ARTICLE IN PRESS

Please cite this article in press as: Wu H, et al., Involvement of p21 (waf1) in merlin deficient sporadic vestibular schwannomas, Neuroscience (2010), doi: 10.1016/j.neuroscience.2010.06.051

Neuroscience xx (2010) xxx

INVOLVEMENT OF P21 (WAF1) IN MERLIN DEFICIENT SPORADIC VESTIBULAR SCHWANNOMAS

H. WU,[a] Y. CHEN,[a] Z. WANG,[a] W. LI,[b] J. LI,[b] L. ZHANG[b] AND Y. LU[b]*

AQ: 1 [a]*Department of Otolaryngology-Head & Neck Surgery, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, 1665 Kongjiang Road, Shanghai 200092, PR China*

[b]*Laboratory of Biochemistry and Molecular Biology, Tongji University, 1239 Siping Road, Shanghai 200092, PR China*

Abstract—Previous studies have demonstrated that merlin acts as a tumor suppressor by blocking Ras-mediated signaling. However, the mechanism by which merlin controls cell proliferation has remained obscure. Here we show that merlin deficient tumors exhibited loss of p21, concomitant with elevated CDKs/cyclin D1 levels in sporadic vestibular schwannomas (VS) from clinic patients. Likewise, silencing of merlin gene expression in the cell lines resulted in downregulation of p21. Furthermore, we find that merlin-enhanced p21 protein stability, rather than increased RNA accumulation, was responsible for the elevated p21 levels. Interestingly, p21 was required to maintain merlin levels and the inhibitory effect of merlin on Ras signaling was partially overridden by knockdown of p21. Consistent with the observation that over-expression of merlin arrested cell growth at G1-phase, the current study indicates 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. **© 2010 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: Merlin, p21 (waf1), CDKs, vestibular schwannomas.

Neurofibromatosis type 2 (NF2) is a cancer predisposition syndrome phenotypically characterized by the occurrence of multiple nervous system tumors, such as schwannomas and meningiomas (Ferner, 2007). Mutations in the *NF2* gene are detected in NF2-related schwannomas, supporting its role as a classical tumor suppressor gene (Lepont et al., 2008). However, the events leading to schwannomas development are still largely unknown.

The 595-amino-acid NF2 protein, merlin, is homologous to a subgroup of band 4.1 proteins, ezrin, radixin, and moesin, collectively known as the ERM protein family. On the basis of homology of the NF2 protein to the ERM proteins, it has been postulated that the NF2 protein likewise functions as a membrane–cytoskeleton linking protein (Johnson et al., 2002). Experiments have also demonstrated that merlin can mediate contact inhibition of growth through interactions with CD44 (Morrison et al., 2001), function as well as in epidermal growth factor receptor (Curto et al., 2007), RhoGTPase (Flaiz et al., 2009), phosphatidylinositol 3-kinase (Rong et al., 2004), ERK (Ammoun et al., 2008; Meloche and Pouyssegur, 2007), PAK (Kissil et al., 2003), and apoptosis (Schulze et al., 2002). Several evidence have implicated that merlin plays an important role in the down-regulation of Ras/Rac1 signaling, which involved in suppressing cell growth at G_0/G_1 (Shaw et al., 2001). These cell biology studies have provided a greater understanding of the role of merlin in regulating cell growth. However, the mechanism by which merlin regulates cell proliferation is not well understood.

Cell cycle progression is driven by cyclin-dependent kinases (CDKs) in association with cyclins. The activities of cyclin–CDK complexes are modulated by two classes of CDK inhibitor (CDKIs). The INK4 CDKI proteins sequester CDKs and inhibit the formation of CDK-cyclin complexes, whereas the p21Cip/p27Kip CDKIs bind to cyclin-CDK complexes (Reynisdóttir et al., 1995). P21 (also known as waf1, cip1 or sdi1), which acts primarily as a transcription target of p53, is an important cellular checkpoint molecule for the inhibition of a range of cyclin-CDKs activities. In previous studies, we and others demonstrated that association of p21 with CDK2/CyclinE blocks cell cycle progression at multiple points (Tang et al., 2006; Lu et al., 1998; Harper et al., 1993).

In the present study, we show that merlin deficiency is correlated with the loss of p21 in sporadic vestibular schwannomas (VS). These data are in agreement with the mode in the cell lines implicate that p21 protein levels is physiologically regulated by merlin.

EXPERIMENTAL PROCEDURES

Study subjects

Five fresh tumors were surgically resected from patients with sporadic VS. All patients were in the Department of Otolaryngology, Xinhua Hospital, between January 1, 2007 and December 31, 2008. Myelinated nerve (MN) and cranial nerve (CN) VIII (the part of a cochlear nerve) were harvested from autopsy patients. Ethical approval for this study and agreement by all patients were obtained from Xinhua Hospital.

Cells and transfection

Human Schwann cells (HSC) were purchased from Sciencell (SC-1700). NIH3T3 cells were cultured in DMEM medium supplemented with 10% FBS (Hyclone, Logan, UT, USA). DNA encoding HA tagged wild-type and A518- or D518-mutant-merlin were kindly provided by Dr. D.H. Gutmann (Washington University, St.

