

# P14ARF deficiency and its correlation with overexpression of p53/MDM2 in sporadic vestibular schwannomas

Ying Chen · Zhao-yan Wang · Hao Wu

Received: 25 February 2014 / Accepted: 3 June 2014 / Published online: 26 June 2014  
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**Abstract** Recent studies have shed considerable light into schwannomas. To date, only merlin has been identified as a hallmark or pathogenesis of both sporadic and NF2-related schwannomas. Here, we show, by immunoblot and immunohistochemical analyses of 58 sporadic vestibular schwannomas, that upregulation of p53 was observed in 90 % of tumors examined. No p53 mutations were found in 12 % tumors analyzed. Expression of p14ARF was negative in 95 % of tumors, while overexpression of MDM2 was found in all specimens. Aberrant DNA hypermethylation of the p14ARF promoter was observed in three of seven tumors examined (43 %), associated with remarkably decreased mRNA levels. The very high degree of concordance in the aberrations of the p14ARF/MDM2/p53 pathway in this tumor may be considered to be a new player in the pathogenesis of sporadic vestibular schwannomas. Moreover, expression of p21 (waf1) was negative in all tumors, suggesting that the aberration of this pathway is associated with greater attenuation of p21-mediated signals that are critical for growth inhibition. These data are in agreement with the model in RT-4 rat schwannoma cells: i.e., overexpression of ARF was associated with accumulation of p21 expression both at protein and mRNA levels. ShRNA knock-down of p53 expression attenuated p21-mediated increase in cellular arrest in the G1-phase, suggesting that p14ARF regulates p21 protein levels through a p53-dependent pathway. Thus, this study reveals a high degree of concordance in the aberrations of the p14ARF/MDM2/p53 pathway with the development of sporadic vestibular schwannomas.

**Keywords** p14ARF · p53 · MDM2 · p21 (waf1) · Sporadic vestibular schwannomas

## Introduction

Schwannomas are benign, and typically encapsulated tumors of the nerve sheaths composed of Schwann cells. Vestibular schwannomas (VS) are usually slow-growing tumors that develop from the vestibular nerves supplying the inner ear. VS can also press on the facial nerve causing facial paralysis. If the tumor becomes large, it will eventually press against nearby brain structures, becoming life-threatening. About 95 % of VS are sporadic and unilateral, and about 5 % occur in the inherited disorder with Neurofibromatosis type 2 (NF2) related [16].

The *Ink4/ARF* locus encodes two tumor suppressor proteins, p16Ink4a and p14ARF, that govern the antiproliferative functions [22]. It has been shown that p14ARF can activate the p53 pathway by interacting with and inhibiting the ubiquitin ligase activity of MDM2, hence, preventing the polyubiquitination, nuclear export, and cytoplasmic degradation of p53 [27]. P53 is a key regulator of cell cycle checkpoints and apoptosis, controlling the transcription of a number of genes, including p21 (waf1) and BAX. P21 (waf1) is an important cellular checkpoint molecule for the inhibition of activities of a range of cyclin-CDKs [7]. Our previous study in vestibular schwannoma indicated that merlin exerts its antiproliferative effect, at least in part, by maintaining p21 expression, and loss of p21 is a prominent feature of merlin-deficient schwannomas [26].

The p14ARF/MDM2/p53 pathway is therefore critical for normal cell cycle progression [23]. P14ARF, MDM2, and p53 have been proved to present frequent mutations or loss of expression in brain tumors and malignant

Y. Chen · Z. Wang · H. Wu (✉)  
Department of Otolaryngology, Head and Neck Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Kongjiang Road #1665, Shanghai 200092, China  
e-mail: wuhao622@sina.cn

mesothelioma [4, 19, 20]. Studies on schwannoma development in patients suggest that a third hit in addition to the alteration of the *NF2* gene may be necessary for the development of this tumor [18, 24, 25]. It has been demonstrated that tumors with inactivated *NF2* show frequent homologous deletions of the *Ink4/ARF* locus, and mesothelioma develops at high incidence in *NF2* and *Ink4/Arf* conditional knockout mice [1, 8, 9, 14]. These observations indicate that functional inactivation of *NF2* leads to tumor development in a permissive (*Ink4/Arf* deficient) background and *Ink4/Arf* may well be a candidate for the site of a third mutational event for the development of schwannomas. Copy gain of *MDM2* gene was observed in vestibular schwannoma and the copy gains of certain tumor-related genes may play a role in its biological behavior [13].

