRNA-specific forward primer, TaqMan® probe and reverse primers were loaded into a PCR reaction plate in quadruplicate and incubated in a thermocycler (BIO-RAD iCycler iQ) for 10 min. at 95°C followed by 40 cycles of denaturing (15 sec. at 95°C), annealing and extension (60 sec. at 60°C). Experiments were set up in quadruplicate and repeated three times. Mean threshold cycles (C_T) were calculated by averaging the technical replicates for each experiment and then by averaging the mean replicate C_T across the three runs. Quadruplicates with a standard deviation greater than 0.50 were eliminated and these assays repeated. The miR-21 expression was normalized to RNU6B (ΔC_T) for each tissue. Relative expression and fold differences were determined by comparing normalized expression levels between tissues ($\Delta\Delta C_T$) using the $2^{-\Delta\Delta C_T}$ method (20). Statistical significance was determined using the Student's t-test assuming unequal variance.

Reverse Transcriptase/PCR

RT/PCR was used to detect transcripts for PTEN, STAT3, the neurotrophic cytokines LIF, CNTF, and IL-6, and their receptor signaling subunits LIFR, IL-6R α , CNTFR α , and gp130. National Center for Biotechnology Information (NCBI) mRNA sequences were obtained from the GenBank database and primer sets were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Gene-specific primer sets and the expected amplicon sizes are listed in Table 1. Approximately 0.5–1 μ g aliquots of total RNA were used to generate cDNA for these experiments. Total RNA extracted from the tumors using TRIzol Reagent (Invitrogen, Carlsbad, CA) was treated with 1 μ l RNase-Free DNase I (Promega, Madison, WI) in 1 μ l RNase-Free DNase 10X Reaction Buffer (Promega, Madison,

WI) and incubated for 10 min at 37°C. DNase I was inactivated by adding 1 µl of DNase Stop Solution (Promega, Madison, WI) followed by heating for 10 min at 65°C. RNA was then reverse transcribed using random hexamer primers and the Superscript III kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reactions in which reverse transcriptase was omitted served as a negative control for PCR amplification. Amplicons were resolved by agarose gel electrophoresis and visualized using GelStar nucleic acid stain (Lonza, Rockland, ME). Restriction enzyme mapping was used to confirm the authenticity of each amplicon (data not shown).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Tissue samples were homogenized and lysed using the TRIzol procedure for protein extraction (Invitrogen, Carlsbad, CA). Approximately twenty micrograms of total protein was resolved on a 12% sodium dodecyl sulfate polyacrylamide gel by electrophoresis, transferred to a nitrocellulose membrane (Amersham International, Little Chalfont, UK), and probed for PTEN using 2 µg/ml polyclonal rabbit anti-human PTEN (ab23694) (Abcam Inc., Cambridge, MA). All western blots were stripped using Re-Blot Plus strong antibody stripping solution (Chemicon International, Temecula, CA) and nonspecific sites were blocked using nonfat milk. Blots were then reprobed for beta actin using the monoclonal anti-actin antibody diluted 1:10,000 (CP01) (Calbiochem, San Diego, CA) to ensure equal loading of total protein in all lanes. All antibodies were diluted in phosphate-buffered saline containing 1% (vol/vol) Tween-20. The signal was detected by chemiluminescence using ECL western blotting detection reagents (Amersham International, Little Chalfont, UK).

Primary VS Cell Cultures

Primary VS cultures (n=4) were prepared as previously described (21,22). Briefly, acutely resected tumors were cut into 1 mm³ fragments, digested with collagenase (2 mg/ml, Sigma, St. Louis, MO) and 0.25% trypsin (Sigma) for 45–60 min at 37°C, and then put in 10% fetal bovine serum (FBS). Following centrifugation at 800 rpm for 3 min, the cells were resuspended and dissociated by trituration through narrow pipettes. Cell suspensions were plated in 4-well culture slides (Nalge Nunc International, Rochester, NY) pretreated with polyornithine, followed by laminin (20 µg/ml). Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) with N2 supplements (Sigma), bovine insulin (Sigma, 1 mg/ml) and 10% FBS. The medium was exchanged two to three days later and when the cells reached 50–70% confluence, they were subsequently maintained in serum-free conditions until used for experiments, typically after 7–10 days. Over 90% of the cells in the cultures were S100-positive. Cultures were maintained in a humidified incubator with 6.0% CO₂ at 37°C.