*Corresponding author. Tel: +86-21-65986230; fax: +86-21-65983793.
E-mail address: yanjunlu@hotmail.com (Y. Lu).
Abbreviations: CDKs, cyclin-dependent kinases; CN, cranial nerve; HSCs, human Schwann cells; MN, myelinated nerve; NF2, neurofibromatosis type 2; VS, vestibular schwannomas.

0306-4522/10 $ - see front matter © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuroscience.2010.06.051

tapraid3/zpn-nsc/zpn-nsc/zpn99907/zpn4876d07z | chindarkara | S=1 | 7/3/10 | 7:06 | Art: 12221

Louis, MO, USA) and Dr. G. H. Xiao (Fox Chase Cancer Center, Philadelphia, PA, USA). Myc-p21-pcDNA4 expression vector were generated as described (Tang et al., 2006). Merlin-target shRNA sequence against NF2 (#1, GCA GCA AGC ACA ATA CCA T; #2, TGG CCA ACG AAG CAC TGA T) (Striedinger et al., 2008; Okada et al., 2005) and p21 shRNA (#1, CTT CGA CTT TGT CAC CGA G; #2, GAC CAT GTG GAC CTG TCA C) (Gevry et al., 2007; Marusyk et al., 2007) were cloned into pSUPER-EGFP1 constructs (OligoEngine, Seattle, WA, USA). The pSUPER-Scramble plasmid (gat ccc cTT CTC CGA ACG TGT CAC GTt tca aga gaA CGT GAC ACG TTC GGA GAA ttt ttg gaa a) was used as the nonsense control (Pager and Dutch, 2005). Cells were transiently transfected with expression constructs using Lipofectamine 2000 reagent (Invitrogen, Carisbad, CA, USA). For half-life experiments, cells were treated with MG132 (Beyotime, Jiangsu, PR China), an inhibitor of proteasome at a final concentration of 20 μM for 2 h and with EST (CalBiochem-Novabiochem, La Jolla, CA, USA), a control inhibitor of non-proteasomal proteases at final concentration of 100 μM in ethanol.

Western blotting

The cells were prepared in lysis buffer of MC-CelLytics Kit (Shenergy Biocolor, Shanghai, PR China). The protein content was determined using the Bradford calorimetric assay method by Protein Quantitative Analysis Kit (Shenergy Biocolor). Antibodies were used against merlin (sc331; Santa Cruz, CA, USA), p-MEK(9121, Cell Signaling, USA), MEK(9122, Cell Signaling), p-ERK(9106, Cell Signaling), ERK(9102, Cell Signaling), p21 (2946, Cell Signaling), CDK2 (PC44, CalBiochem), CDK4 (sc260, Santa Cruz), cyclinD1 (sc20044, Santa Cruz), cyclin E (sc25303, Santa Cruz), HA (05-904, Upstate, USA), Myc (46-0603, Invitrogen), GAPDH (AG019, Beyotime). Then the blot was incubated with a secondary antibody, IRDye 800 conjugated affinity purified anti-mouse or anti-rabbit IgG (Rockland Immunochemicals, PA, USA) and detected with Odyssey Infrared Imaging System (LI-CDR Biosciences, NE, USA).

Real-time PCR analysis

Total RNA of cells and tissues was extracted by homogenization in 1 ml TRIzol reagent (Invitrogen), followed by chloroform re-extraction and isopropanol precipitation. The RNA was quantified with Eppendorf Biophotometer (Eppendorf, Hamburg, Germany). Total RNA was reverse transcribed using PrimeScript™ RT reagent Kit (DRR037A, Takara, Japan). Real-time PCR was done in a final volume of 20 μl containing 1.6 μl of each cDNA template, 0.4 μl of each primer (10 μM), and 10 μl of a SYBR Green master mix (2×) (Takara). Primers were synthesized (Sangon Bio-technology, PR China) as: 5′-GGC AGA CCA GCA TGA CAG ATT-3′ (sense) and 5′-GCG GAT TAG GGC TTCC TCT T-3′ (antisense) for *p21* (waf/cip1); 5′-CGC CCA TGA ACC CAA TTC-3′ (sense) and 5′-GGC TGT CAC CAA TGA GGT TGA-3′ (antisense) for *NF2 (merlin)*; 5′-GAA ATC CCA TCA CCA TCT TCC AGG-3′ (sense) and 5′-GAG CCC CAG CCT TCT CCA TG-3′ (antisense) for human GAPDH. The average of *p21* and *NF2* genes was normalized to GAPDH as endogenous housekeeping gene.

Cell cycle pattern analysis

Cells were washed twice with PBS, trypsinized and resuspended in PBS containing 0.1% Triton X-100 and RNase (1 mg/ml) (Sigma, St. Louis, MO, USA). The cell suspension was incubated at 37 °C for 30 min. Propidium iodide (Molecular Probes, Inc. Eugene, OR, USA) was added at a final concentration of 50 μg/ml and the cell suspension was kept at 4 °C for 1 h. The cells were filtered and the cell cycle was analyzed by flow cytometry with the FACScan system (Becton Dickinson, NJ, USA).

Immunohistochemistry

Four-μm-thick sections were deparaffinized and dehydrated, and then treated with methanol containing 0.3% H_2O_2 for 5 min to inhibit endogenous peroxidase. Sections were washed in PBS (pH 7.2), immersed in citric acid buffer (pH 6.0), microwaved for 5 min for antigen recovery and washed in PBS again. Primary anti-p21 monoclonal antibody (diluted 1:100; 2946, Cell Signaling) was incubated at 4 °C overnight. 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.