In the present study, we provide first evidence that loss of physiologic levels of p14ARF is a prominent feature in sporadic vestibular schwannomas, suggesting that disruption of the p14ARF/MDM2/p53 pathway is a fundamental event underlying the development of sporadic vestibular schwannomas.

## Materials and methods

### Subjects

58 paraffin-embedded tumor specimens, of these, seven fresh tumors were surgically resected from patients with sporadic vs. Myelinated nerve (MN) and Cranial nerve (CN) VIII were harvested from autopsy patients.

### Ethics statement

All patients were in the Department of Otolaryngology, Xinhua Hospital, between January 1, 2007, and December 31, 2008. Written informed consent was obtained from each subject and the approval for the study was provided by the Ethics Committee of the Shanghai Jiaotong University School of Medicine.

### Cells and transfection

Human Schwann cells were purchased from Sciencell (SC-1700). PcDNA3-*p19ARF* was kindly provided by Dr Charles J. Sherr (Department of Genetics, St. Jude Children's Research Hospital, Memphis, USA). pSUPER-p53 was constructed to knock-down *p53* as published previously [3]. The pSUPER-Scramble plasmid was used as the nonsense control [17]. Cells were transiently transfected with expression constructs using Lipofectamine 2000 reagent (Invitrogen, Carisbad, CA).

### Promoter methylation analysis

DNA was extracted from schwannomas and myelinated nerve using Tissue Genomic Isolation Kits (Dingguo Biotech, China). Methylation-specific PCR (MSP) distinguishes unmethylated from methylated alleles based on the sequence changes produced after bisulfite treatment of DNA, which converts unmethylated cytosines to uracil, and subsequent PCR using primers designed for either methylated or unmethylated DNA [6]. DNA methylation patterns in the CpG islands of the p14ARF gene were determined by MSP, according to the protocol of CpGenome™ FAST DNA Modification kit (S7824, Chemicon International, CA).

### MDM2 amplification

Real-time PCR analysis was performed on RotorGene RG-3000 (Corbett Research, Australia). The MDM2 levels were normalized by control gene GAPDH.

### p53 DNA sequencing

DNA was extracted as above and amplified by nest PCR, using four primer sets covering every exon of the p53 CDS [15]. The PCR products were sequenced.

### mRNA expression

Total RNA was extracted by homogenization in 1 mL TRIzol reagent (Invitrogen, Carlsbad CA, USA). The RNA was reverse transcribed using PrimeScript™ RT reagent Kit (Takara). Real-time PCR analysis was done as above, using the primers: p14ARF [11], p21 [2] and MDM2 [21].

### Western blotting analysis

The tissues and cells were prepared in lysis buffer of MC-CellLytics Kit (Shenergy Biocolor, Shanghai, China). Antibodies used as follows, merlin (1:200 sc331; Santa Cruz), p53 (1:500; 2,524, Cell Signaling), p14ARF (1:250; 2,407, Cell Signaling), p19ARF (1:200; sc32748, Santa Cruz), MDM2 (1:200; SPM344, Santa Cruz), p21 (1:500; 2,946, Cell Signaling), CDK2 (1:333; PC44, CalBiochem), CDK4 (1:50; sc260, Santa Cruz), cyclinD1 (1:500; sc20044, Santa Cruz), cyclinE (1:125; sc25303, Santa Cruz), and GAPDH (1:500; AG019, Beyotime). Then, the blot was incubated with a secondary antibody, IRDye 800 conjugated affinity purified anti-mouse or anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA, USA) and detected with Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, Nebraska, USA).

