Downregulation of RNF138 inhibits cellular proliferation, migration, invasion and EMT in glioma cells via suppression of the Erk signaling pathway

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Abstract. Glioma is the most common adult malignant primary brain tumor; however, the effect of chemotherapy is often limited by drug-resistance and poor prognosis is common. Ring finger protein 138 (RNF138) belongs to the E3 ligase family, and has significantly higher expression level in glioma tissue than in noncancerous brain tissues. Epithelial-mesenchymal-transition (EMT) has a critical role in cancer invasion and metastasis, ultimately leading to increased cell motility and resistance to genotoxic agents. Extracellular-signal regulated kinase (Erk) pathways promote the growth of glioma cells and enhance tumor invasion, with a role in the progression of EMT. However, the association between RNF138 and human glioma progression remains poorly understood. Relatively little is known about the association between RNF138, Erk, and EMT in glioma progression. In the current study, experiments were performed to explore the potential roles and mechanisms of RNF138 in glioblastoma in vitro and in vivo. Glioma cell line proliferation, migration and invasion were inhibited by knockdown of RNF138 in vitro. By lowering the RNF138 expression, cleaved caspase3 and E-cadherin were upregulated, while phospho-Erk1/2, vimentin, MMP2, HIF-1α and VEGF were downregulated in U87 and U251 cells in vitro. In vivo findings revealed that the growth of U87 cell-transplanted tumors in nude mice was inhibited in tumors with RNF138 knockdown. These findings suggested that downregulation of RNF138 inhibited glioma cell proliferation, migration, and invasion, and reversed EMT, potentially via Erk signaling pathway. Therefore, RNF138 may be a potential therapeutic target against glioma.

Introduction

Glioblastoma (GBM) is one of the most aggressive types of brain tumor. Despite great progress in the therapeutic strategies for GBM over the past few decades, the prognosis remains poor (1-4). The median overall survival is ~14.6 months from the time of diagnosis and the 5-year survival rates are <9.8% following standard care (5,6). With the continuing advances in molecular biology, gene therapy has become a focus for cancer treatment (7). Thus, deeper research and better understanding of the molecular mechanisms of glioma formation and progression is necessary to establish the diagnostic and therapeutic targets.

E3 ubiquitin-protein is a peptide ligase that have been identified as a bio-marker and therapeutic target of glioblastoma (8). Ring finger protein 138 (RNF138) is a member of the E3 ligase family, which contains an N-terminal Cys(3)-His-Cys(4) ring finger protein domain, three zinc-finger-like domains and a C-terminal ubiquitin-interacting motif (UIM) type (9). Our previous study confirmed that RNF138 expression was significantly increased in glioma tissues and in the glioma cell lines U87 and U251 compared with non-cancerous brain tissues by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (10). However, the mechanism of RNF138 in the progression of glioma has not been fully elucidated.

Epithelial-mesenchymal-transition (EMT) is a developmental process, where polarized epithelial cells undergo multiple biochemical changes and assume a mesenchymal phenotype (11). This subsequently increases the migratory capacity, invasiveness, resistance to apoptosis and expression of extracellular matrix components in cells (12-14). Tumor recurrence in glioma is partly attributed to the increased EMT, enhanced aggressive behavior and treatment resistance of the tumor cells (15,16). The extracellular signal-regulated protein kinases (Erk) signaling...
pathways have been reported to have an important role in modulating cell invasion and progression of EMT. Thus, it is necessary to understand the role of Erk signaling pathways and RNF138 in the EMT process in human glioblastoma.

Therefore, the present study explored whether RNF138 has a role in the proliferation, migration and invasion of glioma. A lentiviral-mediated RNA interference (RNAi) system was used to explore the effect of RNF138 on the migration, proliferation of U87 and U251 cells in vitro. Furthermore, the possible mechanisms involved in this process were also investigated. The study suggested that RNF138 was important for the proliferation, migration and invasion of glioma cells. Downregulation of RNF138 inhibited the process of EMT by suppressing Erk signaling pathway in glioma.

Materials and methods

Cell lines. The cell lines U87 ATCC (glioblastoma cells of unknown origin) and U251 (astrocytoma) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; HyClone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 100 U/ml of penicillin, 100 µg/ml of streptomycin and 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified atmosphere of 5% CO₂ in air.

