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Original Article

The ubiquitin ligase RNF126 promotes glioma cell proliferation by negatively regulating p27

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Abstract: It has been reported that ubiquitin ligase RNF126 (RING finger protein 126) targets tumor suppressor p21 for ubiquitin-mediated degradation, indicating that RNF126 is involved in the growth of breast and prostate cancers. However, the potential role and underlying mechanism of RNF126 in human glioma are still unclear. In the present study, we firstly studied the clinical relevance of RNF126 as well as the cell cycle negative regulation factor p27. We found that RNF126 was up-regulated in human glioma tissues, which was reversely related to the level of p27. Then, the role of RNF126 in glioma cell proliferation was investigated using RNF126 loss- and gain-of-function. The results showed that silencing of RNF126 by short hairpin RNA (shRNA) inhibited glioma cell proliferation, while overexpression of RNF126 significantly promoted it. Molecularly, RNF126 was found to physiologically interact with p27. And it was showed that down-regulation of RNF126 dramatically increased the protein level of p27 by decreasing its ubiquitination. On the contrary, overexpression of RNF126 remarkably reduced the protein level of p27 by aggravating its ubiquitination. Taken together, our findings indicate that RNF126 plays an important role in promoting the growth of glioma cells by negatively regulating p27.

Keywords: Ubiquitin ligase, RNF126, p27, glioma

Introduction

Human malignant glioma is the most ordinary and malignant tumor in the central nervous system [1]. In spite of significant progression in surgery and adjunctive therapy, the median survival time is only 12-15 months [2-4]. The poor prognosis of glioma is largely in virtue of their rapid growth and invasive/migratory nature. Thus, an understanding of the molecular mechanisms underlying the gliomagenesis is pivotal to seek specific molecular targets that could be served as a treatment for glioma.

Protein post-translational modification which includes ubiquitination and phosphorylation is important for activating the proteins and maintain their functions. Protein ubiquitination regulates a multitude of cancer-related cellular processes, including the cell cycle [5]. Ubiquitination of protein is typically sequentially mediated by 3 enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3) that controls substrate specificity [6]. Ubiquitin proteolytic system is a most important regulator of protein degradation. The aberrant accumulation of oncoproteins and abnormal degradation of tumor suppressor contribute to the development and progression of cancers. Thus, E3 ubiquitin ligases are considered as the next wave of molecules for targeted therapy [7-9]. Several specific and effective small molecular inhibitors of Mdm2 (Mouse double minute 2) have been reported to have substantial antitumor effects *in vivo* [7, 8]. A CRL E3 ligase (cullin RING ligase) inhibitor MLN4924 is undergoing testing for cancer treatment in clinical trials [9, 10].

RNF126 is a RING finger ubiquitin ligase that contains a Zn finger near the N-terminus and a RING finger at the C-terminus [11]. It has been

shown that RNF126 might be involved in the tumorigenesis [12-16]. Our preliminary experiment indicates that RNF126 is differentially expressed in glioma tissues and nontumor tissues, which suggests that RNF126 could be a potential protein participated in the development of glioma. Thus, the possible role and underlying mechanism of RNF126 in human glioma needs to be further clarified.

In this study, we aimed to investigate the role of RNF126 in the proliferation of glioma cells and its underlying mechanism. The proliferation and viability of human glioma cells were suppressed by knocking down of RNF126 and were amplified by overexpressing of RNF126. In addition, the effect of RNF126 on cell cycle negative regulation factor p27 was analyzed.

Materials and methods

Antibodies

RNF126 antibody was bought from Abcam (Cambridge, UK). Antibodies specific for p27, ubiquitin and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA).

Tissue samples

Seven specimens of human glioma tissues (age range: 18-70, Average age: 48) and seven specimens of nontumorous brain tissues (age range: 18-70, Average age: 47) were collected at the Shandong University Qilu Hospital (Jinan, China). The nontumor brain tissues were obtained from the patients undergoing surgery for internal decompression in cerebral trauma. All glioma patients had confirmed pathological diagnosis and were classified in accordance with the World Health Organization (WHO) criteria. Surgically resected tissues were subjected for histological diagnosis, and the remaining tissues were immediately frozen in liquid nitrogen and kept at -80°C in the fridge. Written informed consent was permitted by each patient and the use of human samples was authorized by the Research Ethics Committee of the Qilu Hospital of Shandong University.

Cell culture

Human glioma cell lines U251, U118, A172, U87 and human embryonic kidney cell line HEK293T were bought from the Cell bank of Shanghai, Type Culture Collection Committee, Chinese Academy of Sciences. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (TransGen, Beijing, China) in a humidified incubator with 5% CO_2 at 37°C.