## Immunohistochemistry

Four- $\mu\text{m}$ -thick sections were deparaffinized and dehydrated, and then treated with methanol containing  $\text{H}_2\text{O}_2$  for 5 min to inhibit endogenous peroxidase. Sections were washed in PBS, immersed in citric acid buffer, microwaved for 5 min for antigen recovery. Primary antibodies used were as follows: an anti-p21 monoclonal antibody (MoAb) (1:100; 2,946, Cell Signaling, USA), an anti-p53 MoAb (1:50; 2,524, Cell Signaling), an anti-human p14ARF MoAb (1:100; 2,407, Cell Signaling), and an anti-human MDM2 MoAb (diluted 1:50; SPM344, Santa Cruz). Then, the slides were treated with biotinylated secondary antibody. Finally, the slides were treated with Horseradish peroxidase-labeled streptavidin for 10 min. As a chromogen, 3,3-diaminobenzidine tetrahydrochloride was used, and the sections were counterstained with hematoxylin. Slides were evaluated by two or three authors independently.

## Results

Loss of p14ARF expression was a prominent feature of sporadic schwannomas

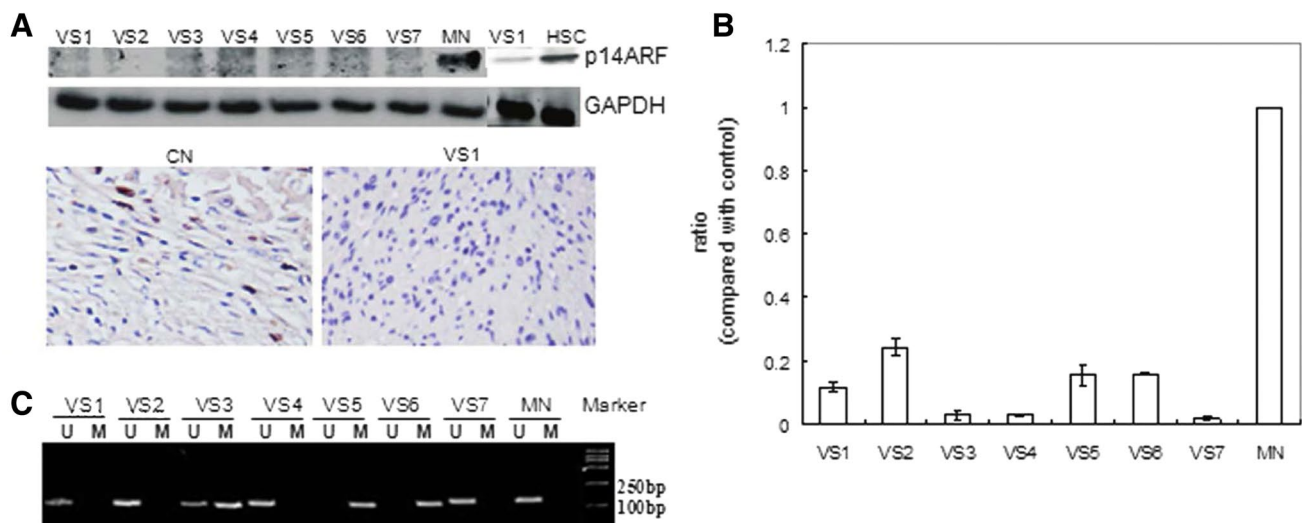
It is well established that the cells achieve replicative immortality by inactivating either p14ARF or p53 genes but not both. We analyzed p14ARF expression in VSs, MN, and Human schwann cells (HSC) as controls by Western

blotting. As shown in Fig. 1a, p14ARF protein levels in VS specimens were much lower than in MN and HSC. Furthermore, loss of p14ARF expression (<5 % immunostaining) was detected in 55 out of the 58 VS specimens, compared to CN VIII (Table 1; Fig. 1a). These results are consistent with our finding that reduced levels of p14ARF protein expression were preceded by >fourfold reduction in the mRNA expression in VS specimens compared with MN (Fig. 1b), suggesting that the reduced level of p14ARF protein is likely due to the reduction in its mRNA in schwannomas.