miR-21 Knock Down

Anti-miR-21 miRCURY LNATM knockdown probe and control, scrambled miR probe were designed and synthesized by Exiqon (Vedbaek, Denmark). The miR-21 probe sequence (Exiqon 138102-04) was: TCAACATCAGTCTGATAAGCTA. The corresponding micro RNA targeting sequence was: UAGCUUAUCAGACUGAUGUUGA. The scrambled miR probe sequence (Exiqon 199002-04) was: GTGTAACACGTCTATACGCCCA. When the VS cultures reached 60–80% confluency, they were maintained in medium without antibiotics for 24 h. Subsequently, the cultures were transfected using LipofectamineTM RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, anti-miR-21 and scrambled probes (2.5 mM) were diluted in Opti-MEM medium (Opti-MEM, Invitrogen) at 1:50 and mixed with equal volume of RNAiMAX pre-diluted at 1:50 in Opti-MEM. After 20 min incubation at room temperature, the complexes were added to the cultures for 12–20 h prior to media exchange. Preliminary studies using Cy-3-labeled oligonucleotides confirmed that over 90% of VS cells are transfected by RNAiMAX. Both the

anti-miR-21 probe and scrambled probes were 5'-fluorescein labeled and only cultures demonstrating over 85% of transfected cells were analyzed. Following transfection, cultures were maintained in serum-free conditions until fixation. PDGF-BB (20 ng/ml) was added to the indicated cultures 24 h prior to fixation.

Immunostaining

The cultures were fixed with 4% paraformaldehyde, washed with phosphate-buffered saline (PBS), permeabilized with PBS with 0.1% Triton X-100 for 10 min, blocked with blocking buffer (5% goat serum, 2% bovine serum albumin [BSA], 0.1% Triton- X in PBS) for 30–60 min, and then incubated with anti-BrdU (1:800; clone G3G4, Hybridoma core, University of Iowa, Iowa City, IA) and anti-S100 (1:400, Sigma # S-2644) antibodies diluted in blocking buffer overnight at 4°C. Following washing, Alexa 546 and Alexa 647 labeled secondary antibodies (1:800; Invitrogen, Carlsbad, CA) were incubated at 37°C for 1 h. Nuclei were labeled with Hoechst 33342 (10 µg/ml, Sigma) for 15 min.

Fluorescence images were captured using an inverted Leica DMRIII microscope (Leica, Bannockburn, IL) equipped with epifluorescence filters and a charge coupled device camera using Leica FW4000 software and prepared for publication using Adobe Photoshop (Adobe, San Jose, CA).

VS Cell Proliferation and Apoptosis

VS cultures were labeled with 5-bromo-2-deoxyuridine (BrdU 10 µM, Sigma) for 24 h prior to fixation. Fixed cultures were treated with 2N HCl for 15 min. prior to immunostaining and BrdU uptake was detected by immunostaining as above. The percent of BrdU positive VS cells (S100-positive) nuclei was determined by counting 10 randomly selected fields for each condition. Only S100-positive cells were scored. Apoptotic cells were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) using the In Situ Cell Death Detection Kit, TMR red kit (Roche Diagnostics) according to the manufacturer's instructions. The percent of apoptotic VS cell (S100-positive) nuclei was determined by counting 10 randomly selected fields for each condition. Criteria for scoring were a TUNEL-positive nucleus with typical condensed morphology in an S100-positive cell. Each condition was repeated on four VS cultures derived from separate tumors.

Results

Quantitative real-time RT/PCR comparing miR-21 expression in eight VSs and fourteen normal nerve specimens showed that miR-21 was consistently over-expressed in all VSs when compared to the mean level of expression in control normal nerve tissues (Fig. 1). Since the level of miR-21 expression in greater auricular nerve was not statistically different from that in normal vestibular nerve, we combined these negative controls for statistical purposes. On average, VSs exhibited ~6.5 fold (calculated with the $2^{-\Delta\Delta CT}$ method) higher level of expression of miR-21 (ΔCT 7.53 $\pm$ 0.274 SE) as compared to the normal nerve control group (ΔCT 4.81 $\pm$ 0.897 SE) and this difference was statistically significant ($p < 0.01$) (Fig. 1).

Since miR-21 targets and inhibits tumor suppressor PTEN expression in other cancers, we examined PTEN expression using RT/PCR to detect mRNA and western blots to assess PTEN protein and found altered expression patterns. Because of available tumor volume, we were unable to extract adequate protein to perform the western blot experiments in four samples. Both PTEN mRNA and western blot data are needed in combination to show a transcription and expression relationship, therefore we were only able to make this comparison in four of the individual VS specimens. Although PTEN mRNA was easily detectable by RT/PCR in the four vestibular schwannomas (Fig. 2A), western blot analysis showed that PTEN protein was

barely detectable in three out of the four tumors (Fig. 2B). VS4 showed much higher levels of PTEN protein compared to VS1, VS2, and VS3. Interestingly, VS4 also had the lowest level of miR-21 expression compared to the other three tumors. Rat brain extract served as a positive control for PTEN expression. Beta-actin was used as a sample loading control. Thus, elevated miR-21 expression correlates with decreased levels of PTEN protein, but not mRNA, levels in vestibular schwannomas consistent with the observation that miR-21 reduces PTEN protein expression in tumor cells (12).