Constructs and production of the lentivirus

For silencing of RNF126, three sets of shRNA duplexes were designed and synthesized as follows: shRNF126 #1 F, 5'-GATCGCACTCAAACCCTATGGACTTCAAGAGAGTCCATAGGGTTTGAGTGCTTTTTTG-3'; shRNF126 #1 R, 5'-AATTCAAAAAAGCACTCAAACCCTATGGACTCTCTTGAAGTCCATAGGGTTTGAGTGC-3'; shRNF126 #2 F, 5'-GATCGTCTAACCTCACCCTCTAATTCAAGAGATTAGAGGGTGAGGTTAGACTTTTTTG-3'; shRNF126 #2 R, 5'-AATTCAAAAAAGTCTAACCTCACCCTCTAATCTCTTGAATTAGAGGGTGAGGTTAGAC-3'; shRNF126 #3 F, 5'-GATCGCTTTGAAATAAACGGACGTTCAAGAGACGTCCGTTTATTTCAAAGCTTTTTTG; shRNF126 #3 R, 5'-AATTCAAAAAAGCTTTGAAATAAACGGACGTCTCTTGAACGTCCGTTTATTTCAAAGC; Control shRNA F, 5'-GATCTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTTG-3'; Control shRNA R, 5'-AATTCAAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAA-3'.

The RNF126 shRNAs and control shRNA oligomers were annealed and then subcloned into the pLV-shRNA plasmid using the *BamH* I and *EcoR* I cloning sites. To overexpress RNF126 in glioma cells, the RNF126 cDNA was inserted into the pWPXLd plasmid by using *BamH* I and *Mlu* I cloning sites. Cell transfection was performed with PolyJet (SignaGen, Gaithersburg, MD, USA) as described in the manufacturer's protocol. The lentiviruses were produced in HEK293T cells by co-transfecting the core plasmid and the helper plasmids.

Development of the stable cell lines

The stable cell lines was developed as Shi et al previously described [17, 18]. For stable silencing or overexpression of RNF126, the U251 cells were infected by control, shRNF126 #1, GFP, GFP-RNF126 lentiviruses, respectively. Forty-eight hours after infection, the cells were continuously provided with the medium containing 2.5 μg/ml puromycin (Sigma, St. Louis,

MO, USA). The survived cells were developed into cell lines that stably expressing control shRNA, shRNF126 #1, GFP or GFP-RNF126.

EdU assay

The cells that down-regulation or overexpression of RNF126 were seeded into 96-well plate at 4×10^3 cells per well. At the designated time point, the cells were reacted with 50 μM of 5-ethynyl-20-deoxyuridine (EdU; Ribobio, Guangzhou, China) for additional 2 h at 37°C. The cells were then fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.5% Triton-X-100 for additional 20 min at room temperature. After washing with PBS for five times, the cells were incubated with 100 μl of 1× Apollo® reaction cocktail for 30 min. Thereafter, the cells were stained with 100 μl of Hoechst 33342 (5 μg/ml) for 20 min and visualized with a fluorescent microscope (IX71; Olympus, Tokyo, Japan).

MTT assay

The MTT assay was performed as Wu et al previously described [19]. Two thousand cells in 200 μl of medium were seeded into 96-well plate and cultured under normal conditions. At the designated time point, 3-(4,5-Dime-thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) was applied into the medium, bringing its concentration to 0.5 mg/ml. After incubation for 4 h at 37°C, medium was sucked and 150 μl of DMSO was added to dissolve the crystals. The absorbances at 490 nm were measured by a SynergyMx Multi-Mode Microplate Reader (Biotek, Winooski, VT). The cell viability was calculated according to the absorbances.

Plate colony formation

The plate colony formation assay was performed as Wu et al previously described [19]. Four hundred cells that stably silencing or overexpressing of RNF126 were plated in 6 cm dish and subjedted for continuously culture for ten days. Then the cells were washed with PBS and fixed with 100% methanol. Finally, the cells were stained with 0.05% crystal violet to assess colony staining. After washing with PBS, the plates were taken a photograph with a camera for colony counting.

Western blotting

Equal amount of protein lysates were subjected for SDS-PAGE and then transferred to PVDF membrane. After blocking with 5% non-fat milk, the PVDF membrane was hybridized with primary antibodies (RNF126, p27, Ubiquitin and β-actin) at 4°C overnight and secondary antibodies at room temperature for 1 h. Bound antibodies were measured by the ECL Plus Western Blotting Substrate (Thermo Fisher, Waltham, MA, USA). The densities of bands were quantified by Image J Software (Wayne Rasband, National Institutes of Health, MD), and the relative level of protein was determined by normalizing the optical density value of interest to that of the loading control.

Immunoprecipitation and ubiquitination

The cells were pre-treated with MG-132 for 6 h before harvesting, and then lysed in a Triton-X-100-based lysis buffer (1% Triton-X-100, 150 mM NaCl, 20 mM HEPES, pH 7.4, 2 mM EDTA, 5 mM $MgCl_2$) for 1 h on ice. The lysates were incubated with indicated antibody for 16 h and then bound to protein A/G beeds for 4 h at 4°C with gentle agitate. The immunoprecipitates were washed three times in lysis buffer, and the proteins on the beads were recovered by boiling in SDS loading buffer and detected by western blotting. Ubiquitin-conjugated p21 was detected by immunoblotting with anti-ubiquitin antibody.

Statistical analysis

The results are from representative of experiments that repeated at least three times, and the quantitative data were expressed as means ± S.E.M. Differences in two groups were determined using a Student's t-test. P values < 0.05 were considered as statistically significant ($*P < 0.05$).