Here, the data indicate that deficiency of p14ARF expression is a prominent feature of schwannomas. P14ARF normally functions to safeguard cells against sustained and potentially oncogenic hyperproliferative signals, and its loss strongly predisposes to tumor development. Thus, loss of physiologic levels of p14ARF may play an important role in sporadic vestibular schwannomas formation.

Aberrant methylation of the p14ARF promoter in sporadic schwannomas

Next, we examined VS specimens and MN to see if there was methylation alteration in a CpG-rich region of the transcription initiation site of the *p14ARF* gene. As shown in Fig. 1, promoter hypermethylation of the *p14ARF* gene was detected in three specimens, whereas no detectable hypermethylation was found in the remaining of VS specimens and MN.



**Fig. 1** Loss of p14ARF expression with aberrant hypermethylation in p14ARF gene promoter in sporadic schwannomas. P14ARF protein levels were assessed by Western blotting analysis (**a**, above) or by immunohistochemistry at  $\times 40$  magnification (**a**, bottom). Real-time PCR was performed to assess *p14ARF* mRNA (**b**) in sporadic VS and MN, HSC or CN VIII as controls, and normalized to GAPDH

expression. Error bar indicates the standard error of the mean of three independent experiments. The presence of a PCR product in *Lanes M* indicates the presence of methylated promoter of p14ARF in VS3, 5 and 6; the presence of product in *Lanes U* indicates the presence of unmethylated promoter in the remaining of schwannomas and myelinated nerve (**c**)

**Table 1** Alteration of ARF, p53, MDM2 and p21 in sporadic VS

Characteristics	Number	Age (years)	Tumor size (mm)	Patients (male/female)
Overall	58	45.3 ± 12.9	2.9 ± 1.3	26/32
p53				
Protein				
Normal	6	59.3 ± 6.3	2.9 ± 1.0	3/3
Over <sup>a</sup> (%)	52 (90 %)	43.7 ± 12.5	2.9 ± 1.3	23/29
cDNA				
WT	7	44.0 ± 12.2	2.8 ± 1.2	3/4
MT(%)	0 (0 %)	/	/	/
ARF				
Protein				
Normal	3	42.7 ± 3.1	2.2 ± 0.8	1/2
Deficiency <sup>b</sup> (%)	55 (95 %)	45.5 ± 13.2	2.9 ± 1.2	25/30
DNA methylation				
U	4	40.8 ± 8.5	2.8 ± 1.3	2/2
M(%)	3 (43 %)	48.3 ± 16.9	2.8 ± 1.3	1/2
MDM2				
Protein				
Normal	0	/	/	/
Over <sup>a</sup> (%)	58 (100 %)	45.3 ± 12.9	2.9 ± 1.3	26/32
DNA				
Normal	7	44.0 ± 12.2	2.8 ± 1.2	3/4
Amplification	0 (0 %)	/	/	/
p21				
Protein				
Normal	0	/	/	/
Deficiency <sup>b</sup> (%)	58 (100 %)	45.3 ± 12.9	2.9 ± 1.3	26/32

<sup>a</sup> The slide was judged to exhibit aberrant overexpression of p53 or MDM2 when the percentage of the cells with positive nuclear staining exceeded 10 %

<sup>b</sup> The slide was judged to exhibit deficiency expression of ARF or p21 when the percentage of the cells with positive nuclear staining <5 %

### Elevation of p53 expression and lack of gene mutations in merlin-deficient vestibular schwannomas

To investigate the relationship between merlin deficiency and p53 status in schwannomas, we examined merlin and p53 expression in sporadic VS and MN or HSC as controls by Western blotting analysis. As seen in Fig. 2, merlin protein levels in VSs were much lower but p53 protein levels were much higher than in MN and HSC. These results are consistent with our finding of prominent expression of p53 (>10 % immunostaining-positive) in 52 out of 58 paraffin-embedded VS specimens as compared to the CN VIII (Table 1; Fig. 2a).

To evaluate the p53 mutational status in these schwannoma tumors, DNA sequencing was performed for p53 DNA isolated from VS specimens. No mutations were detected (Table 1).

