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miR-1 association with cell proliferation inhibition and apoptosis in vestibular schwannoma by targeting *VEGFA*

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ABSTRACT. A growing body of research has demonstrated the tumor suppressive function of microRNA (miR)-1 in many cancers. Our study aimed to investigate its role in vestibular schwannoma (VS). We examined miR-1 expression in 95 VS specimens and 79 normal vestibular nerves using quantitative real-time polymerase chain reaction. Moreover, miR-1 mimics, miR-1 inhibitors, and negative control oligonucleotides were transfected into HEI-193 human VS cells to investigate the functional significance of miR-1 expression in this condition at a cellular level. Finally, the role of vascular endothelial growth factor A (VEGFA) in miR-1-mediated HEI-193 cell growth was confirmed. miR-1 levels were significantly reduced in VS specimens compared with normal vestibular nerve tissues ($P < 0.001$). In addition, low levels of miR-1 were associated with larger

tumor volumes. In functional assays, miR-1 suppressed HEI-193 cell proliferation and colony formation, and enhanced apoptosis. *VEGFA* was verified as a target gene of miR-1, and *VEGFA* overexpression partially negated the effects of miR-1 on HEI-193 cells. These findings suggest that miR-1 suppresses VS growth by targeting *VEGFA*, and should be considered as a potential therapeutic target for treatment of this condition.

Key words: miR-1; Vestibular schwannoma; Proliferation; Apoptosis

INTRODUCTION

Vestibular schwannomas (VSs), benign intracranial tumors arising from the Schwann cell sheath of the vestibulocochlear nerve, are the most common tumors of the cerebellopontine angle. VSs frequently go undiagnosed until large enough to result in significant clinical symptoms. Current treatment modalities include microsurgical removal and stereotactic radiosurgery, but complications from such interventions are not uncommon (Arthurs et al., 2011). Recently, several genes and pathways associated with VS, including *NF2* (Chen et al., 2014), vascular endothelial growth factor A (*VEGFA*; Koutsimpelas et al., 2012; Dilwali et al., 2015), and PI3K/AKT (Jacob et al., 2008), have been identified. However, the molecular events involved in the development of this condition are not well understood. There is an urgent need to investigate new therapeutic targets for VS and develop novel treatment options.

MicroRNAs (miRs) comprise a family of endogenous short (19 to 22 nucleotides) noncoding RNAs that bind to the 3'-untranslated regions (UTRs) of target mRNAs, which are then cleaved or translationally repressed as a result (Bartel, 2004). miRs play pivotal roles in a wide array of biological processes, including tumor development (Chen, 2005; Cech and Steitz, 2014). Up-regulation of oncogenic miRs and/or down-regulation of tumor-suppressive miRs may contribute to the progression of many human tumors, including that of VSs (Cioffi et al., 2010; Wu et al., 2014; Shi et al., 2015; Xu and Zhao, 2016). For instance, Cioffi et al. (2010) found miR-21 to be overexpressed in VS specimens compared to normal vestibular nerve tissues. These authors also showed that anti-miR-21 decreases the proliferation and increases the apoptosis of VS cells, highlighting the potential value of miR-21 as a drug target in VS therapy.

As a candidate tumor-suppressive miR, miR-1 is down-regulated in breast cancer (Liu et al., 2015), esophageal squamous cell carcinoma (Jiang et al., 2016), lung cancer (Mataki et al., 2015), gastric cancer (Han et al., 2015), colorectal tumors (Taniguchi et al., 2016), hepatocellular carcinoma (Wang et al., 2015), clear cell renal cell carcinoma (Xiao et al., 2015), endometrial cancer (Yamamoto et al., 2015), and osteosarcoma (Zhu and Wang, 2016). miR-1 overexpression can suppress tumor cell growth, colony formation, migration, and invasion. However, the exact role of miR-1 in VS remains unknown. In the current study, we measured miR-1 expression in VS specimens and investigated its activity using *in vitro* gain- and loss-of-function approaches. Furthermore, to elucidate the molecular mechanisms behind miR-1-mediated VS growth, we assessed *VEGFA* as a downstream target of miR-1.

MATERIAL AND METHODS

Patients and tissue samples

This study was approved by the institutional review board of Xinxiang Central Hospital and Nanyang Central Hospital, and all patients provided their written informed consent. Fresh VS specimens (N = 95) were collected during surgical resection of unilateral sporadic VSs between January 2008 and December 2015. Normal vestibular nerve samples (N = 79) were obtained during vestibular neurectomies. Tissues were frozen immediately in liquid nitrogen and stored at -80°C until use.