Results

RNF126 is conversely related to the level of p27 in human glioma tissues

It has been reported that tumor suppressor p21 is a substrate of RNF126 [12]. However, whether RNF126 could regulate the cell cycle negative regulation factor p27 is not clear. In order to address our hypothesis, we firstly ana-

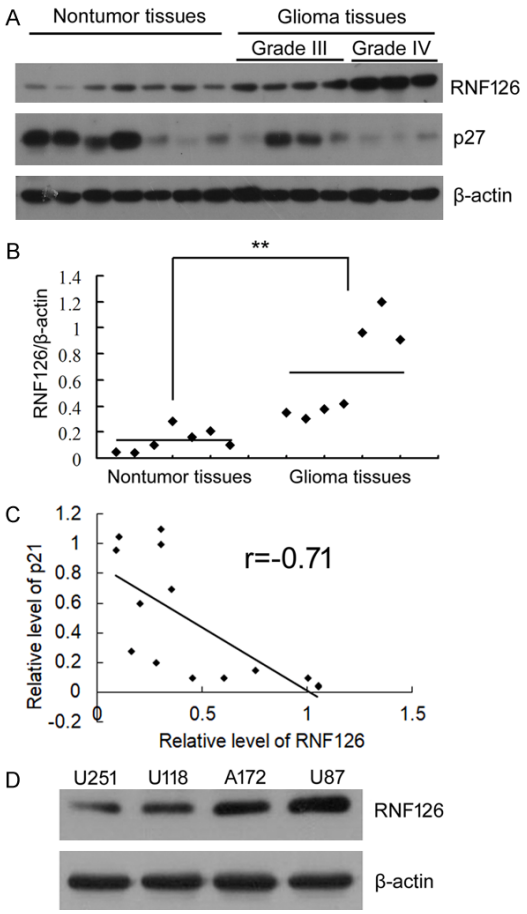


Figure 1. Expression of RNF126 and p27 in human glioma tissues. A. Total proteins isolated from nontumor brain tissue and glioma tissues were analyzed by western blotting for assessment of RNF126 and p27. B. Statistical chart showed the expression level of RNF126 in non-tumorous brain tissue and glioma tissues. C. The correlation analysis of RNF126 and p27 in human glioma tissues, r=-0.71. D. Western blotting assay showed the protein level of RNF126 in U251, U118, A172 and U87 cells. $^{**}P < 0.01$.

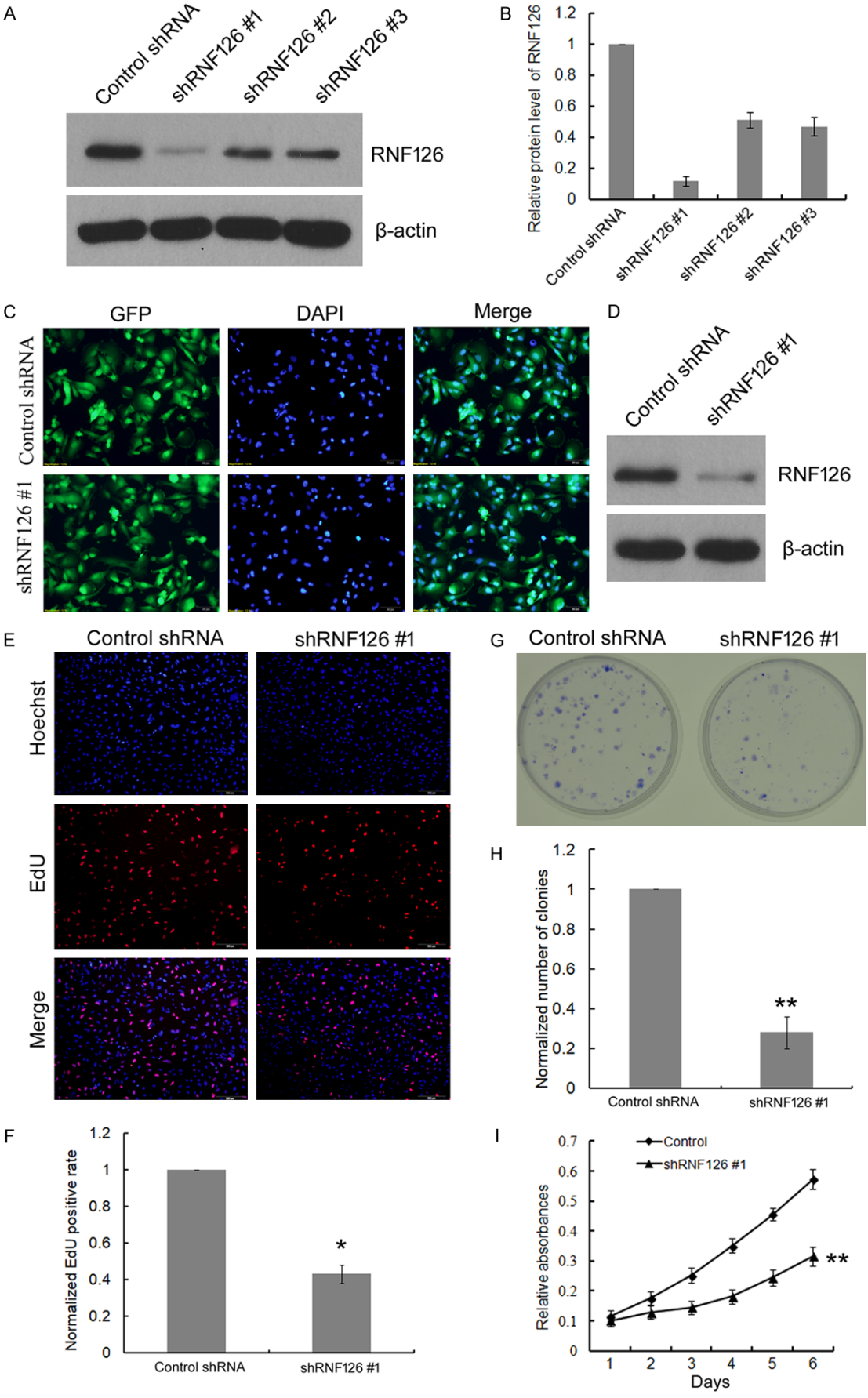
lyzed the expression of RNF126 and p27, and their relationship in clinical patients. The expression of RNF126 and p27 was detected by western blotting in 7 specimens of glioma tissues and 7 specimens of nontumor brain tissues. The representative blots showed that, compared with the nontumor brain tissues, RNF126 expression was significantly up-regulated in glioma tissues, especially in high grade glioma tissues (**Figure 1A** and **1B**). Interestingly, the up-regulation of RNF126 was associated with the loss of p27, indicating that the expression of RNF126 was conversely related to p27 in clinical samples (**Figure 1A** and **1C**). Additionally, we found that expression level of RNF126 was variable in glioma cell lines with different tumor malignancy (**Figure 1D**). Taken together, these results indicate that RNF126 expression is increased in human glioma tissues and RNF126 might be involved in the regulation of p27 in the development of human glioma.