RNF138 small interfering RNA (siRNA) lentiviral vectors. siRNAs sequences targeting human RNF138 gene were designed by Shanghai GenePharm Co., Ltd. (Shanghai, China). The selected template was 5'-CTG TAA CAG TAA TCA CCT A-3'. Following confirmation by sequencing, the sequences were cloned into pGCSIL-green fluorescent protein (GFP; Shanghai GenePharm Co., Ltd., Shanghai, China) to generate the lentiviral vectors. The following sequences were used: RNF138, sense 5'-ATG TCC TAT TTG TGT GTC TCT TCC-3', antisense 5'-GCA GTT TGG ATG GAT TTT TTT TTT GT-3'; product size was 144 bp. GAPDH was used as the internal control gene and the primer pairs used are as follows: Sense 5'-CAAGGT CATCCATGACAACCTTTG-3', antisense 5'-GTCCACCACT CCTGTGCTGTAG-3'; product size was 496 bp. The number of cycles of PCR was optimized to ensure the product intensity to be within the linear phase of amplification. The PCR protocol consisted of an initial denaturation step at 95°C for 5 min, followed by 30 cycles of three-step program at 95°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec, and a final extension step of 72°C for 10 min to amplify RNF138. The RNF138 and GAPDH genes were amplified with SYBR Master Mixture (Takara, Japan). Results are presented as quantitation cycle (Cq) values, and were defined as the PCR cycle quantification number at which an amplified product was first detected. The average Cq was calculated for RNF138 and GAPDH, and 2^(-ΔΔCq) was determined as the mean of the triplicate Cq values for RNF138 minus the mean of the triplicate Cq values for GAPDH. The 2^(-ΔΔCq) method (17) was used to analyze the relative changes in the gene expression.

Colony suppression assay. For colony formation assay, 200 cells/well were seeded into 6-well plates for 48 h after transduction. Then the cells were cultured at 37°C in a 5% CO₂ incubator with the medium changed every 2 days. On day 14, the culture medium was removed, and the cells were washed twice with PBS. Subsequently, the colonies were fixed in 10% methanol at room temperature for 15 min, stained with 1% crystal violet (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at room temperature for 30 min, and then washed. Colonies were counted and photographed.

Analysis of cell proliferation. Cells (at a density of 2x10⁴/100 µl) were seeded into 96-well plates following transduction with siRNA or the negative control lentivirus for 12 h. Then, the Cell Counting kit-8 (CCK-8; Beyotime Institute of Biotechnology, Haimen, China) reagent (10 µl) was added to each well at 24, 48 and 72 h post-transduction. The plates were then incubated for 4 h at 37°C. Absorbance values were measured at a wavelength of 450 nm in an ELISA reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the proliferation rate of each group was analyzed.

Wound healing assay. Cells were seeded into 6-well plates and cultured till the density reaches 80-90% confluence. A wound was made using a 1 ml plastic pipette tip then cells were washed twice with PBS. Subsequently, the medium was replaced by DMEM containing 2% FBS. The size of the wound was
measured under a microscope (Olympus Corporation, Tokyo, Japan) at 0 and 24 h after wounding. For each wound, the width of the wound at 0 h was designated as 100%, and after 24 h the relative width of the open wound was calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA) (18).

Transwell invasion assay. Cell invasion assay was carried out using a 24-well Transwell chamber (Corning Incorporated, Corning, NY, USA). Matrigel (BD Biosciences, San Jose, CA, USA) was diluted to 1:8 with cold DMEM without FBS and used to coat the upper compartment chamber. Different groups of cells (2x10^4) were then plated into the upper wells with 100 µl serum-free DMEM and the bottom chamber was filled with DMEM containing 10% FBS. After incubation at 37°C for 24 h, noninvasive cells on the top chambers were gently wiped with cotton wool. Invasive cells on the bottom surface were fixed with 4% paraformaldehyde for 30 min at 37°C and stained with 0.1% crystal violet for 2 h at room temperature, then counted under a light microscope. The experiment for each group was performed in triplicate and the results were averaged.

Flow cytometry analysis of apoptosis. Apoptosis was measured with Annexin V-phycocerythrin (PE) Apoptosis Detection kit (BD Biosciences). The cells (2x10^5 cells/well) were plated in 6-well plates for 24 h. Then, the cells were washed with cold PBS and resuspended in 100 µl 1X binding buffer, followed by the addition of 5 µl Annexin V-PE and 5 µl 7-aminoactinomycin D (7-AAD). The cells were incubated for 15 min at room temperature in the dark. Finally, 380 µl 1X binding buffer was added to the cells. Cell apoptosis was analyzed using a flow cytometer and CXP analysis software 2.2 (Beckman Coulter, Inc., Brea, CA, USA).