Cell culture and transfection

The human vestibular schwannoma cell line HEI-193 was obtained from the House Ear Institute (Los Angeles, CA, USA). Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37°C in a humidified incubator. miR-1 mimics, miR-1 inhibitors (anti-miR-1), and the negative control (NC) were synthesized and purified by GenePharma (Shanghai, China). The *VEGFA*-expressing vector pcDNA3.1-VEGFA (lacking the *VEGFA* 3'-UTR) was obtained from Sangon Biotech (Shanghai, China). Transfection experiments were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using an RNeasy Mini kit (QIAGEN, Valencia, CA, USA), and 1 μg was reverse transcribed to complementary DNA with a PrimeScript reverse transcriptase reagent kit (TaKaRa, Dalian, China). Amplification and detection were carried out with SYBR *Premix Ex Taq* (TaKaRa) on the ABI Prism 7700 system (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions: 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 60 s. Relative expression of miR-1 was normalized to that of *U6* using the $2^{-\Delta Ct}$ method.

Cell proliferation assay

In vitro cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. HEI-193 cells were seeded on 96-well plates (5 x 10^3 cells/well) containing 100 mL medium in each well, and treated with 20 μL MTT (5 mg/mL; Sigma, St. Louis, MO, USA) for 4 h at 37°C. Subsequently, the MTT-containing medium was removed, and the cells were lysed with dimethyl sulfoxide (200 μL/well). Absorbance at 490 nm was then measured using a microplate reader (BioTek Instruments, Winooski, VT, USA).

Colony formation assay

Twenty-four hours after transfection, HEI-193 cells were seeded on six-well plates (1 x 10^3 cells/well) and incubated for a further 10 days, with the medium being replaced on day 5. Following incubation, the cells were fixed with 3.7% methanol and stained with 0.1% crystal violet.

Cell apoptosis analysis

Flow cytometric analysis was performed using an annexin V-fluorescein isothiocyanate (FITC) kit (Beckman Coulter, Brea, CA, USA). HEI-193 cells were washed twice with cold phosphate-buffered saline, resuspended in 500 μL cold annexin binding buffer, and stained with annexin V-FITC and propidium iodide (PI) for 15 min in the dark. The apoptotic index was calculated as the percentage of annexin V-positive/PI-negative cells.

Luciferase reporter assays

Two *VEGFA* 3'-UTR sequences were amplified and inserted into pGL3-control luciferase reporter plasmids, one containing the wild-type putative miR-1-binding site and a homologous sequence carrying mutations in this site. For the luciferase assay, HEI-193 cells were co-transfected with the *VEGFA* 3'-UTR reporter and miR-1 mimics on a 24-well plate using Lipofectamine 2000. After 48 h, the activities of firefly and *Renilla* luciferases in cell lysates were determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western blot analysis

Cells were harvested after transfection and proteins were extracted. Cell lysates were transferred to polyvinylidene fluoride membranes, which were then blocked for 2 h in 5% skim milk. The membranes were subsequently probed with diluted rabbit VEGFA or GAPDH antibodies overnight at 4°C, and incubated with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G antibody. Protein expression levels were measured using an enhanced chemiluminescence detection system (Millipore, Bedford, MA, USA).

Statistics

Statistical analyses were performed using the SPSS 15.0 software (SPSS Inc., Chicago, IL, USA), and differences between groups were assessed by the Student *t*-test. P values < 0.05 were considered statistically significant.

RESULTS

Decreased miR-1 expression in VS specimens

qRT-PCR revealed that miR-1 levels in VS tissues were significantly reduced (mean ± standard deviation: 8.51 ± 1.83) compared to those in normal vestibular nerve specimens (18.20 ± 3.65; $P < 0.001$; Figure 1A). We divided the 95 VS patients into high and low miR-1 groups according to the 50th percentile (median) of miR-1 expression. Low miR-1 levels were associated with larger VS tumor volumes ($P < 0.01$; Figure 1B).

miR-1 overexpression suppresses HEI-193 cell proliferation and colony formation, and promotes apoptosis

miR-1 mimics and anti-miR-1 were employed to alter miR-1 expression in HEI-

193 cells, and the expected effects were confirmed by qRT-PCR (Figure 2A). MTT and colony formation assays showed that both cell viability and colony formation ability were significantly suppressed in miR-1 mimic transfectants in comparison to NC cells (Figures 2B and 2C). As Figure 2D demonstrates, the percentage of apoptotic cells was significantly increased by elevated miR-1 presence in HEI-193 cells. Conversely, inhibition of miR-1 led to a significant increase in cell proliferation and colony formation, as well as a significant decrease in apoptosis.