RESULTS

Merlin deficiency is accompanied by loss of p21 in sporadic vestibular schwannomas

Recent studies have shed considerable light into the cause of VS. Merlin is commonly lost in VS, concomitant with displayed heightened Ras/Rac signal transduction. Some studies have shown that merlin may control cell proliferation by interfering with cell cycle progression (Xiao et al., 2005). P21 is considered the most important cell cycle checkpoint protein. That led us to ask if p21 was involved in merlin deficient tumor formation. We first examined merlin and p21 expression in sporadic VS, and human normal MN or HSC as controls by Western blotting analysis. As seen in Fig. 1A above, merlin and p21 protein levels in sporadic VS specimens were much lower than in MN and HSC. Furthermore, loss of p21 expression (<5% immunostaining) was detected in the VS specimen by immunohistochemistry, as compared to human CN VIII (Fig. 1A bottom). These results suggest that the loss of physiologic levels of p21 is well correlated with merlin deficiency in sporadic VS.

We then evaluated *p21* mRNA by real-time RT-PCR (qPCR) analysis to determine whether mRNA accumulation contributed to elevated p21 levels in VSs and control. In contrast to the large differences in protein levels, *p21* mRNA levels were modestly different between VS tumors and human normal MN (Fig. 1B).

P21 inhibits cell proliferation in part 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 VS specimens and control. High levels of CDK2, CDK4 and cyclin D1 were detected in the VS specimens, whereas cyclin E expression was comparable between VS specimens and MN (Fig. 1C). These results are consistent with our previous finding that p21 suppressed cell growth by arresting cell cycle at G1 or G2 phase through binding and inhibition of CDK2/4 activity (Tang et al., 2006; Lu et al., 1996, 1998, 2000; Ma et al., 2008), and suggest that the loss of p21 may be causative for tumor formation in merlin deficient cells.

Merlin regulates p21 expression in cell line

To examine the role of merlin in p21 expression, we used merlin-targeted (NF2) shRNA to silence merlin expression in NIH3T3 and HSCs. As seen in Fig. 2A, B, cells transfected with merlin-targeted (NF2) shRNAs showed a

UNCORRECTED PROOF

tapraid3/zpn-nsc/zpn-nsc/zpn99907/zpn4876d07z | chindarkara | S=1 | 7/3/10 | 7:06 | Art: 12221

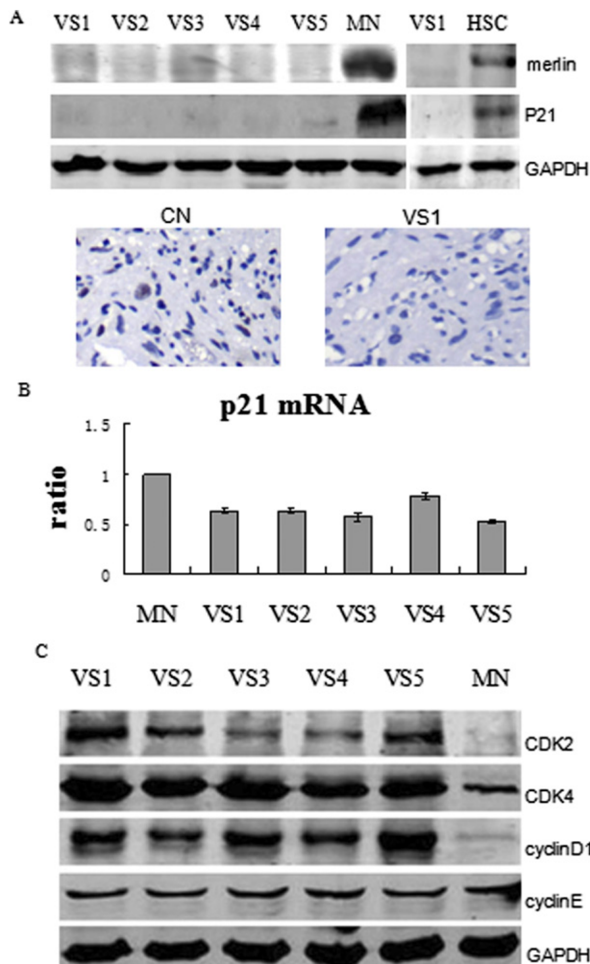

Fig. 1. Loss of p21 correlated with merlin deficient in VS. P21 and merlin protein levels were assessed by Western blotting analysis (A, above) or p21 protein levels were assessed by immunohistochemistry at 40×magnification (A, bottom), or p21 mRNA expression was determined by real-time PCR, normalized to GAPDH expression (B), or western blotting analysis was performed to assess CDK2, CDK4, cyclinD1 and cyclin E protein levels (C) in sporadic VSs and human normal (MN), CN VIII or HSC as controls. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

strong reduction of endogenous merlin expression, accompanied by a significant decrease in p21 protein level compared to the cells without reduction of merlin expression. These data suggested that the level of p21 protein is physiologically regulated by merlin in the 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 NIH3T3 cells. High levels of CDK2, CDK4 and cyclin D1, cyclin E were detected in the cells transfected with merlin-targeted (NF2) shRNA1, as compared to the cells transfected with the nonsense control (Fig. 2C).