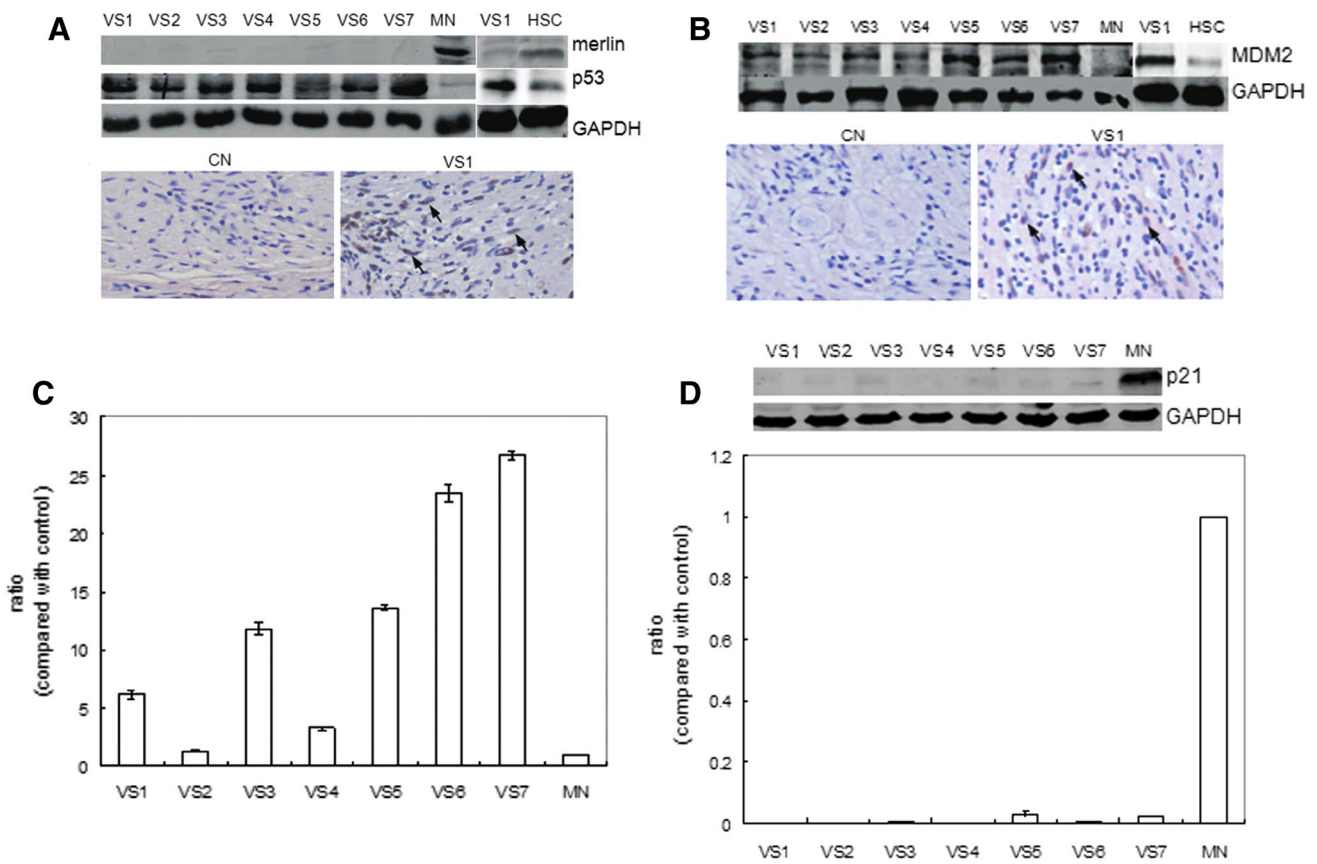
Aberrant overexpression of MDM2 is accompanied by loss of p21 (waf1) in p14ARF-deficient schwannomas

The p14ARF/MDM2/p53 pathway is important for tumor surveillance. To examine this point, we assessed MDM2

expression in seven sporadic VSs and MN or HSC as controls by Western blotting analysis. As shown in Fig. 2b, MDM2 protein levels in sporadic VS specimens were much higher than in MN and HSC. Furthermore, overexpression of MDM2 protein (>10 % immunostaining-positive) was detected in all 58 VS specimens as compared to CN VIII (Table 1; Fig. 2b). Immunofluorescence staining of VS showing nuclear localization is indicated by the arrows in Fig. 2b.