Down-regulation of RNF126 suppresses the proliferation of human glioma cells

In order to explore the possible role of RNF126 in the proliferation of human glioma, we firstly knocked down RNF126 with RNF126 specific shRNAs and observed its effects on cell proliferation of U251 cells. For silencing of RNF126, three shRNA targets (shRNF126 #1, shRNF126 #2 and shRNF126 #3) were subcloned into the pLV-shRNA lentiviral vector and screened for their efficacy in suppressing RNF126 expression. As shown in **Figure 2A** and **2B**, the silencing efficiency of shRNF126 #1 was ~85%. Thereafter, we produced the lentivirus with shRNF126 #1 and the control shRNA vectors, which were used to develop the stable cell line that silencing of RNF126 (**Figure 2C** and **2D**). Next, we observed whether the cell proliferation was affected by silencing of RNF126 with the stable cell lines. The EdU incorporation assay displayed that, compared with the control group, EdU incorporated cells of the RNF126 down-regulated group reduced 57% (**Figure 2E** and **2F**). The ability of colony formation was also obviously decreased upon silencing of RNF126 (**Figure 2G** and **2H**). In addition, MTT assay showed that the cell viability of shRNF126 #1 group was significantly reduced, compared with the control group (**Figure 2I**).

Overexpression of RNF126 promotes the proliferation of human glioma cells

To further determine the role of RNF126, we examined the effect of RNF126 overexpression on cell proliferation of U251 cells. The stable cell lines that overexpressing GFP or GFP-RNF126 were established and identified by GFP imaging and western blotting (**Figure 3A** and **3B**). Then, we observed whether the cell proliferation was promoted upon RNF126 overexpression with the stable lines overexpressing GFP or GFP-RNF126. The EdU incorporation assay displayed that, compared with the GFP group, EdU incorporated cells of the GFP-RNF126 group increased by 110% (**Figure 3C**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Control shRNA
shRNF126 #1
shRNF126 #2
shRNF126 #3
RNF126
β-actin
B
Relative protein level of RNF126
1.2
1
0.8
0.6
0.4
0.2
0
Control shRNA
shRNF126 #1
shRNF126 #2
shRNF126 #3
C
GFP
DAPI
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Control shRNA
shRNF126 #1
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Figure 2. Knocking down of RNF126 inhibits the proliferation of human glioma cells. A. The silencing efficiency of RNF126 shRNAs was examined by western blotting. B. Histogram showed the silencing efficiency of RNF126 shRNAs. C. The GFP photograph showed the stable cell line that silencing of RNF126 in U251 cells, bar=50 μm. D. The stable cell line that silencing of RNF126 was verified by western blotting. E and F. EdU assay showed that RNF126 down-regulation inhibited cell proliferation in U251 cells. G and H. Plate colony formation assay showed that RNF126 down-regulation decreased the ability of colony formation in U251 cells. I. MTT assay showed that RNF126 down-regulation decreased the proliferative rate of U251 cells. $*P < 0.05$ and $**P < 0.01$.

and **3D**). The ability of colony formation was also obviously increased upon overexpressing of GFP-RNF126 (**Figure 3E** and **3F**). In addition, MTT assay showed that the cell viability was increased upon overexpression of GFP-RNF-126, compared with the GFP control (**Figure 3G**).