Reciprocally, the over-expression of merlin (NF2) in NIH3T3 and HSCs increased endogenous p21 protein levels (Fig. 3A, B). P21 inhibits CDKs/cyclins activities to block cell cycle progression at G1-phase. To examine the alteration in the cell cycle pattern of NIH3T3 cells transfected with merlin expression vector, the flow cytometry analysis was carried out. As expected, a fraction of G1-phase cells was increased after over-expression of merlin, as compared to the cells with control vector (Fig. 3C). Thus, we suggested that the cell cycle underwent to arrest at G1-phase induced by merlin.

We then evaluated *p21* mRNA by real-time RT-PCR (qPCR) analysis to determine whether mRNA accumulation contributed to elevated p21 levels. In contrast to the large differences in protein levels, *p21* mRNA levels were modestly different between merlin-transfected cells and the control cells (Fig. 3D left). More, no significant effect of merlin-targeted (NF2) shRNA1 on p21 mRNA level was confirmed in the cells (Fig. 3D right). Therefore p21 levels were elevated mainly due to the protein stabilization other than mRNA accumulation.

The effect of merlin on p21 protein levels was confirmed by co-expression of ectopic Myc-tagged p21 and HA-tagged wild-type merlin, A518- or D518-mutant-merlin. Merlin is phosphorylated at Ser518 by Rac/PAK signaling. It has been shown that the A518 phosphorylation-defective form of merlin inhibits Ras or Rac-induced activation as well as or better than the wild-type protein. D518 phosphorylated form of merlin is growth permissive and therefore represents the functionally inactive tumor suppressor (Kim et al., 2002). Immunoblot analyses showed strong ectopic Myc-p21 expression in wild-type and A518 merlin transfected cells (lane 4 and 5), but not in D518 phosphorylated form of merlin (lane 3), as compared to transfected with Myc-tagged p21 alone in NIH3T3 cells, (lane 2, Fig.

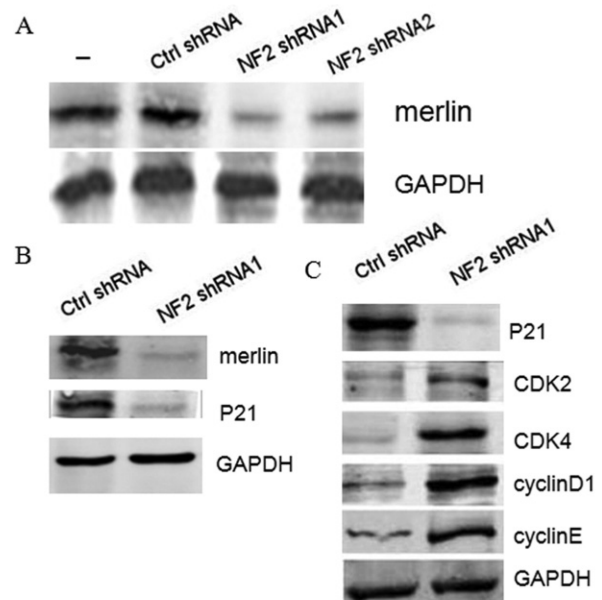

Fig. 2. Merlin regulated p21 expression in the cell lines. Western blotting analysis was performed to assess merlin and p21 levels after transfected with merlin-targeted (NF2) shRNA1 and 2 or nonsense control for 48 h in NIH3T3 cells (A), or in HSCs (B). Expression of levels of p21, CDK2, CDK4, cyclinD1 and cyclin E were assessed with or without merlin-targeted (NF2) shRNA1 in NIH3T3 cells (C).