Western blot analysis. Cells were harvested, washed twice with PBS, lysed in ice-cold radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) with a freshly added 0.01% protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA) and incubated on ice for 30 min. Protein concentration was measured by bichoninic acid protein assay kit (Beyotime Institute of Biotechnology). Equal amounts of protein (30 µg) were subjected to 10% SDS-PAGE and then transferred onto the polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with Western Blocking Buffer (Beyotime Institute of Biotechnology) for 2 h at room temperature and incubated with primary antibodies at 4°C overnight. The membranes were washed with PBS with 0.1% Tween-20 before incubating them with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (1:5,000; cat. no. 7074; Cell Signaling Technology, Inc., Danvers, MA, USA) or HRP-conjugated anti-mouse secondary antibodies (1:3,000; cat. no. XR-9720; ProSci Incorporated, Poway, CA, USA) for 2 h at room temperature. After washing again, protein levels were analyzed by Pierce™ Fast Western Blot Kit Enhanced Chemiluminescence Substrate (ECL; cat. no. 3050; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and western blotting detection system (Odyssey CLx; Gene Company, Ltd., Hong Kong, China). The bands were quantified by densitometry using ImageJ 1.46r software (National Institutes of Health, Bethesda, MD, USA). The following antibodies were used in this study: GAPDH (1:1,000; cat. no. sc-69778) and β-actin (1:1,000; cat. no. sc-58673; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used as internal controls. Bcl2 associated X apoptosis regulator (BAX; 1:1,000; cat. no. sc-4239 WB; Santa Cruz Biotechnology, Inc.), apoptosis regulator Bcl2 (Bcl2; 1:1,000; cat. no. sc-23960; Santa Cruz Biotechnology, Inc.); vascular endothelial growth factor (VEGF; 1:1,000; cat. no. sc-365578) and hypoxia-inducible factor-1α (HIF-1α; 1:1,000; cat. no. sc-13515; Santa Cruz Biotechnology, Inc.), cleaved caspase3 (cat. no. 9664), caspase3 (cat. no. 9662), Erk1/2 (cat. no. 4695), phospho (p)-Erk1/2 (cat. no. 4370), matrix metalloproteinase 2 (MMP2; cat. no. 40994), E-cadherin (cat. no. 14472) and vimentin (cat. no. 5741) were acquired from Cell Signaling Technology, Inc. and RNF138 (cat. no. DF4025; Affinity Biosciences, Cincinnati, OH, USA) at a dilution of 1:1,500.

Nude mouse model of intracranial glioma. All experimental protocols were approved by the Institutional Review Board of the Department of Laboratory Animal Science of the First Affiliated Hospital of Soochow University (Suzhou, China). All animal manipulations were performed under sterile conditions. BALB/c-A nude female mice at 4 weeks of age were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China) were randomly assigned into two groups (n=11 per group). U87 cells (3x10^5 cell transduced with lentivirus containing siRNA-RNF138 or negative control sequences) in 4 µl DMEM/F12 were injected 3 mm deep into the frontal lobe, 1.8-2.0 mm to the right side of the sagittal suture and 1.8-2 mm anterior to the coronal suture using an astereotactic frame. Mice were observed daily, weighed twice a week until severe neurological deficit and/or 20% weight-loss and the following day was recorded as the survival date (19,20). For mice found dead, the previous day was recorded as the survival date.

Histology and immunohistochemical staining. Tissues were fixed in 4% buffered formaldehyde for 24 h at 4°C. Paraffin-embedded tissue sections (5 µm thickness) were placed on positively charged slides and air-dried. Following drying, slides were incubated in a 60°C oven for 30 min. Sections were deparaffinized with two xylene washes for 10 min each at room temperature, then rehydrated using two incubations in absolute alcohol of 5 min each at room temperature, then dehydrated using two washes in 1% acid alcohol for 30 sec at room temperature then performed in 0.2% ammonia water for 30 sec at room temperature. Sections were washed in running tap water for 5 min at room temperature. Differentiation was performed in 1% acid alcohol for 30 sec at room temperature then sections were washed in running tap water for 1 min. Bluing was performed in 0.2% ammonia water for 30 sec at room temperature. Sections were washed in running tap water for 5 min at room temperature and rinsed in 95% alcohol, 10 dips at room temperature. Counterstaining was performed using eosin-phloxine solution for 30 sec at room temperature; then sections were dehydrated through 95% alcohol, two changes of
absolute alcohol for 5 min each at room temperature. Sections were cleared in two incubations with xylene for 5 min each at room temperature. Digital images were acquired using a light microscope (Olympus BX40; Olympus Corporation).