RNF126 promotes glioma cell proliferation by regulating the ubiquitination and degradation of p27

It has been reported that tumor suppressor p21 is a substrate of RNF126 [12]. In this report, it also mentioned that RNF126 knock down could increase the level of p27 [12]. Our above results have indicated that RNF126 was reversely related to the level of p27 in human glioma tissues. Thus, we asked whether RNF126 could regulate the ubiquitination and degradation of p27 in human glioma cells. Firstly, we investigated the interaction between RNF126 and p27 by co-immunoprecipitation assay. When 3×FLAG tagged RNF126 was over-expressed in cells, immunoprecipitation with an anti-FLAG antibody showed that 3×FLAG-RNF126 associated with p27 (**Figure 4A**). Then, the interaction between RNF126 and p27 was confirmed by the immunoprecipitation assay with the endogenous proteins (**Figure 4B**). Next, we showed that silencing of RNF126 significantly increased the protein level of p27, while overexpression of RNF126 obviously reduced it (**Figure 4C**). Similarly, down-regulation of RNF-126 remarkably decreased the ubiquitination of p27, whereas overexpression of RNF126 led to a much stronger ubiquitination of p27 (**Figure 4D**). In addition, we demonstrated that RNF126 mediated degradation of p27 could be blocked by proteasome inhibitor MG132 (**Figure 4E**). We also investigated whether purified GST-RNF126 could directly ubiquitinate 6*His-p27 *in vitro*. However, we failed to detect the ubiquitiantion of p27 by GST-RNF126 *in vitro*, suggesting that additional factors, such as p27 modifications, appropriate E2s, or other cofactors/proteins may be required for p27 ubiquitination by RNF-126 (data not shown). In summary, the above results suggest that RNF126 promotes glioma cell proliferation by regulating the ubiquitination and degradation of p27.

Discussion

With the deepening understanding of the causes of cancer, the interest of research has shifted to genetics and proteomics. The role of E3 ubiquitin ligases in tumorigenesis is constantly emerging with distinct biological activities. Multiple ubiquitin ligases, including Neural Precursor Cell Expressed, Developmentally Down-Regulated 4 (NEDD4) and WW domain-containing protein 2 (WWP2) etc, have been identified to be related with the tumorigenesis [20-25]. To find the glioma related E3 ligases, we examined the expression of RNF126 in glioma patients and nontumor brain tissues and discovered the candidate molecule, RNF126. However, there is large variation in the RNF126 expression in the glioma tissues. This might be due to the individual difference or different tumor grades. In fact, the last three patients with higher level of RNF126 are all grade four gliomas, which has even higher RNF126 expression. Accordingly, different level of expression of RNF126 in multiple glioma cell lines further indicates that variation of RNF126 level might represent different tumor malignancy in glioma.

Several evidences have already showed that RNF126 is associated with the tumorigenesis. First, the substrates of RNF126, like p21 [12], Bag6 (BCL2 associated athanogene 6) [26], and EGFR [13] are involved in oncogenesis. Second, RNF126 contains similar protein domains and structures as the E3 ligases, BCA2 (Breast cancer-associated gene 2), which plays an important role in the development and progression of breast cancer [27]. RNF126 is thus suspected to play a similar role with BCA2. In addition, it was also determined that RNF126 was expressed increasingly in several breast cancer cell lines [14]. In the present study, we