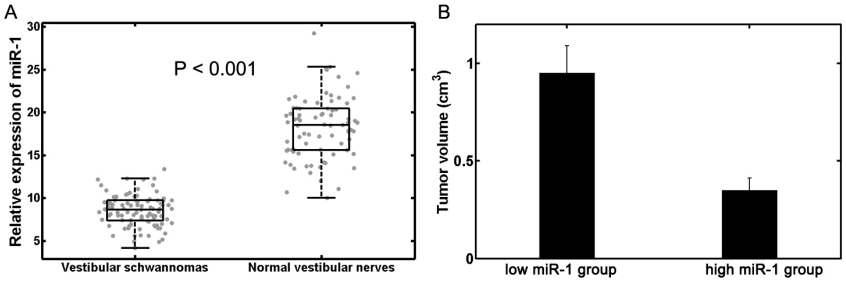

Figure 1. Relative miR-1 expression in human vestibular schwannoma (VS) samples and normal vestibular nerves. **A.** miR-1 expression was significantly lower in VS specimens compared to normal vestibular nerves ($P < 0.001$). **B.** Low miR-1 expression was associated with larger VS volumes ($P < 0.01$).

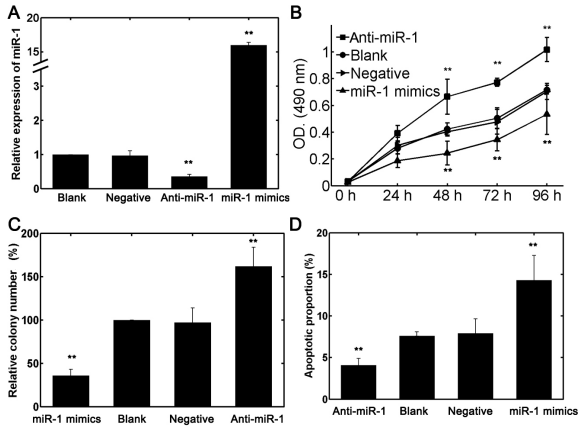

Figure 2. Effects of miR-1 on HEI-193 cell proliferation, colony formation, and apoptosis. **A.** Quantitative real-time polymerase chain reaction confirmed increased and decreased miR-1 expression in HEI-193 cells after transfection with miR-1 mimics and anti-miR-1, respectively. **$P < 0.01$ compared with the negative control. **B.** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays showed that miR-1 reduced cell proliferation *in vitro*. **$P < 0.01$ compared with the negative control. OD = optical density. **C.** Colony formation ability was notably weakened after transfection with miR-1 mimics. **$P < 0.01$ compared with the negative control. **D.** Flow cytometric analysis indicated that miR-1 promoted cell apoptosis. **$P < 0.01$ compared with the negative control.

VEGFA is a miR-1 target

As shown in Figure 3A, *VEGFA* contains one miR-1-binding site in its 3'-UTR. Up-regulation of miR-1 significantly decreased the relative luciferase activity associated with the wild-type *VEGFA* 3'-UTR construct compared with the NC, but had no effect on that associated

with the mutant *VEGFA* 3'-UTR sequence (Figure 3B). Furthermore, VEGFA protein levels in HEI-193 cells were significantly down-regulated following transfection with miR-1 mimics (Figure 3C). These data indicate that miR-1 directly targets *VEGFA* expression by binding to its 3'-UTR.

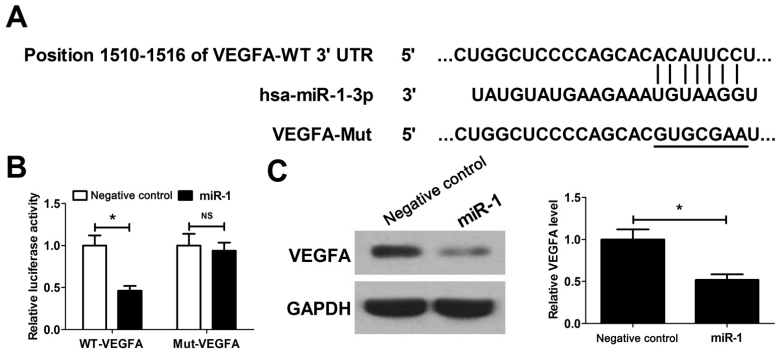

Figure 3. Vascular endothelial growth factor A (*VEGFA*) is a direct target of miR-1. **A.** Predicted miR-1 target sequence in the 3'-untranslated region (UTR) of *VEGFA*. **B.** Analysis of relative luciferase activity associated with VEGFA-WT and VEGFA-Mut. *P < 0.05. NS = not significant. **C.** miR-1 up-regulation in HEI-193 cells was associated with decreased VEGFA protein levels. *P < 0.05.