As summarized in Table 2, STAT3, CNTF, IL-6, LIF, IL6R α , LIFR, CNTFR α , and gp130 were found to be expressed in each of the four tumors analyzed. However, IL-6, IL-6R α , LIF and LIFR transcripts could not be detected in pooled total RNA isolated from three different normal vestibular nerve samples.

We were unable to perform western blot analysis on all specimens due to limited protein yields. However, we were able to demonstrate that activation of STAT3 occurred in VS2, which is also the tumor that had the highest expression of miR-21. Examination of STAT3 by western blot in VS2 using an antibody that recognizes STAT3 protein regardless of activation state and an antibody that specifically detects the activated (phosphorylated) form of STAT3 (PSTAT3) showed the presence of PSTAT3, which correlates with elevated miR-21 expression in this tumor (Fig. 3). Because we were unable to perform western blot analyses on the remaining seven VSs, we could not complete a correlation between activated STAT3 and miR-21 levels in all tumors.

To determine the functional significance of miR-21 overexpression in VS cells, we adopted a knock-down approach using anti-miR-21 oligonucleotide probes. We first verified the ability to transfect primary VS cultures with oligonucleotide probes by transfecting cultures with Cy-3 tagged probes. Over 90% of primary VS cells were transfected with the tagged oligonucleotides (Fig. 4).

Transfection of anti-miR-21 significantly reduced the percent of BrdU-positive VS cells in cultures stimulated with PDGF-BB, a VS cell mitogen (23,24), more than 2-fold, but did not decrease proliferation in cultures maintained in basal medium (Fig. 5). The average percent of BrdU-positive VS cells for these four cultures in the control condition was 0.74 ± 0.32 (mean $\pm$ SD). Thus, miR-21 contributes to the proliferative potential of primary VS cells. Anti-miR-21 increased the percent of TUNEL-positive, apoptotic VS cells maintained in basal medium by more than 3 fold (Fig. 6), indicating that miR-21 supports VS cell survival. The average percent of TUNEL-positive VS cells for these four cultures in the control condition was 1.16 ± 0.47 (mean $\pm$ SD). These observations demonstrate that miR-21 contributes to VS cell proliferation and survival raising the possibility that over-expression of miR-21 contributes to VS growth.

Discussion

Using quantitative real-time RT/PCR, we confirmed our previous microarray findings that showed that miR-21 is over-expressed in VSs. Consistent with our results, miR-21 has been shown to be over-expressed in a variety of solid tumors (25). In addition, important links between miR-21 expression and cancer-related cellular processes such as proliferation, migration, apoptosis and tumor growth have been demonstrated in human breast and hepatocellular cancer cells (12,13). Inhibition of miR-21 in hepatocellular carcinoma cell lines increased expression of tumor suppressor PTEN, and decreased cellular proliferation, migration and invasion; while enhancing miR-21 expression had the opposite effects (12). Despite demonstrating the presence of PTEN mRNA in the four VSs tested, we observed very low PTEN protein levels in three of the four tumors tested. This may be due to translational suppression by miR-21. VS4 was an exception in that it displayed the highest level of PTEN

protein; however, this tumor also exhibited the lowest relative level of miR-21 expression. Alternatively, PTEN mRNA in VS4 may be missing the miR-21 binding site in the 3' untranslated region due to either gene mutations or alternative splicing and thereby escape post-transcriptional regulation. Since PTEN is a strong negative regulator of the AKT pathway, decreased expression of PTEN would enhance signaling through this pathway leading to increased cellular proliferation, decreased apoptosis, or both. Interestingly, the PI3 kinase/AKT pathway has recently been shown to be activated in human VSs (15).