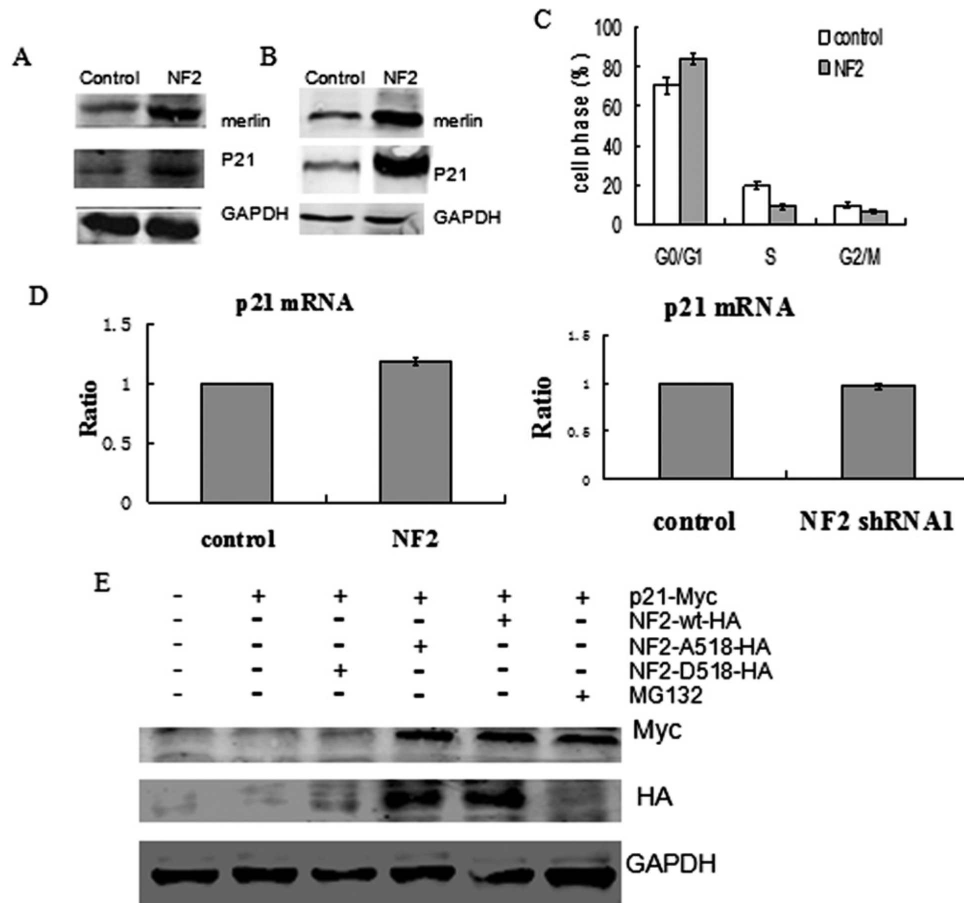

Fig. 3. Merlin increased p21 accumulation in a proteasome-dependent manner in the cell lines. Cells were transiently over-expressed with or without merlin (NF2) for 48 h. Merlin and p21 protein levels were measured by Western blotting analysis in NIH3T3 cells (A), or in HSCs (B), flow cytometry analysis was carried out in NIH3T3 cells (C). P21 mRNA expression was determined by real-time PCR, normalized to GAPDH expression after the over-expression of merlin (NF2) (D, left), or after knockdown of merlin (NF2) in NIH3T3 cells (D, right). (E) NIH3T3 cells were transfected with HA-tagged wild-type merlin, HA-tagged A518- and D518-mutant merlin and Myc-tagged p21 as indicated. Cells were either treated or untreated with proteasome inhibitor MG-132 for 2 h. After transfection for 48 h, ectopic p21 and merlin proteins were assessed using anti-Myc or anti-HA antibodies.

3E). In addition, to check the transfection efficiency among the samples, we used plasmid expressing non-relevant protein GFP to co-transfect with wild-type merlin, A518- or D518-mutant-merlin into the cells, and the results showed that they had the same transfection rate (data not shown). Thus, the data suggested that the A518 mutant can act as a constitutively active form of merlin as well as wild-type protein in regulation of p21, but not D518 tumor type merlin. The elevation of p21 protein level was also observed in the presence of the proteasome inhibitor MG-132 (lane 6, Fig. 3E). These data suggest that merlin causes the accumulation of p21 protein levels possibly through the inhibition of proteasome-mediated protein degradation.

P21 promotes merlin elevation in NIH3T3 cells

To further explore the relationship between merlin and p21, we next examined the effect of p21 on merlin levels in the cell lines. As seen in Fig. 4A, B, the endogenous merlin protein levels were increased following p21 over-expression in both NIH3T3 and HSCs. This effect was confirmed by the co-expression of ectopic HA-tagged-wild-type or -A518-mutant-merlin and Myc-tagged p21. Immunoblot analyses showed strong expression of HA-merlin in the two co-transfected cell lines, as compared to transfected with wild-type or A518 merlin alone in NIH3T3 cells (lane 2, 3 and 4, Fig. 4C). We found that p21 mediated induction of merlin stability was more effective by co-transfection with the A518 merlin, a constitutively active form of merlin than with wild-type merlin (lane 3 and 4). Expression of wild-type merlin alone in the presence of MG-132, but not EST, a control inhibitor of non-proteasomal proteases, resulted in its stabilization (lane 5 and 9). Immunoblot analyses also showed strong expression of HA-merlin in NIH3T3 cells that were transfected with both the wild-type merlin and p21 and the MG-132 (lane 6), and with the active merlin mutant (A518), p21 and MG132 (lane 7), and with the inactive merlin mutant (D518), p21, and MG132 (lane 8, Fig. 4C), implying that p21 promotes accumulation of merlin possibly through inhibition of the proteasome-mediated protein degradation in NIH3T3 cells.

Likewise, when silencing of p21 expression by p21 shRNAs, reduced levels of endogenous merlin was found