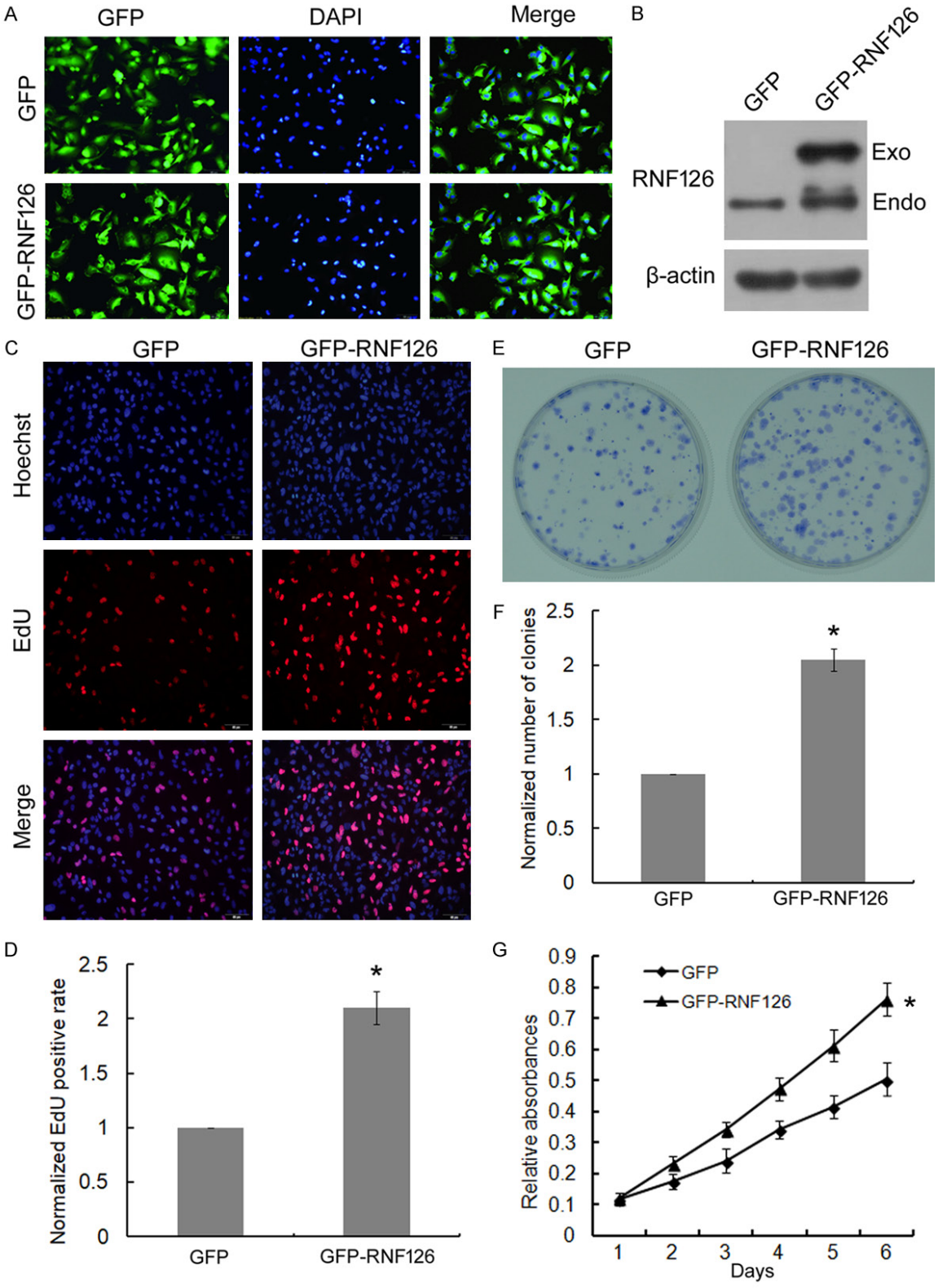

Figure 3. Overexpression of RNF126 promotes the proliferation of human glioma cells. A. The GFP photographs showed the stable cell line that overexpressing of RNF126 in U251 cells, bar=50 μm. B. The overexpressing efficiency of RNF126 was examined by western blotting with RNF126 antibody. C and D. EdU assay showed that RNF126 overexpression promoted cell proliferation both in U251 cells. E and F. Plate colony formation assay showed that RNF126 overexpression increased the ability of colony formation both in U251 cells. G. MTT assay showed that RNF126 overexpression increased the proliferative rate of U251 cells. *$P < 0.05$.

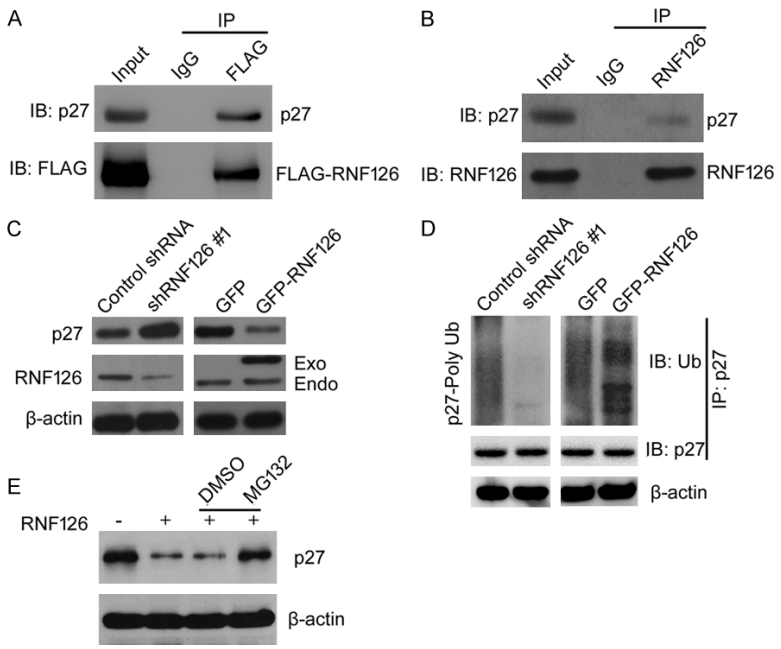

Figure 4. RNF126 regulated p27 by ubiquitination and degradation. A. Co-immunoprecipitation assay showed that overexpressed RNF126 interacted with p27. B. Co-immunoprecipitation assay showed that endogenous RNF126 interacted with p27. C. Silencing of RNF126 significantly up-regulated the protein level of p27, while overexpressing of RNF126 down-regulated it. D. Silencing of RNF126 significantly decreased the ubiquitination of p27, while overexpressing of RNF126 increased it. MG132 (20 μM) were added to the cells before harvest to prevent p27 degradation. E. RNF126 targeted degradation of p27 could be blocked by proteasome inhibitor MG132.

aimed to explore the role and underlying mechanism of RNF126 in the proliferation of glioma. Firstly, we revealed that the expression of RNF126 and p27 was conversely related in the human glioma tissues. Then, we determined the role of RNF126 in human glioma cell proliferation by knocking down and overexpressing approaches. Finally, we examined the effects of RNF126 on the ubiquitination and degradation of p27. Our existing findings indicate that RNF126/p27 pathway may be associated with the development of human glioma.

