RIT1 Promotes Glioma Proliferation and Invasion via the NF- κ B/p65 pathway

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Research Article

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Abstract

RIT1, a member of the Ras family, has been identified as an oncogene in several malignancies. However, the expression and function of RIT1 in glioma remains to be addressed. In this study, we found RIT1 was upregulated in glioma and was associated with poor prognosis of glioma patients. Manipulating RIT1 levels in glioma cells via RNA interference significantly inhibited glioma cell proliferation and invasion in vitro whereas RIT1 overexpression exhibited the opposite effects. Mechanistically, we demonstrate that RIT1 engaged in the activation of the NF- κ B pathway in vitro and in vivo. Furthermore, treating RIT1-overexpressing glioma cells with the p65 siRNA partially restrained their proliferation and invasion. Together these results indicate RIT1 contributes to the development and metastasis of glioma via the NF- κ B pathway and suggest that targeting RIT1 may be a treatment strategy for this disease.

Introduction

Glioma is the most common primary malignant brain tumor and is characterized by its aggressiveness and significant mortality [1]. The standard clinical treatment for glioma consists of surgical resection combined with radiotherapy and/or chemotherapy with temozolomide (TMZ). Despite the emergence of advanced clinical treatments for many other cancers, patients with glioma still have poor prognosis with a median survival time of less than 2 years [2]. Therefore, in order to find effective treatment strategies and improve the prognosis of these patients, it has become of utmost importance to uncover the molecular mechanisms driving glioma progression.

RIT1 (Ras-like-without-CAAX-1) is a small GTP-binding protein that belongs to the Ras superfamily and shares a similar sequence and domain organization with KRAS, HRAS and NRAS. RIT1 possesses intrinsic GTP hydrolysis activity and functions in the cell as a guanine nucleotide-regulated molecular switch that changes between active GTP-bound and inactive GDP-bound states [3]. Previous studies have indicated that RIT1 serves as a critical player in neurogenesis via regulating the transcription of Sox2 [4]. Other studies also indicate that RIT1 promotes the survival of immature hippocampal neurons following brain injury through the P38-MAPK signaling pathway [5]. In the context of cancer, there is accumulating evidence to indicate that RIT1 is overexpressed in various cancers and implicated in tumorigenesis and progression [6-10]. For example, RIT1 acts as an oncogene in lung adenocarcinoma where it is related to the activation of the P13K/AKT and MAPK pathways [8]. RIT1 is also induced by HIF-1 α and enhances the proliferation, migration and invasion in hepatocellular carcinoma [9]. Conversely, recent evidence suggests that RIT1 functions as a tumor suppressor in esophageal squamous cell carcinoma (ESCC) through inactivation of multiple signaling pathways [10]. However, presently there are few studies concerning the role of RIT1 in glioma.

On this basis, we investigated the expression and function of RIT1 in glioma. Our results showed that RIT1 is upregulated in glioma and associated with glioma proliferation and invasion. Moreover, we demonstrated that RIT1 enhances glioma proliferation and invasiveness through the NF- κ B/p65 pathway. These results support the prospect of using RIT1 as a potential treatment target in glioma.

Materials And Methods

Bioinformatics analysis

The expression profiles and associated prognostic data of RIT1 in glioma from the TCGA and CGGA databases were analyzed via GlioVis (<http://gliovis.bioinfo.cnio.es/>).

Patient samples

A total of 12 tissue samples were obtained from the Department of Neurosurgery at the Second Affiliated Hospital of Soochow University (Suzhou, China). The samples consisted of 3 normal brain tissues and 9 glioma tissues (grade ≥ 3 , grade ≥ 3 , grade ≥ 3 , respectively, according to the WHO classification criteria). The tissues were immediately frozen and stored in liquid nitrogen. No patients received chemotherapy or radiotherapy before sample collection. All patients signed informed consent forms and the study was conducted with the approval of the Research Ethics Committee of the Second Affiliated Hospital of Soochow University.

Cell culture and transfection

Human glioblastoma cell lines (U87, GBM12 and LN229) were purchased from the ATCC (USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, USA) and maintained at 37 °C in a humidified atmosphere with 5% CO₂. To knockdown RIT1, glioma cells were transfected with small interfering RNAs (siRNAs) (Genepharma, Suzhou) using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. The siRNA sequences used were as follows: siNC

TGTTTCGCATTATCCGAACCAT; siRIT1 1# CGAGAATTCAGCTGTCCCTT; siRIT1 2#CACGTCTGA-AGTTTCCATGAAGT.

Lentiviral transfection

To construct stable U87 and LN229 cell lines with silencing or overexpression of RIT1, lentiviral vectors containing shRNA sequences targeting RIT1 or RIT1 cDNA were purchased from Genepharma (Suzhou, China). Two days after transduction, the infected cells were treated with 2 μ g/mL puromycin for 14 days. The efficiency of knockdown and overexpression was evaluated by Western blot. The shRIT1 target sequence was CCGGCGAGAATTCAGCTGTCCCTTTCTCG. The RIT1 expression plasmid was purchased from Genechem (Shanghai, China).