We then evaluated the levels of MDM2 mRNA by real-time RT-PCR analysis. As shown in Fig. 1c, compared with MN, there was >threefold increase of MDM2 mRNA expression in six out of seven VS specimens examined, while one tumor showed >1.2-fold increase of MDM2 mRNA expression, yielding an 86 % concordance rate between elevated MDM2 mRNA and protein expression in VS specimens. We also examined the *MDM2* gene copies by real-time PCR analysis. As shown in Table 1, compared with myelinated nerve, there was no apparent amplification of *MDM2* copies in the VS specimens.

To determine if loss of p14ARF expression affected cellular checkpoint protein p21 expression, we analyzed p21



**Fig. 2** Merlin deficiency accompanied by elevation of p53 and MDM2 expression, with reducing p21 expression in sporadic schwannomas. Western blotting analysis was performed to assess merlin and p53 protein levels (a, above) and immunostaining for p53 (arrows) at  $\times 40$  magnification (a, bottom), to assess MDM2 protein levels (b, above) or by immunohistochemistry (arrows) at  $\times 40$

magnification (b, bottom). Real-time PCR was performed to assess *MDM2* mRNA normalized to GAPDH expression (c). To assess p21 protein (d) or mRNA normalized to GAPDH expression in VS and MN, HSC or CN VIII as controls. Error bar indicates the standard error of the mean of three independent experiments

protein and mRNA levels in seven VS specimens and control by Western blotting and real-time PCR. As shown in Fig. 2D, p21 protein and mRNA levels in sporadic VS specimens were much lower than in MN. These results are consistent with our finding (Table 1) that loss of p21 expression (<5 % immunostaining) was detected in all 58 VS specimens, and indicated that loss of p14ARF may have led to the lost p21 expression in schwannomas, possibly through p53 inactivation.

These results are consistent with our previous finding that p21 suppressed cell growth through inhibition of CDK2/4 activity, and suggest that loss of p21 may be causative for the attenuation of cell cycle arrest in schwannomas.

#### Regulation of p21 expression by P14ARF and activation of cell cycle arrest through a p53-dependent pathway in RT-4 rat schwannoma cells

To examine the role of p14ARF in p21 expression, we ectopically over expressed p19ARF in RT-4 rat schwannoma

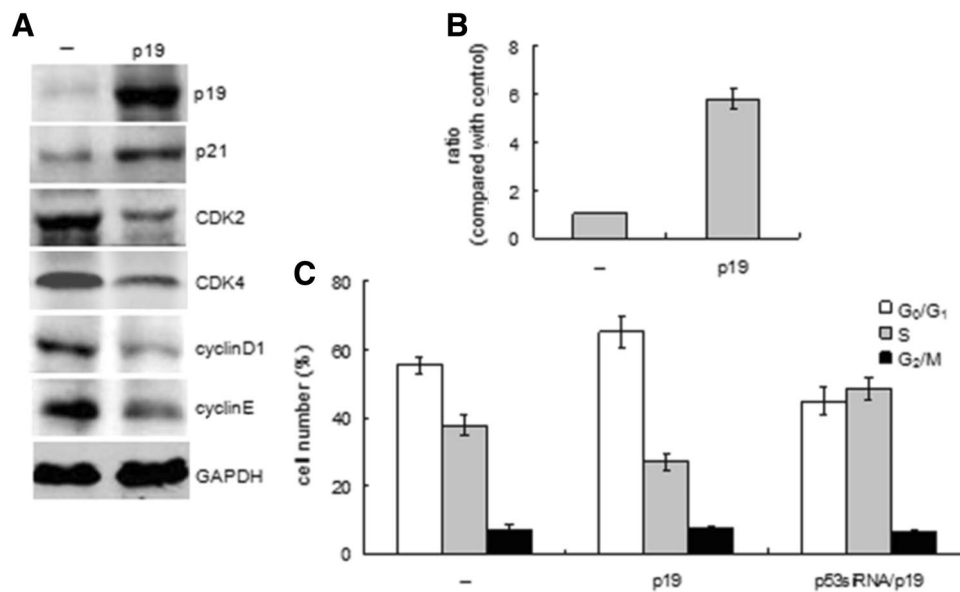
cells. As shown in Fig. 3a, cells overexpressing p19ARF showed remarkably increased endogenous p21 protein levels. These data suggest that level of p21 protein is physiologically regulated by ARF in RT-4 rat schwannoma cells.