The cell cycle negative regulation factor p27 governing CDK activity is critical for regulating the cell cycle transition from the G0/G1 to the S phase [28]. The p27 levels are mainly regulated through poly-ubiquitination. Till now, it has been reported that the ubiquitination of p27 could be mediated by several ubiquitin ligases including S-phase kinase associated protein 2 (Skp2), p53-induced protein with a RING (Really Interesting New Gene)-H2 domain (PirH2), Kip1 ubiquitination-promoting complex (KPC1), seven in absentia homolog 1 (Siah1) and constitutively photomorphogenic 1 (COP1) [29-33]. Here, we discovered that RNF126 might be a newly identified ubiquitin ligase involving in the ubiquitination of p27. In our cancer model study, RNF126 knock down inhibited glioma cell proliferation by decreasing the ubiquitination and degradation of p27, while overexpression of RNF126 had opposite effect. Thus, these studies recapitulated the relationship between RNF126 and p27 *in vitro*. We also found that RNF126 overexpression is very common in different glioma patients. In addition, p27 down-regulation is frequently observed in RNF126 overexpressed patients. Together, our results demonstrated a link between RNF126 regulation, p27 stability, and glioma cell proliferation. The role of RNF126 in attenuating p27 might offer a new bridge for knowledge gap regarding the function of RNF126 in the development of glioma.

In summary, our present study discovered that RNF126 promoted glioma cell proliferation by targeting the cell cycle negative regulation factor p27 protein for ubiquitin-mediated degradation. More interestingly, the expression of RNF126 and p27 was found to be reversely related in human glioma patients. These findings suggest that RNF126 might be an oncogenic protein in gliomagenesis, which could be served as a potential glioma therapeutic target. It is possible that we can design specific inhibitor for RNF126 to suppress its function for those patients with high expression of RNF126. Also, the shRNA of RNF126 could be introduced to tumors to down-regulate the RNF126 expression so as to achieve the treatment of glioma patients.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

[1] Furnari FB, Fenton T, Bachoo RM, Mukasa A, Stommel JM, Stegh A, Hahn WC, Ligon KL, Louis DN, Brennan C, Chin L, DePinho RA and Cavenee WK. Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev 2007; 21: 2683-2710.

[2] Mrugala MM, Kesari S, Ramakrishna N and Wen PY. Therapy for recurrent malignant glioma in adults. Expert Rev Anticancer Ther 2004; 4: 759-782.

[3] Norden AD and Wen PY. Glioma therapy in adults. Neurologist 2006; 12: 279-292.

[4] Meyer MA. Malignant gliomas in adults. N Engl J Med 2008; 359: 1850; author reply 1850.

[5] Ohta T and Fukuda M. Ubiquitin and breast cancer. Oncogene 2004; 23: 2079-2088.

[6] Ciechanover A and Iwai K. The ubiquitin system: from basic mechanisms to the patient bed. IUBMB Life 2004; 56: 193-201.

[7] Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N and Liu EA. In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. Science 2004; 303: 844-848.

[8] Shangary S, Qin D, McEachern D, Liu M, Miller RS, Qiu S, Nikolovska-Coleska Z, Ding K, Wang G, Chen J, Bernard D, Zhang J, Lu Y, Gu Q, Shah RB, Pienta KJ, Ling X, Kang S, Guo M, Sun Y, Yang D and Wang S. Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. Proc Natl Acad Sci U S A 2008; 105: 3933-3938.

[9] Soucy TA, Smith PG and Rolfe M. Targeting NEDD8-activated cullin-RING ligases for the treatment of cancer. Clin Cancer Res 2009; 15: 3912-3916.

[10] Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, Brownell JE, Burke KE, Cardin DP, Critchley S, Cullis CA, Doucette A, Garnsey JJ, Gaulin JL, Gershman RE, Lublinsky AR, McDonald A, Mizutani H, Narayanan U, Olhava EJ, Peluso S, Rezaei M, Sintchak MD, Talreja T, Thomas MP, Traore T, Vyskocil S, Weatherhead GS, Yu J, Zhang J, Dick LR, Claiborne CF, Rolfe M, Bolen JB and Langston SP. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature 2009; 458: 732-736.

[11] Delker RK, Zhou Y, Strikoudis A, Stebbins CE and Papavasiliou FN. Solubility-based genetic screen identifies RING finger protein 126 as an E3 ligase for activation-induced cytidine deaminase. Proc Natl Acad Sci U S A 2013; 110: 1029-1034.

[12] Zhi X, Zhao D, Wang Z, Zhou Z, Wang C, Chen W, Liu R and Chen C. E3 ubiquitin ligase RNF126 promotes cancer cell proliferation by targeting the tumor suppressor p21 for ubiquitin-mediated degradation. Cancer Res 2013; 73: 385-394.

[13] Smith CJ, Berry DM and McGlade CJ. The E3 ubiquitin ligases RNF126 and Rabring7 regulate endosomal sorting of the epidermal growth factor receptor. J Cell Sci 2013; 126: 1366-1380.

[14] Wang Y, Deng O, Feng Z, Du Z, Xiong X, Lai J, Yang X, Xu M, Wang H, Taylor D, Yan C, Chen C, Difeo A, Ma Z and Zhang J. RNF126 promotes homologous recombination via regulation of E2F1-mediated BRCA1 expression. Oncogene 2016; 35: 1363-1372.

[15] Wang L, Wang X, Zhao Y, Niu W, Ma G, Yin W and Shi C. E3 Ubiquitin ligase RNF126 regulates the progression of tongue cancer. Cancer Med 2016; 5: 2043-2047.