For immunoreactivity staining, 5 µm deparaffinized polylysine-coated sections were treated with 3% hydrogen peroxide for 5 min at room temperature to remove endogenous oxidizes. The cells were treated using citrate buffer (BioGenex Laboratories, Fremont, CA, USA) for 5 min at 100˚C, and permeabilized with 0.3% Triton X-100. The slides were incubated with primary antibodies, including Ki-67 (1:50; cat. no. sc-56319; Santa Cruz Biotechnology, Inc.) and MMP2 (1:150; cat. no. 40994), E-cadherin (1:100; cat. no. 14472), VEGF (1:100; cat. no. sc-365578) and vimentin (1:100; cat. no. 5741; Cell Signaling Technology, Inc.) at 4˚C overnight, followed by incubation with a biotin-labeled secondary antibody (1:100 dilution; cat. no. ab64264; Abcam, Cambridge, MA, USA) for 1 h at room temperature. 5% diaminobenzidine (Abcam) solution for 10 min at room temperature was used for brown color development. Immunohistochemical signals were observed under a microscope (Olympus Corporation).

Statistical analysis. All results are expressed as the mean±standard deviation from at least three replicates. One-way analysis of variance and Tukey’s test, or Student’s unpaired t-test was used to analyze significance using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). The Kaplan-Meier method was used to plot the survival curves followed by a log-rank test. Survival analysis was performed using GraphPad Software version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Successful establishment of stable RNF138 expression downregulation in U87 and U251 cell lines. According to our previous research (10), U87 and U251 cell lines exhibited relatively higher RNF138 expression compared with noncancerous brain samples, and hence, these two cell lines were chose for RNF138 silencing experiments. To determine the effect of RNF138, lentiviral siRNA was used to stably and specifically reduce the expression of RNF138 in U87 and U251 cells established from high-grade tumors.

The transfection efficiency of U87 and U251 cells transfected with RNF138-siRNA and the negative control lentivirus was determined by fluorescence inverted microscope and flow cytometry. The phase contrast fluorescence inverted microscopic evaluation revealed >95% transfected cells (Fig. 1A) and flow cytometry analysis revealed >95% GFP-positive cells (Fig. 1B). RNF138 mRNA and protein expression levels were measured by RT-qPCR and western blotting analyses, respectively. Results revealed that RNF138 protein (Fig. 2A and B) and mRNA expression levels (Fig. 2C) were significantly attenuated compared with the negative control lentiviral groups (P<0.01).

Stable downregulation of RNF138 expression inhibits malignant glioma cell proliferation, migration and invasion in vitro. The biological function of RNF138 in malignant glioma was analyzed. U87 and U251 cell lines were selected to investigate the effects of RNF138 on cell proliferation, migration and invasion following transduction with RNF138-siRNA and negative control lentiviruses. Colony formation and CCK-8 assays were performed after transfection of the cells. Colony formation assay (Fig. 3A) demonstrated that U87 and U251 cells infected with RNF138-siRNA decreased the colony formation compared with the negative control lentiviral groups (P<0.01; Fig. 3B). CCK-8 assay revealed that the downregulation of RNF138 led to a significant reduction of cell proliferation in U251 and U87 cells compared with negative control lentiviral groups (P<0.01; Fig. 3C and D). These results indicated that silencing of RNF138 suppresses glioma cell proliferation.

To investigate whether the downregulation of RNF138 in U87 and U251 cells affected their migratory and invasive
abilities in vitro, wound healing and Transwell assays were performed. Results of wound healing assay (Fig. 4A) demonstrated that downregulation of RNF138 resulted in a decrease in the migration compared with negative control.
lentivirus groups (P<0.01; Fig. 4B), suggesting the reduced migration-promoting abilities of RNF138 in the knockdown samples. Additionally, the experimental results of the Transwell assay demonstrated that the number of cells invading through the chamber was decreased significantly by RNF138-siRNA compared to negative control lentiviral groups (P<0.01; Fig. 4C and D), indicating that RNF138 has an important role in glioma cell invasion. To further elucidate the detailed mechanism, the protein level changes of HIF-1α, VEGF and MMP2 were determined by western blotting (Fig. 5A). Western blot
analysis revealed significant decrease of HIF-1α, VEGF and MMP2 protein levels by downregulating RNF138 compared with the negative control (P<0.01; Fig. 5B-D). These data demonstrated that knockdown of RNF138 inhibited glioma cell migration and invasion, potentially via downregulation of HIF-1α, VEGF and MMP2 expression levels. The data implied that RNF138 has a critical role in glioma cell proliferation, migration and invasion.