P21 inhibits cell proliferation by suppressing the expression of CDKs/cyclins. To examine this point, the levels of CDK2, CDK4, cyclin D1 and cyclin E were measured by Western blotting in RT-4 cells. The significant decrease levels of CDK2, CDK4 and cyclin D1, cyclin E were detected in the cells transfected with *p19ARF*, as compared to the cells transfected with the control vector (Fig. 3a bottom).

We then evaluated p21 mRNA by real-time RT-PCR to determine whether mRNA accumulation contributed to elevated p21 levels. As seen in Fig. 3b, overexpression of p19ARF induced significantly increased *p21* mRNA levels compared to the control vector, suggesting that p19ARF may regulate p21 expression at the level of mRNA synthesis or stability in RT-4 cells.

To address whether p19ARF-induced p21 expression was p53-dependent, we used a pSUPER-p53 targeting





**Fig. 3** Upregulation of p21 by Overexpression of *ARF* in RT-4 rat schwannoma cells. Cells were transfected with or without *p19ARF* expression for 48 h. P19ARF, p21, CDK2, CDK4, cyclin D1 and cyclin E protein levels were assessed by Western blotting analysis (a), or *p21* mRNA expression was determined by real-time PCR, normalized to *GAPDH* expression. Error bar indicates the standard error of the

mean of three independent experiments (b). Cells were co-transfected with or without *p53* or control shRNA and *p19ARF* expression vector for 48 h. The flow cytometry analysis was carried out. Error bar indicates the standard error of the mean of three independent experiments (c)

the *p53* gene to silence *p53* expression in the cells overexpressing p19ARF. P21 inhibits CDKs/cyclins activities and blocks cell cycle progression at the G1-phase. To examine the alteration in the cell cycle pattern of RT-4 cells transfected with *p19ARF* or *p53* shRNA expression vectors, flow cytometry analysis was carried out. As shown in Fig. 3c, the fraction of G1-phase cells was increased after overexpression of p19ARF but decreased following the expression of *p53* shRNA and ARF, as compared to the cells transfected with control vector, indicating knock-down of p53 expression attenuated p21-mediated increase in the G1-phase and the cell cycle underwent an arrest at the G1-phase induced by ARF through a p53-dependent pathway. These data suggested that a restoring ARF function in schwannomas would properly upregulate p21 through p53-dependent pathway to activate the cell cycle arrest.

## Discussion

Here, we show that loss of p14ARF expression both at the mRNA and protein levels appears to be a near universal event in merlin-deficient sporadic vestibular schwannomas isolated from human patients. The schwannomas lacking p14ARF exhibit relatively high levels of wt p53 correlated with MDM2 overexpression. This aberration of the p14ARF/MDM2/p53 pathway is associated with greater

attenuation of p21 expression in schwannoma patients. These data are in agreement with the mode of action of ARF in RT-4 rat schwannoma cells: i.e., ARF regulates p21 protein levels in a p53-dependent manner. Thus, we provide evidence for a critical role of the p14ARF/MDM2/p53 pathway in the pathogenesis of sporadic vestibular schwannomas.