STAT3 is a critical regulator of gene expression in response to many growth factors and cytokines (17). The neurotrophic cytokines IL-6, CNTF, and LIF use the common signal transducing gp130 subunit to mediate STAT1/3 signaling and play important roles in nerve regeneration and repair (18,26). IL-6 has been shown to mediate the activation of STAT3 in adult Schwann cells and rat schwannoma cells (27). Furthermore, activation of STAT3 by IL-6 induces miR-21 expression in multiple myeloma cells (16). We postulated that VSs may be secreting neurotrophic cytokines and stimulating their own growth through an autocrine pathway mediated, in part, by the overexpression of miR-21. The detection of mRNAs encoding these cytokines and their receptor subunits in VS supports this possibility. That we were unable to detect the expression of IL-6, IL-6R α , LIF and LIFR in normal vestibular nerve may reflect the absence or low abundance of these mRNAs in this quiescent, non-proliferative control tissue. Membrane tyrosine kinase receptors such as the platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), and erbB receptors have also been implicated in the activation of STATs (17,28). The EGFR and neuregulin-1/erbB signaling pathways contribute to cell proliferation in NF2-deficient cells and VS primary cultures, respectively (22,29). However, it is not clear to what extent STAT3 activation and miR-21 overexpression mediate this effect. A recently published case study demonstrating some effectiveness of the EGFR inhibitor erlotinib on VS progression in an NF2 patient suggests that targeted therapies for NF2-related tumors hold promise (30). We demonstrated STAT3 activation using western blot analysis in one tumor (VS2) since its expression of miR-21 was the highest compared to the seven other tumors. Our objective was to verify that STAT3 was activated, i.e., phosphorylated (PSTAT3), in this tumor. Our finding suggest that STAT3 activation may be required for miR-21 expression in these VSs.

Recent studies shed additional light on the role of miR-21 in cancer-related processes. MCF-7 breast cancer cells transfected with anti-miR-21 oligonucleotides resulted in increased apoptosis and inhibition of cell growth *in vitro* and tumor growth in a xenograft mouse model (13). Using similar *in vitro* approaches, we found that miR-21 contributes to the proliferative potential and survival of VS cells, confirming the functional significance of miR-21 overexpression in these cells. The tumor suppressor PDCD4 (programmed cell death 4) is an important functional target of miR-21 and the down-regulation of PDCD4 by miR-21 in colorectal cancer stimulated invasion, extravasation, and metastasis (31,32). It would be interesting to know whether PDCD4 is a target of miR-21 in VSs. In addition to supporting VS cell proliferation and survival, there is some evidence suggesting that miR-21 may also contribute to the cystic phenotype that is occasionally observed in more aggressive tumors. MiR-21 increases matrix metalloproteinase-2 (MMP-2) expression in cardiac fibroblasts via the PTEN pathway and MMP-2 has been implicated in cyst development in VSs (33,34).

It is clear that miR-21 plays a significant role in the regulation of multiple pathways controlling cell proliferation in many cancers and this attribute makes it a very attractive target for the development of new therapies. Future experiments aimed at manipulating miR-21 expression in xenograft or genetic animal schwannoma models will allow us to test the relative importance of miR-21 on VS growth *in vivo*. Furthermore, the application of proteomics strategies in these cells should help identify new molecular targets of miR-21 as has been demonstrated in the MCF-7 breast cancer cell line (20,35). It has also been shown that miR-21 plays a role in non-

neoplastic processes. Cai et al. outlined the potential role of miR-21 in the molecular management of heart disease (36). The application of these methodologies and findings in other biologic systems may shed new light on the molecular pathways controlling tumor growth and help identify novel therapeutic targets for the treatment of NF2 or sporadic VS.

Should miR-21 be confirmed to be integral to the growth and regulation of VS, it is possible to clinically manipulate this biologic phenomenon. Using molecules that can bind and interfere with these microRNAs, i.e., RNA interference (RNAi), would be one strategy to down regulate this pathway. Early clinical trials using this approach have been used in patients with macular degeneration and respiratory syncytial virus infections (37). A miR-21 specific RNAi knockdown could be achieved systemically by administering a viral gene expression vector; however, the side-effects on a patient may be undesirable because of off target effects. A more focused delivery via an endoscopic direct injection, or delivery during an intentionally incomplete resection of the VS could be an alternative method.

Conclusion

Since PTEN regulates signaling through the growth-promoting PI3K/AKT pathway, our findings suggest that miR-21 may be a suitable molecular target for therapies aimed specifically at reducing VS growth.

Acknowledgments

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Figure 1.

A, Quantitative real-time RT/PCR results showing relative fold increase in expression of miR-21 in each of the eight vestibular schwannomas compared to control pooled normal vestibular and greater auricular nerve samples. *B*, Delta C_T values were used to determine a statistically significant difference in miR-21 expression in tumors ($\Delta C_T 7.53 \pm 0.274$ SE) compared to normal controls ($\Delta C_T 4.81 \pm 0.897$ SE) ($p < 0.01$) (asterisk).