Knockdown of RNF138 increased apoptosis of glioma cells through caspase pathway. Annexin V-PE apoptosis detection and flow cytometry analysis were used to examine whether knockdown of RNF138 inhibited glioma cell proliferation by inducing cell apoptosis. Annexin V-PE and 7-AAD, 7-aminoactinomycin D.
protein of caspase3 was unchanged in U87 and U251 cells with RNF138 knockdown compared with the negative control lentivirus group (P<0.01; Fig. 7B). Additionally, the protein expression of BAX and cleaved caspase3 (Fig. 7C and D) were upregulated, while Bcl2 (Fig. 7E) was downregulated in the RNF138-siRNA group compared with the negative lentivirus group. Taken together, these data suggested that downregulation of RNF138 inhibited glioma cell proliferation, increased apoptosis of glioma cells through caspase signaling pathway.

Downregulation of RNF138 inhibited EMT in U87 and U251 cells and suppressed the Erk signaling pathway. EMT is a reversible biological process that occurs in cells during normal development and aberrantly activated in various cancer types (21). Previous studies have reported that EMT is closely associated with malignant tumor cell invasion capacity. To further explore whether downregulation of RNF138 expression level could influence EMT in glioma cell lines, the EMT markers E-cadherin and vimentin were measured using western

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**Figure 7.** Protein levels of apoptotic-associated factors in U87 and U251 cells transduced with RNF138-siRNA and negative control lentivirus. (A) Expression of caspase3, cleaved caspase3, BAX and Bcl2 were detected by western blot analysis. β-actin served as the loading control. Densitometry analysis of (B) caspase3, (C) cleaved caspase3, (D) BAX and (E) Bcl2, relative to β-actin expression in U87 and U251 cells with ImageJ analysis. Data are presented as the mean ± standard deviation for three independent experiments. **P<0.01. RNF138, ring finger protein 138; siRNA, small interfering RNA; BAX, Bcl2 associated X apoptosis regulator; Bcl2, Bcl2 apoptosis regulator; NS, not significant.
blot analysis (Fig. 8A). Compared with the negative control lentiviral cells, the expression level of E-cadherin was significantly increased (P<0.01; Fig. 8B), while the vimentin levels were decreased in RNF138-siRNA cells (P<0.01; Fig. 8C).

There are several cell signaling pathways implicated in the process of EMT. Erk signaling pathways has been reported to have a role in modulating cell invasion and progression of EMT (22,23). Western blot analysis was performed to further investigate whether inhibition of RNF138 expression could influence EMT via the Erk signaling pathway. These results revealed that downregulation of RNF138 significantly inhibited the activation of Erk1/2 (P<0.01; Fig. 8D), however, total Erk1/2 levels were unchanged (P>0.01; Fig. 8E). These data indicate that the downregulation of RNF138 may reverse the EMT process via the Erk signaling pathway in glioblastoma.

Knockdown of RNF138 suppresses tumor growth in an orthotopic nude mice model. As described above,
downregulation of RNF138 suppressed the tumorigenicity of glioma cells in vitro and further analysis is required in vivo. Tumorigenicity was determined in an orthotopic nude mouse model to confirm the function of RNF138 knockdown in vivo. U87 cells with stable expression of RNF138-siRNA or negative control lentivirus were intracranially injected into nude mice. Eleven mice were collected for RNF138-siRNA cells treated group (experiment group), and another eleven mice were treated with Sci-siRNA as control group. Hematoxylin and eosin (H&E) staining of the tumors resulting from injection with U87 cells revealed decreased cell motion in the RNF138-siRNA group compared with the scramble controls (Fig. 9A), Kaplan-Meier analysis showed survival was prolonged in the RNF138-siRNA group compared with the negative control group (P<0.01; Fig. 9B). Immunohistochemistry revealed that the expression of Ki-67, E-cadherin, MMP2, Vimentin and VEGF were lower in the RNF138-siRNA group compared with the negative group, while E-cadherin expression was notably higher than the negative control group (Fig. 9C). These results were consistent with the in vitro results, indicating that knockdown of RNF138 suppressed tumorigenesis of malignant glioma in vivo.