[16] Yoshino S, Hara T, Nakaoka HJ, Kanamori A, Murakami Y, Seiki M and Sakamoto T. The ERK signaling target RNF126 regulates anoikis resistance in cancer cells by changing the mitochondrial metabolic flux. Cell Discov 2016; 2: 16019.

[17] Hiyoshi H, Goto N, Tsuchiya M, Iida K, Nakajima Y, Hirata N, Kanda Y, Nagasawa K and Yanagisawa J. 2-(4-Hydroxy-3-methoxyphenyl)-benzothiazole suppresses tumor progression and metastatic potential of breast cancer cells by inducing ubiquitin ligase CHIP. Sci Rep 2014; 4: 7095.

[18] Shi H, Zheng B, Wu Y, Tang Y, Wang L, Gao Y, Gong H, Du J and Yu R. Ubiquitin ligase Siah1 promotes the migration and invasion of human glioma cells by regulating HIF-1alpha signaling under hypoxia. Oncol Rep 2015; 33: 1185-1190.

[19] Wu Y, Wang L, Bao H, Zou S, Fu C, Gong H, Gao Y, Tang Y, Yu R and Shi H. Nrdp1S, short variant of Nrdp1, inhibits human glioma progression by increasing Nrdp1-mediated ErbB3 ubiquitination and degradation. J Cell Mol Med 2016; 20: 422-9.

[20] Zeng T, Wang Q, Fu J, Lin Q, Bi J, Ding W, Qiao Y, Zhang S, Zhao W, Lin H, Wang M, Lu B, Deng X, Zhou D, Yin Z and Wang HR. Impeded

Nedd4-1-mediated Ras degradation underlies Ras-driven tumorigenesis. Cell Rep 2014; 7: 871-882.
[21] Boase NA and Kumar S. NEDD4: the founding member of a family of ubiquitin-protein ligases. Gene 2015; 557: 113-122.
[22] Luo ZL, Luo HJ, Fang C, Cheng L, Huang Z, Dai R, Li K, Tian FZ, Wang T and Tang LJ. Negative correlation of ITCH E3 ubiquitin ligase and miRNA-106b dictates metastatic progression in pancreatic cancer. Oncotarget 2016; 7: 1477-1485.
[23] Jung JG, Stoeck A, Guan B, Wu RC, Zhu H, Blackshaw S, Shih Ie M and Wang TL. Notch3 interactome analysis identified WWP2 as a negative regulator of Notch3 signaling in ovarian cancer. PLoS Genet 2014; 10: e1004751.
[24] Fukumoto C, Nakashima D, Kasamatsu A, Unozawa M, Shida-Sakazume T, Higo M, Ogawara K, Yokoe H, Shiiba M, Tanzawa H and Uzawa K. WWP2 is overexpressed in human oral cancer, determining tumor size and poor prognosis in patients: downregulation of WWP2 inhibits the AKT signaling and tumor growth in mice. Oncoscience 2014; 1: 807-820.
[25] Mei Z, Zhang D, Hu B, Wang J, Shen X and Xiao W. FBXO32 targets c-Myc for proteasomal degradation and inhibits c-Myc activity. J Biol Chem 2015; 290: 16202-16214.
[26] O'Connor HF, Lyon N, Leung JW, Agarwal P, Swaim CD, Miller KM and Huibregtse JM. Ubiquitin-Activated Interaction Traps (UBAITs) identify E3 ligase binding partners. EMBO Rep 2015; 16: 1699-1712.
[27] Zheng M, Morgan-Lappe SE, Yang J, Bockbrader KM, Pamarthy D, Thomas D, Fesik SW and Sun Y. Growth inhibition and radiosensitization of glioblastoma and lung cancer cells by small interfering RNA silencing of tumor necrosis factor receptor-associated factor 2. Cancer Res 2008; 68: 7570-7578.
[28] Polyak K, Lee MH, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P and Massague J. Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 1994; 78: 59-66.
[29] Carrano AC, Eytan E, Hershko A and Pagano M. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. Nat Cell Biol 1999; 1: 193-199.
[30] Hattori T, Isobe T, Abe K, Kikuchi H, Kitagawa K, Oda T, Uchida C and Kitagawa M. Pirh2 promotes ubiquitin-dependent degradation of the cyclin-dependent kinase inhibitor p27Kip1. Cancer Res 2007; 67: 10789-10795.
[31] Kamura T, Hara T, Matsumoto M, Ishida N, Okumura F, Hatakeyama S, Yoshida M, Nakayama K and Nakayama KI. Cytoplasmic ubiquitin ligase KPC regulates proteolysis of p27 (Kip1) at G1 phase. Nat Cell Biol 2004; 6: 1229-1235.
[32] Nagano Y, Fukushima T, Okemoto K, Tanaka K, Bowtell DD, Ronai Z, Reed JC and Matsuzawa S. Siah1/SIP regulates p27 (kip1) stability and cell migration under metabolic stress. Cell Cycle 2011; 10: 2592-2602.
[33] Choi HH, Phan L, Chou PC, Su CH, Yeung SC, Chen JS and Lee MH. COP1 enhances ubiquitin-mediated degradation of p27Kip1 to promote cancer cell growth. Oncotarget 2015; 6: 19721-19734.