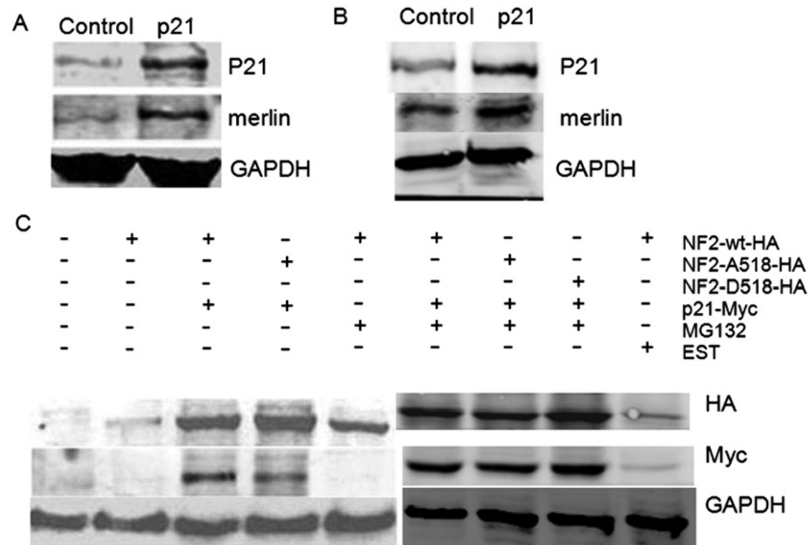

Fig. 4. P21 promoted merlin elevation in the cells. Cells were transiently over-expressed with or without p21 for 48 h P21 and merlin protein levels were measured by Western blotting analysis in NIH3T3 cells (A), or in HSCs (B). NIH3T3 cells were transfected with HA-tagged wild-type merlin, HA-tagged A518- or D518-mutant merlin and Myc-tagged p21 as indicated. Cells were either treated or untreated with proteasome inhibitor MG-132 or non-proteasomal proteases inhibitors EST. for 2 h. After transfection for 48 h, ectopic merlin and p21 proteins were assessed using anti-HA or anti-Myc antibodies (C).

both in NIH3T3 and HSCs (Fig. 5A, B), and increased phosphorylation of MEK and ERK by p21 shRNA were detected in the high cell density in NIH3T3 cells (Fig. 5C).

We then evaluated *merlin (NF2)* mRNA by real-time RT-PCR (qPCR) analysis to determine whether mRNA accumulation contributed to elevated merlin levels. In contrast to the large differences in protein levels, *merlin (NF2)* mRNA levels were modestly different between p21-transfected cells and the control cells (Fig. 5D left). More, no significant effect of p21 shRNA1 on *merlin (NF2)* mRNA level was confirmed in the cells (Fig. 5D right). Therefore merlin levels were elevated mainly due to the protein stabilization other than mRNA accumulation in NIH3T3 cells.

Collectively, these data indicate that there may be a positive induction loop between p21 and merlin and that a

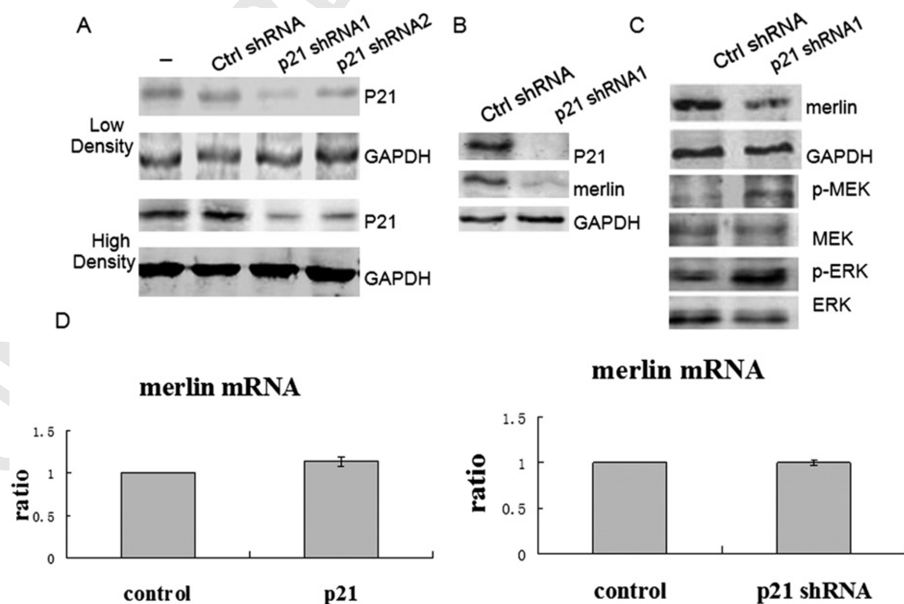

Fig. 5. Negative regulation of Ras signaling by merlin was partially overridden by knockdown of p21 in the cells. After transfected with p21-target shRNAs or nonsense control for 48 h, p21 were assessed by Western blotting analysis in low or high cell density in NIH3T3 cells (A), p21 and merlin protein levels were measured in HSCs (B), the levels of merlin, p-MEK and p-ERK were assessed with or without p21 knockdown in the high cell density in NIH3T3 cells (C). Merlin mRNA expression was determined by real-time PCR, normalized to GAPDH expression after the over-expression of p21 (D, left), or after knockdown of p21 (D, right).

Please cite this article in press as: Wu H, et al., Involvement of p21 (waf1) in merlin deficient sporadic vestibular schwannomas, Neuroscience (2010), doi: 10.1016/j.neuroscience.2010.06.051

ARTICLE IN PRESS

delicate balance must be maintained between these two molecules to control cellular proliferation.

DISCUSSION

In this report, we show that merlin deficient sporadic VS exhibited loss of p21. These data are in agreement with the mode of action of merlin in the cell lines: that is it regulates p21 protein levels in both NIH3T3 and HSCs. Consistent with our findings that p21 suppresses cell growth through inhibition of CDKs activities; the current study indicates that merlin exerts its antiproliferative effect, at least in part, via the induction of p21 expression. The results also led us to suggest that the loss of p21, a negative regulator of cell progression, is involved in tumor formation in merlin deficient Schwann cells.