Discussion

Ubiquitylation is a dynamic and reversible post-translational modification that regulates a wide range of biological processes, including cell cycle progression, transcription, apoptosis and inflammation (24). RNF138 is characterized as an E3 ubiquitin ligase that contains a RING, zinc finger and UIM domains. RNF138 was originally reported to act as a negative regulator of Wnt signaling through Nemo-like kinase (25). Although the upregulation of RNF138 in human glioma tissues has been previously identified (10), the biological functions of RNF138 in glioma cell lines remain largely unknown.

Therefore, CCK-8 proliferation assay, colony formation assay and flow cytometry were used to explore the effects of RNF138 on migration, proliferation and apoptosis of U87 and U251 cells in vitro. The current study demonstrated decreased migration and invasion, and increased the apoptosis of U87 and U251 glioma cells with RNF138 knockdown. However, the exact molecular mechanisms on how tumor cell proliferation, migration, apoptosis and invasive potential are affected remained unclear.

Apoptosis is a well-orchestrated cellular mechanism of programmed cell death that is mediated through a caspase cascade (26,27). Therefore, the protein levels of apoptosis-associated factors were determined in RNF138-siRNA-infected glioblastoma cells. The levels of both cleaved caspase3 and BAX were upregulated in U87 and U251 cells with RNF138 silencing. Conversely, the expression levels of Bcl2 were downregulated by RNF138 knockdown. The current study demonstrated the critical role of regulatory role of RNF138 in apoptosis via the caspase3 pathway in U87 and U251 cells. Apoptotic assay further confirmed the apoptosis inducing function of downregulated RNF138. These findings are supported by the fact that RNF138 silencing induced an increase in the abundance of cleaved caspase3 protein.
Hou et al (28) suggested that cancer cells obtain invasion ability via EMT, whereby epithelial cells lose their cell-cell adhesion and attain mesenchymal characteristics. This process has a critical role in the development and progression, invasion and migration of diverse human tumors (28-31). Therefore, to elucidate the precise mechanisms involved in cell migration and invasion, the effects of RNF138 on EMT-associated proteins were examined. The suppression of RNF138 expression resulted in elevated expression of E-cadherin and reduced expression of vimentin, which acts as a crucial step for cancer cell migration and invasion in various cancer types (32-34). These findings indicate that the knockdown of RNF138 potentially reduced EMT in the glioma cell lines.

Erk signaling is associated with the process of EMT, and an essential component of the mitogen-activated protein kinase signal cascades. Erk is associated with the regulation of glioma proliferation, differentiation, migration and apoptosis (35-38). The effect of RNF138 on Erk signaling pathway was also investigated. The level of p-Erk1/2 was notably decreased in RNF138-siRNA glioma cells compared with negative control glioma cells, and cell migration was suppressed following RNF138 knockdown. Thus, the data confirmed that lower expression of RNF138 in glioma cells reversed the EMT process, potentially via the Erk pathway.

In addition, RNF138 knockdown notably decreased MMP2, HIF-1α and VEGF protein expression levels. MMP2, HIF-1α and VEGF have been reported to participate in EMT progression in different types of cancer, which was regulated via Erk signaling (39-41). HIF-1α is stabilized by hypoxia-induced reactive oxygen species, which results in the enhanced expression of several hypoxia-associated genes, including the VEGF, which is an angiogenic activator (42). Furthermore, immunohistochemistry staining revealed that vimentin, VEGF and MMP2-positive cells were reduced, while E-cadherin was higher in tumors produced from RNF138 knockdown cells than the negative control group. Taken together, these results suggest that suppression of RNF138 reduced the invasion and migration of glioma cells, and regulated the protein levels of HIF-1α, VEGF and MMP2 potentially by reversing EMT via Erk signaling.