Mutations in the *NF2* gene are detected in schwannomas and meningiomas, supporting its role as a classical tumor suppressor gene. The analyses of malignant mesotheliomas from asbestos-treated *NF2* ( $\pm$ ) mice reveal homozygous deletion of *Ink4/ARF* locus [1, 8, 9]. A synergy of *NF2* and *Ink4/ARF* mutations, but not p53 is found in meningiomas [10]. Thus, alterations of the *Ink4/ARF* cell cycle regulatory pathways and *NF2*/merlin-AKT signal transduction pathways seem to be critical events that cooperate to drive tumorigenesis. These data are in agreement with our observation in sporadic vestibular schwannomas suggesting that *NF2*/merlin mutations combined with *p14ARF* deficiency may induce this tumor formation. These findings are consistent with cancer being a multistep process involving the accumulation of somatic genetic changes that enable tumor cells to override fail-safe mechanisms regulating normal cell proliferation.

Methylation-induced gene silencing is an important alternative form in the progression to cancer. It has also been shown that aberrant methylation is the most frequent way for expression changes of the *p14ARF* gene in the

human tumors [12]. Here, we found that 43 % (3 of 7) VSs were aberrantly hypermethylated in *p14ARF* gene promoter region, partially correlating with decreased *p14ARF* mRNA in schwannoma patients (Fig. 1c). Other studies have shown that epigenetic modification of genes occurs in a high frequency in merlin-deficient schwannomas [28]. Thus, our results suggest that epigenetic modification of *p14ARF* via hypermethylation represents a meaningful mechanism for the inactivation of this gene, at least, in some of sporadic vestibular schwannomas. However, p14ARF methylation in schwannomas requires further studying.

It is a well-accepted notion that cells infected with oncogenic protein myc genotypes for p53 mutation or p14ARF loss but not both. Although p53 mutations are the most common molecular lesions in human cancer, overexpression of p53 but no p53 mutations are reported in human schwannomas [5]. These are comparable to our observations in this study that schwannoma tumors lacking p14ARF exhibited relatively high levels of p53 but no p53 mutations (Figs. 1, 2 and Table 1). On the other hand, we found aberrant upregulation of MDM2 correlated with p53 overexpression in p14ARF-deficient schwannomas (Fig. 2 and Table 1). This aberration of the p14ARF/MDM2/p53 pathway may play an important role in sporadic vestibular schwannoma formation.

Our findings demonstrate that p14ARF-deficient schwannomas were accompanied by a loss of p21 (Fig. 2 and Table 1). Thus, consistent with the model in RT-4 rat schwannoma cells that ARF regulates p21 expression (Fig. 3a, b), the present study indicates that p14ARF-deficient schwannomas have lost p21 expression, thus unable to regulate normal cell proliferation. The p14ARF protein can physically interact with p53 and activate p53-dependent transcription. P21 protein plays a critical role in cell cycle control by arresting cell cycle at the G1-phase. Knock-down of p53 expression attenuated p14ARF-mediated increase in the G1-phase fraction (Fig. 3c) and it suggests that p14ARF may be required for p53 function in suppressing cell proliferation in Schwann cells. Taken together, our working hypothesis is that p14ARF deficiency abrogates p53-dependent transcriptional activity on p21 induction and leads to defects in cell cycle regulation, and confers a selective growth advantage for vestibular schwannomas.

Schwannoma is the third most common tumor of the central nervous system, after gliomas and meningiomas. The anatomical location of vestibular schwannomas being close to facial nerves and brain structures makes operations difficult [1]. New treatment is needed. It is well established that activation of p14ARF can decrease levels of MDM2 which in turn activates p53 transcription. Our study suggests that development of schwannomas lacking p53-inducible cell cycle arrest may critically depend on the loss of p14ARF. These findings are consistent with the data of the

RT-4 cell line, suggesting that restoring ARF functions in schwannoma cells may have several functions in schwannomas: (1) it may upregulate p21 expression and activate the cell cycle arrest through p53-dependent transcription. (2) it may properly downregulate MDM2, activate the cell cycle arrest or apoptotic machinery downstream of the sensory signals that normally lead to p53-dependent activation and help eliminate schwannomas by repairing the impaired growth regulatory pathway.

**Acknowledgments** This work was supported by the Grant 2009CB521703 from 973 Program of China to Hao Wu and the Natural Science Foundation of China (30801286 and 30973307) to Hao Wu.

**Conflict of interest** The authors declare that they have no competing interests.

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