Merlin is known to be a tumor suppressor by down-regulation of Ras or Rac related signaling (Kim et al., 2002; Morrison et al., 2007; Shaw et al., 2001). However, the mechanism by which merlin controls cell proliferation has not been clearly defined. Here, we show that the loss of merlin may be a fundamental event in tumor formation in clinical sporadic schwannomas patients. We further demonstrate that merlin deficiency was accompanied by the loss of p21, and the elevated cyclin D1, CDK2, CDK4 levels in sporadic schwannomas (Fig. 1A–C). These data indicate that merlin may be a tumor suppressor that blocks cell cycle progression in a p21-related manner.

The causal relationship between merlin and p21 was further confirmed by knockdown and over-expression studies pointing to a physiological role for merlin in the regulation of p21 expression in both NIH3T3 and HSCs (Figs. 2 and 3A, B). We also found that negative regulation of Ras signaling caused by merlin can be partially overridden by knockdown of p21 in the high cell density (Fig. 5C). These data are in agreement with the observation in the merlin deficient sporadic VS suggesting that merlin exerts its anti-proliferative effect, at least in part, in a p21-dependent manner.

In this study, we have found that *p21* mRNA levels were modestly different in merlin (NF2) over-expressed or knockdown cells compared with the control cells (Fig. 3D left and right). More, the presence of the proteasome inhibitor MG-132 was sufficient to mediate stabilization of p21 (Fig. 3E). The results led us to suggest that merlin may increase p21 accumulation in a proteasome-dependent manner.

Recently, two groups showed that over-expression of merlin are associated with accumulation of p21 and p27 (Xiao et al., 2005; Morrison et al., 2007). These are comparable to our observations in this study and emphasize the importance of p21 regulation by merlin. We showed that p21 inhibited cell proliferation by arresting cell cycle at the G_1 (Fig. 3C), in correlation with its ability to bind CDKs and inhibit their activities (Fig. 2C). Taken together, we suggest that p21 is required for merlin's function in suppressing cell proliferation.

Here, we also observed a physiological role for p21 in the regulation of merlin expression by knockdown and over-expression studies in both NIH3T3 and HSCs (Figs. 4 and 5A, B). More, *merlin (NF2)* mRNA levels were modestly different in p21 over-expressed or knockdown cells compared with the control cells (Fig. 5D, left and right). The presence of the proteasome inhibitor MG-132, but not EST, a control inhibitor of non-proteasomal proteases, was sufficient to mediate stabilization of merlin (Fig. 4C). Therefore merlin levels were elevated mainly due to the protein stabilization other than mRNA accumulation in NIH3T3 cells. Thus, we have provided evidence for a novel mechanism in which merlin and p21 require each other to maintain their protein levels to control cell proliferation.

Our findings here indicate that there is a positive induction loop between p21 and merlin and that a fine dynamics must be maintained between these two molecules to control cellular proliferation. Both merlin and p21 defects could cause transforming alterations leading to the development of schwannomas. Thus, this study fills the knowledge gap in the causes and detailed events in schwannoma development.

Schwannomas is the second most common tumor of the CNS. The anatomical location of VS being close to facial nerves and brain structures makes operations difficult. New treatment is needed. It is well understood that p21 suppresses cell growth. Our study here suggests that development of schwannomas may critically depend on lacking p21-induced cell-cycle arrest. These findings are consistent with the data of the cell lines, suggesting that restoring p21 (waf1/cip1) functions in schwannoma cells may activate the cell cycle arrest. Thus, p21 (waf1/cip1) is a molecule that should be explored for its potential in targeted gene therapy for schwannomas.

CONCLUSION

In conclusion, we have provided evidence for a novel phenotype in which both merlin and p21 require each other to maintain their protein elevation to control cell proliferation. This growth regulatory circuit would be disrupted, such as in schwannomas. These data highlight the importance of further studies into the role of merlin and p21 in both post-translational regulation of protein expression and its contribution to the transformed phenotype.

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

REFERENCES

Ammoun S, Flaiz C, Ristic N, Schuldt J, Hanemann CO (2008) Dissecting and targeting the growth factor–dependent and growth factor–independent extracellular signal-regulated kinase pathway in human schwannoma. Cancer Res 68:5236–5245.

Curto M, Cole BK, Lallemand D, Liu CH, McClatchey AI (2007) Contact-dependent inhibition of EGFR signaling by Nf2/Merlin. J Cell Biol 177:893–903.

Ferner RE (2007) Neurofibromatosis 1 and neurofibromatosis 2: a twenty first century perspective. Lancet Neurol 6:340–351.

Flaiz C, Ammoun S, Biebl A, Hanemann CO (2009) Altered adhesive structures and their relation to RhoGTPase activation in merlin-deficient schwannoma. Brain Pathol 19:27–38.

Please cite this article in press as: Wu H, et al., Involvement of p21 (waf1) in merlin deficient sporadic vestibular schwannomas, Neuroscience (2010), doi: 10.1016/j.neuroscience.2010.06.051

tapraid3/zpn-nsc/zpn-nsc/zpn99907/zpn4876d07z | chindarkara | S=1 | 7/3/10 | 7:06 | Art: 12221

ARTICLE IN PRESS

Gevry N, Chan HM, Laflamme L, Livingston DM, Gaudreau L (2007) p21 transcription is regulated by differential localization of histone H2A.Z. Genes Dev 21:1869–1881.

Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin dependent kinases. Cell 75:805–816.

Johnson KC, Kissil JL, Fry JL, Jacks T (2002) Cellular transformation by a FERM domain mutant of the Nf2 tumor suppressor gene. Oncogene 21:5990–5997.

Kim H, Lim JY, Kim YH, Kim H, Park SH, Lee KH, Han H, Jeun SS, Lee JH, Rha HK (2002) Inhibition of Ras-mediated activator protein 1 activity and cell growth by merlin. Mol Cells 14:108–114.

Kissil JL, Wilker EW, Johnson KC, Eckman MS, Yaffe MB, Jacks T (2003) Merlin, the product of the Nf2 tumor suppressor gene, is an inhibitor of the p21-activated kinase, Pak1. Mol Cell 12:841–849.

Lepont P, Stickney JT, Foster LA, Meng JJ, Hennigan RF, Ip W (2008) Point mutation in the NF2 gene of HEI-193 human schwannoma cells results in the expression of a merlin isoform with attenuated growth suppressive activity. Mutat Res 637:142–151.

Lu YJ, Tatsuka M, Takebe H, Yagi T (2000) Involvement of cyclin-dependent kinases in doxorubicin-induced apoptosis in human tumor cells. Mol Carcinog 29:1–7.

Lu YJ, Yamagishi N, Miyakoshi J, Noda A, Yagi T, Hiraku T (1996) Sites and types of UV-induced mutations leading to inactivation of the growth-arresting activity in p21 (waf1)(sdi1/cip1/waf1) cDNA. Carcinogenesis 17:2343–2345.

Lu YJ, Yamagishi N, Yagi T, Takebe H (1998) Mutated p21WAF1/CIP1/SDI1 lacking CDK-inhibitory activity fails to prevent apoptosis in human colorectal carcinoma cells. Oncogene 16:705–712.

Ma SL, Tang JJ, Feng JG, Xu YP, Yu XM, Deng QH, Lu YJ (2008) Induction of p21 by p65 in p53 null cells treated with doxorubicin. Biochim Biophys Acta 1783:935–940. AQ: 4

Marusyk A, Wheeler LJ, Mathews CK, DeGregori J (2007) p53 mediates senescence-like arrest induced by chronic replicational stress. Mol Cell Biol 27:5336–5351.

Meloche S, Pouyssegur J (2007) The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1 to S-phase transition. Oncogene 26:3227–3239.

Morrison H, Sherman LS, Legg J, Banine F, Isacke C, Haipek CA, Gutmann DH, Ponta H, Herrlich P (2001) The NF2 tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44. Genes Dev 15:968–980.

Morrison H, Sperka T, Manent J, Giovannini M, Ponta H, Herrlich P (2007) Merlin/neurofibromatosis type 2 suppresses growth by inhibiting the activation of Ras and Rac. Cancer Res 67:520–527.

Okada T, Lopez-Lago M, Giancotti FG (2005) Merlin/NF-2 mediates contact inhibition of growth by suppressing recruitment of Rac to the plasma membrane. J Cell Biol 171:361–371.

Pager CT, Dutch RE (2005) Cathepsin L is involved in proteolytic processing of the Hendra virus fusion protein. J Virol 79: 12714–12720.

Reynisdóttir I, Polyak K, Iavarone A, Massague J (1995) Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-b. Genes Dev 9:1831–1845.

Rong R, Tang X, Gutmann DH, Ye K (2004). Neurofibromatosis 2 (NF2) tumor suppressor merlin inhibits phosphatidylinositol 3-kinase through binding to PIKE-L. Proc Natl Acad Sci U S A 101:18200–18205.

Schulze KM, Hanemann CO, Muller HW, Hanenberg H (2002) Transduction of wild-type merlin into human schwannoma cells decreases schwannoma cell growth and induces apoptosis. Hum Mol Genet 1:69–76.

Shaw RJ, Paez JG, Curto M, Yaktine A, Pruitt WM, Saotome I, O'Bryan JP, Gupta V, Ratner N, Der CJ, Jacks T, McClatchey AI (2001) The Nf2 tumor suppressor, merlin, functions in Rac-dependent signaling. Dev Cell 1:63–72.

Striedinger K, VandenBerg SR, Baia GS, McDermott MW, Gutmann DH, Lal A (2008) The neurofibromatosis 2 tumor suppressor gene product, merlin, regulates human meningioma cell growth by signaling through YAP. Neoplasia 10:1204–1212.

Tang JJ, Shen C, Lu YJ (2006) Requirement for pre-existing of p21 to prevent doxorubicin-induced apoptosis through inhibition of caspase-3 activation. Mol Cell Biochem 291:139–144.

Xiao GH, Gallagher R, Shetler J, Skele K, Altomare DA, Pestell RG, Jhanwar S, Testa JR (2005) The NF2 tumor suppressor gene product, merlin, inhibits cell proliferation and cell cycle progression by repressing cyclin D1 expression. Mol Cell Biol 25:2384–2394.

(Accepted 23 June 2010)

UNCORRECTED PROOF

tapraid3/zpn-nsc/zpn-nsc/zpn99907/zpn4876d07z | chindarkara | S=1 | 7/3/10 | 7:06 | Art: **12221**