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Ethics approval and consent to participate

All experimental protocols were approved by the Institutional Review Board of the Department of Laboratory Animal Science of the First Affiliated Hospital of Soochow University (Suzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Malzkorn B and Reifenberger G: Practical implications of integrated glioma classification according to the World Health Organization classification of tumors of the central nervous system 2016. Curr Opin Oncol 28: 494-501, 2016.
2. Winter SF, Loebel F and Dietrich J: Role of ketogenic metabolic therapy in malignant glioma: A systematic review. Crit Rev Oncol Hematol 112: 41-58, 2017.
3. Ostrom QT, Gittleman H, Liao P, Rouse C, Chen Y, Dowling J, Wolinsky Y, Kruchko C and Barnholtz-Sloan J: CBTRUS statistical report: Primary brain and central nervous system tumors diagnosed in the United States in 2007-2011. Neuro Oncol 16 (Suppl 4): iv1‑iv63, 2014.
4. Tanaka S, Louis DN, Curry WT, Batchelor TT and Dietrich J: Diagnostic and therapeutic avenues for glioblastoma: No longer a dead end? Nat Rev Clin Oncol 10: 14‑26, 2013.
5. Stupp R, Hegi ME, Mason WP, van den Bent MJ, Taphoorn MJ, Janzer RC, Ludwig SK, Allgeier A, Fisher B, Belanger K, et al: Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase IIIB study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol 10: 459-466, 2009.
6. Carlsson SK, Brothers SP and Wahlestedt C: Emerging treatment strategies for glioblastoma multiforme. EMBO Mol Med 6: 1359-1370, 2014.
7. Liang J, Wang WF, Xie S, Zhang XL, Qi WF, Zhou XP, Hu JX, Shi Q and Yu RT: Expression of β-transducin-repeat-containing E3 ubiquitin protein ligase in human glioma and its correlation with prognosis. OncoLett 9: 2651-2656, 2015.
8. Liu Y, Wang F, Liu Y, Yao Y, Lv X, Dong B, Li J, Ren S, Yao Y and Xu Y: RNF135, RING finger protein, promotes the proliferation of human glioblastoma cells in vivo and in vitro via the ERK pathway. Sci Rep 6: 20642, 2016.
9. Xu L, Lu Y, Han D, Yao R, Wang H, Zhong S, Luo Y, Han R, Li K, Fu J, et al: Rn138 deficiency promotes apoptosis of spermatogonia in juvenile male mice. Cell Death Dis 8: e2795, 2017.
10. Zhou YX, Chen SS, Wu TF, Ding DD, Chen XH, Chen JM, Su ZP, Li B, Chen GL, Xie XS, et al: A novel gene RNF138 expressed in human gliomas and its function in the glioma cell line U251. Anal Cell Pathol (Amst) 35: 167-178, 2012.
11. Kalluri R and Weinberg RA: The basics of epithelial-mesenchymal transition. J Clin Invest 119: 1420-1428, 2009.
12. Kang Y and Massagué J: Epithelial-mesenchymal transitions: Twist in development and metastasis. Cell 118: 277-279, 2004.
13. Thiery JP: Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer 2: 442-454, 2002.

14. Thiery JP, Acloque H, Huang RY and Nieto MA: Epithelial-mesenchymal transitions in development and disease. Cell 139: 871-890, 2009.

15. Han MZ, Huang B, Chen AJ, Zhang X, Xu R, Wang J and Li XG: High expression of RAB43 predicts poor prognosis and is associated with epithelial-mesenchymal transition in gliomas. Oncol Rep 37: 903-912, 2017.

16. Zhang L, Zhang W, Li Y, Alvarez A, Li Z, Wang Y, Song L, Lv D, Nakano I, Hu B, et al: SHP-2-upregulated ZEB1 is important for PDGF-Ralpha-driven glioma epithelial-mesenchymal transition and invasion in mice and humans. Oncogene 35: 5641-5652, 2016.

17. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, et al: The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622, 2009.

18. Liu X, Zhang G, Wang Y, Elgehama A, Sun Y, Li L, Gu Y, Guo W and Xu Q: Loss of periplakin expression is associated with the tumorigenesis of colorectal carcinoma. Biomed Pharmacother 87: 366-374, 2017.

19. Li X, Zhang G, Wang Y, Elgehama A, Sun Y, Li L, Gu Y, Guo W and Xu Q: Loss of periplakin expression is associated with the tumorigenesis of colorectal carcinoma. Biomed Pharmacother 87: 366-374, 2017.

20. Frampton GM, Ding L, Wang G, Welch JS, Polesskaya O, Brisken C, et al: The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622, 2009.

21. Li X, Zhang G, Wang Y, Elgehama A, Sun Y, Li L, Gu Y, Guo W and Xu Q: Loss of periplakin expression is associated with the tumorigenesis of colorectal carcinoma. Biomed Pharmacother 87: 366-374, 2017.