Figure 2.

A, Semi-quantitative RT/PCR results showing PTEN gene expression in tumors VS1 to VS4. B, PTEN protein detection by western blot in VS1 to VS4. β -actin served as a loading control.

Figure 3.

Examination of STAT3 by western blot in VS2 using an antibody that recognizes STAT3 protein regardless of the activation state and an antibody that specifically detects the activated (phosphorylated) form of STAT3 (PSTAT3) showed the presence of PSTAT3, which correlates with elevated miR-21 expression in this tumor. Because of this association, and relative differences in miR-21 expression, this miR may contribute to vestibular schwannoma cell proliferation.

Figure 4. Transfection of siRNA oligonucleotides in primary vestibular schwannoma cultures. Vestibular schwannoma cultures were transfected with a Cy3-labeled (red) siRNA oligonucleotide. Nuclei were labeled with Hoechst. Over 90% of cells were transfected. Scale bar=50 μ m.

Figure 5.

Anti-miR-21 suppresses PDGF induced VS cell proliferation. VS cultures were transfected with scrambled or anti-miR-21 probes and maintained in the presence or absence of PDGF-BB (20 ng/ml) for 24 hr in the presence of BrdU (10 μ M) *A and B*, Cultures transfected with scrambled (*A*) or anti-miR-21 (*B*) probes were immunostained with anti-S100 (green) and anti-BrdU (red) antibodies. Nuclei were labeled with Hoechst (blue). Scale bar=100 μ m. *C*, Mean percent of BrdU positive, S100 positive nuclei for four cultures from separate patients for each condition. Error bars present standard deviation. Significance for difference in means determined by two-tailed Student's t-test.

Figure 6.

Anti-miR-21 induces VS cell apoptosis. VS cultures were transfected with scrambled or anti-miR-21 probes. Condensed apoptotic nuclei were identified by TUNEL. *A and B*, Cultures transfected with scrambled (*A*) or anti-miR-21 (*B*) probes were immunostained with anti-S100 antibody (green) and labeled with TUNEL (red). Nuclei were labeled with Hoechst (blue). Scale bar=100 μ m. *C*, Mean percent of TUNEL positive, S100 positive nuclei for four cultures from separate patients for each condition. Error bars present standard deviation. Significance for difference in means determined by two-tailed Student's t-test.

Table 1

Gene specific reverse transcriptase polymerase chain reaction primer pairs and expected amplicon size.

Gene	RT/PCR Primers		
	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon Size (BP)
B2M	TCGCGCTACTCTCTCTTTCTGG	AAAGCAAGCAAGCAGAAATTTGG	401
PTEN	TTTGAGTTCCCTCAGCCGTTAC	CTGCTAGCCCTCTGGATTGACG	362
STAT3	GCAAGATCTGAATGGAAACAACC	CCCCTTTGTAGGAAACTTTTTC	332
CNTF	CAGGCCTGAACAAGAACAT	AAACGAAGGTCATGGATGGA	410
CNTR	ATGAGATTGGGACATGGAGTGAC	AGGGTCACAGATCTTCGTGGTAG	158
IL6	AGCCAGCTATGAACCTCCTTCTC	CAGGAACTGGATTCAGGACTTTTG	471
IL6Ra	TTTCAGGGTTGTGGAATCTTGC	TCAGAACAAATGGCAATGCAGAG	538
GPI30	ATACACAGATGAAGGTGGGAAAGG	TTGAAAGGATCTGGAAACATTAGGC	197
LIF	GTCACAACAACCTCATGAACCAG	GAAGACATCCTTACCCGAGGTG	416
LIFR	GGGCTCCTCATGATTTGAAGTG	CATATCTGAGGCCCAACTCCAG	485

BP = base pairs.

Table 2

Summary of RT/PCR gene expression results for normal vestibular nerve tissue, and VS1 to VS4.

	Normal Vestibular Nerve	VS1	VS2	VS3	VS4
<i>B2M</i>	+	+	+	+	+
<i>STAT3</i>	+	+	+	+	+
<i>CNTF</i>	+	+	+	+	+
<i>CNTFRα</i>	+	+	+	+	+
<i>IL6</i>	–	+	+	+	+
<i>IL6Rα</i>	–	+	+	+	+
<i>GPI30</i>	+	+	+	+	+
<i>LIFR</i>	–	+	+	+	+
<i>LIF</i>	–	+	+	+	+
<i>PTEN</i>	+	+	+	+	+

+ = Robust amplification of authentic amplicon; – = Unable to detect amplicon.