VEGFA overexpression partially negates the effects of miR-1 on HEI-193 cells

To verify the reversal of the effects of the tumor suppressor miR-1 by VEGFA, HEI-193 cells were transfected with miR-1 alone or co-transfected with miR-1 and *VEGFA*. As shown in Figure 4A, transfection with a *VEGFA* sequence lacking the 3'-UTR increased *VEGFA* expression in miR-1-overexpressing HEI-193 cells. In addition, up-regulation of VEGFA partially negated the effects of miR-1 on HEI-193 cell proliferation, colony formation, and apoptosis (Figures 4B-4D). These findings suggest that VEGFA is involved in miR-1-mediated HEI-193 cell growth.

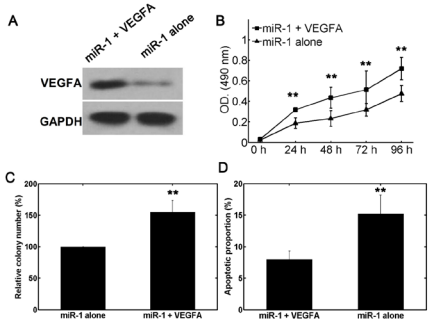

Figure 4. Vascular endothelial growth factor A (*VEGFA*) overexpression partially negated the effects of miR-1 in HEI-193 cells. **A.** Transfection with a *VEGFA* sequence lacking the 3'-untranslated region increased *VEGFA* expression in miR-1-overexpressing HEI-193 cells. **B.** *VEGFA* attenuated the effect of miR-1 on HEI-193 cell proliferation. **P < 0.01. **C.** *VEGFA* promoted colony formation in miR-1-overexpressing HEI-193 cells. **P < 0.01. **D.** Flow cytometric analysis indicated decreased cell apoptosis in HEI-193 cells co-transfected with miR-1 and *VEGFA* compared to those transfected with miR-1 alone. **P < 0.01.

DISCUSSION

Identification of new molecules involved in VS growth is of crucial importance to develop novel therapeutic strategies for this disease. Although Torres-Martin et al. (2013) reported decreased expression of miR-1 in human VS tissues in 2013, its effect on VS cell growth and associated molecular mechanisms have not yet been described. In the present study, we confirmed the down-regulation of miR-1 in VS specimens, with this phenomenon being particularly noticeable in those isolated from large tumors. Further *in vitro* experiments revealed that miR-1 overexpression can suppress VS growth through targeting of *VEGFA*.

In recent years, a growing body of research has demonstrated the tumor suppressive function of miR-1 in many cancers. This miR suppresses the growth of esophageal squamous cell carcinoma via down-regulation of MET, cyclin D1, and CDK4 expression (Jiang et al., 2016). Restoration of miR-1 in breast cancer cells inhibits their proliferation and motility, and increases their apoptosis *in vitro* by targeting *K-RAS* and *MALAT1* (Liu et al., 2015). In addition, down-regulation of miR-1 promotes tumor cell migration and invasion in laryngeal and lung squamous cell carcinomas (Wang et al., 2011; Mataki et al., 2015), chordoma (Osaka et al., 2014), and endometrial cancer (Yamamoto et al., 2015). A recent clinical investigation revealed miR-1 to be significantly down-regulated in renal cancer samples, an event significantly associated with advanced clinical stage and poor overall survival (Xiao et al., 2015). Moreover, miR-1 enhances the chemosensitivity of A549 lung cancer cells to doxorubicin (Nasser et al., 2008). Taking these observations together, miR-1 can be seen to affect the biological behavior of tumor cells in several different aspects, and its tumor suppressive properties warrant further study in other malignancies.

We also investigated the mechanisms of miR-1-mediated tumor suppression in VS. VEGFA is a key regulator of angiogenesis and exerts important effects on tumor growth and metastasis (Carmeliet, 2005). Previous study has revealed that VEGFA signaling is aberrantly up-regulated in VS (Cayé-Thomasen et al., 2005; Koutsimpelas et al., 2012), and knockdown of VEGFA decreases proliferation in primary human VS cell cultures (Dilwali et al., 2015). In our study, raised miR-1 expression in HEI-193 cells reduced VEGFA protein levels, and a dual-luciferase reporter assay showed that miR-1 directly bound the 3'-UTR of *VEGFA*. Moreover, overexpression of VEGFA attenuated the tumor suppressive function of miR-1 in HEI-193 cells. Thus, VEGFA is involved in miR-1-mediated VS growth.

In conclusion, our study revealed that miR-1 may play an important role in VS growth by affecting cell proliferation and apoptosis; hence, it should be considered as a potential therapeutic target for treatment of this condition.

Conflicts of interest

The authors declare no conflict of interest.

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