22. Frampton GM, Ding L, Wang G, Welch JS, Polesskaya O, Brisken C, et al: The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622, 2009.

23. Li X, Zhang G, Wang Y, Elgehama A, Sun Y, Li L, Gu Y, Guo W and Xu Q: Loss of periplakin expression is associated with the tumorigenesis of colorectal carcinoma. Biomed Pharmacother 87: 366-374, 2017.

24. Frampton GM, Ding L, Wang G, Welch JS, Polesskaya O, Brisken C, et al: The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622, 2009.

25. Li X, Zhang G, Wang Y, Elgehama A, Sun Y, Li L, Gu Y, Guo W and Xu Q: Loss of periplakin expression is associated with the tumorigenesis of colorectal carcinoma. Biomed Pharmacother 87: 366-374, 2017.

26. Frampton GM, Ding L, Wang G, Welch JS, Polesskaya O, Brisken C, et al: The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622, 2009.

27. Li X, Zhang G, Wang Y, Elgehama A, Sun Y, Li L, Gu Y, Guo W and Xu Q: Loss of periplakin expression is associated with the tumorigenesis of colorectal carcinoma. Biomed Pharmacother 87: 366-374, 2017.

28. Frampton GM, Ding L, Wang G, Welch JS, Polesskaya O, Brisken C, et al: The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622, 2009.

29. Li X, Zhang G, Wang Y, Elgehama A, Sun Y, Li L, Gu Y, Guo W and Xu Q: Loss of periplakin expression is associated with the tumorigenesis of colorectal carcinoma. Biomed Pharmacother 87: 366-374, 2017.

30. Frampton GM, Ding L, Wang G, Welch JS, Polesskaya O, Brisken C, et al: The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. Clin Chem 55: 611-622, 2009.

31. Pietilä M, Ivaska J and Mani SA: Whom to blame for metastasis, the epithelial-mesenchymal transition or the tumor microenvironment? Cancer Lett 380: 359-368, 2016.

32. De Craene B and Berx G: Regulatory networks defining EMT during cancer initiation and progression. Nat Rev Cancer 13: 97-110, 2013.

33. Micalizzi DS, Farabaugh SM and Ford HL: Epithelial-mesenchymal transition in cancer: Parallels between normal development and tumor progression. J Mammary Gland Biol Neoplasia 15: 117-134, 2010.

34. Vivanco I and Sawyers CL: The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2: 489-502, 2002.

35. Zeng Z, Leng T, Feng X, Sun H, Inoue K, Zhu L and Xiong ZG: Silencing TRPM7 in mouse cortical astrocytes impairs cell proliferation and migration via ERK and JNK signaling pathways. PLoS One 10: e0119912, 2015.

36. Heigener DF, Gandara DR and Reck M: Targeting of MEK in lung cancer therapeutics. Lancet Respir Med 3: 319-327, 2015.

37. Liu Z, Dai H, Jia G, Li Y, Liu X and Ren W: Insufficient radiofrequency ablation promotes human hepatoma SMMC7721 cell proliferation by stimulating vascular endothelial growth factor overexpression. Oncol Lett 9: 1893-1896, 2015.

38. Dong F, Tian H, Yan S, Li L, Dong X, Wang F, Li J, Li C, Cao Z, Liu X and Liu J: Dihydroartemisinin inhibits endothelial cell proliferation through the suppression of the ERK signaling pathway. Int J Mol Med 35: 1381-1387, 2015.

39. Zhang D, Lu C and Ai H: Rab5a is overexpressed in oral cancer and promotes invasion through ERK/MMP signaling. Mol Med Rep 16: 4569-4576, 2017.

40. Morishita Y, Okawara S, Hirahara I, Muto S and Nagata D: HIF-1α mediates Hypoxia-induced epithelial-mesenchymal transition in peritoneal mesothelial cells. Ren Fail 38: 282-289, 2016.

41. Mori H, Yao O, Learman BS, Kurozumi K, Ishida J, Ramakrishnan SK, Overmyer KA, Xue X, Cawthorn WP, Reid MA, et al: Induction of WNT11 by hypoxia and hypoxia-inducible factor-1α regulates cell proliferation, migration and invasion. Sci Rep 6: 21520, 2016.

42. Park SY, Jang WJ, Yi EY, Jang JY, Jung Y, Jeong JW and Kim YJ: Melatonin suppresses tumor angiogenesis by inhibiting HIF-1α stabilization under hypoxia. J Pineal Res 48: 178-184, 2010.