Cell proliferation assays

Cells were seeded in 96-well plates at 2000 cells per well (n=5 replicate wells) and cell proliferation assessed at indicated days after seeding using the CCK-8 reagent according to the manufacturer's protocol (Beyotime Biotechnology). Absorbance was measured at a wavelength of 450 nm using a GENios Pro (Tecan).

Colony formation assays

Cells were seeded into 6-well plates at 300~500 cells per well and incubated for two weeks before washing the cells twice with PBS, fixation with 4% formaldehyde for 15 minutes and staining with Crystal violet. The number of colonies was quantitated using ImageJ software.

Cell cycle analysis

After cells were transfected for 48 hours, cell suspensions were prepared and fixed using 70% ethanol at 4°C overnight before incubating with 500uL propidium iodide/RNASE FREE solution at 37°C in the dark for 30 minutes. The cells were then analysed by flow cytometry and the percentages of cells in different phase of the cell cycle estimated.

Invasion assays

For invasion assays, upper Transwell membrane surfaces were pre-coated overnight with 10% Matrigel diluted with OptiMem media. Cell suspensions in serum-free DMEM medium were seeded into the upper chamber (20000 cells/100 uL) and the lower chamber filled with DMEM containing 10% FBS (8um in 24 well format, Transwell, Falcon). After incubation at 37°C for 24 hours, the upper membrane surface was wiped gently with a cotton swab and the membrane then fixed with 4% formaldehyde for 15 minutes before staining with Crystal violet solution. The membranes were washed with PBS and dried before imaging the lower surface of the membrane.

Western blotting

Tissues and cells were lysed in RIPA lysis buffer for 30 minutes and then centrifuged in 13500 g for 15 minutes at 4°C. Protein concentrations were determined using the BCA protein kit (Beyotime) and equal protein amounts prepared using 5x SDS-loading buffer. After heating protein lysates at 100°C for 5 minutes, 30 ug protein/sample was separated by 10% SDS-PAGE gels before transfer to PVDF membranes (Millipore). Membranes were blocked with 2% BSA and primary antibodies added against p65, P-p65, c-Myc, H3, β -actin (1:1000, Cell Signaling), RIT1 (1:1000, GeneTex) and GAPDH (1:10000, Abcam) overnight as indicated. The membranes were washed three times for 5 minutes and incubated with the corresponding secondary antibodies for 2 hours at room temperature. ECL reagent (Thermo) was applied to visualize the protein bands using a Syngene GeneGnome XRQ (USA) imager.

Tumor xenografts

Female nude mice (5-week old BALB/c) were randomly divided into two groups. Mice were injected subcutaneously into the right forelimb with 5×10^6 U87-shRIT1 RIT1 knockdown cells or U87-shNC control cells. Mice were sacrificed and tumor weights measured. Part of the tumor was used for Western blot analysis. Approval for this experiment was received from the Research Ethics Committee of the Second Affiliated Hospital of Soochow University.

Statistics

All data were analyzed by GraphPad 7.0 software and data presented as mean±standard deviation. Student's t-test and one-way ANOVA were used to determine significant differences between two groups and among multiple groups respectively. $P<0.05$ (*), <0.01 (**), <0.001 (***) was considered statistically significant.

Results

RIT1 is highly expressed in glioma and correlates with poor prognosis

We first examined the expression of RIT1 in glioma tissues from the TCGA, Rembrandt, Gravendeel and CGGA datasets. This analysis showed that RIT1 was highly expressed in glioma compared with normal brain tissues (Fig. 1a and b) and moreover, that RIT1 levels were positively associated with glioma grade (Fig. 1c and d). Furthermore, this analysis showed that lower RIT1 expression was associated with better patient prognosis compared to high expression cases (Fig. 1e and f). Next, we measured RIT1 protein expression levels in freshly isolated patient tissues. Western blotting data indicated that RIT1 was not only highly expressed in glioma compared with normal brain tissues, but also that its expression in advanced grade gliomas was higher than in lower grade tumors (Fig. 1g).

RIT1 promotes glioma proliferation and invasion

To investigate the role of RIT1 in glioma proliferation and metastasis, a series of measures were employed including cell proliferation (CCK-8 and colony formation assays), cell cycle (flow cytometry) and invasion (Transwell assays). To test the effects of RIT1 expression, U87 and GBM12 cells were first transfected with RIT1 or control (NC) siRNAs. Assessing the expression of RIT1 using Western blot confirmed that RIT1 was efficiently knocked down in both cell lines (Fig. 2a). Assessing cell proliferation using CCK-8 assays showed that the growth of U87 and GBM12 cells transfected with siRIT1 was significantly lower than cells in the NC treatment group (Fig. 2b). Cell cycle analyses also indicated more U87 and GBM12 cells in the G0/G1 phase after siRIT1 treatment compared with cells in the NC group (Fig. 2c). Similarly, the number and size of colonies was also reduced in siRIT1-transfected U87 and GBM12 cells using colony formation assays (Fig. 2d). Transwell assays indicated the invasion of U87 and GBM12 cells was suppressed after treatment with RIT1 siRNA (Fig. 2e). Conversely, we tested the effects of RIT1 overexpression on cell growth and invasion by transduction of RIT1 cDNA into the LN229 glioma cell lines. Overexpression efficiency was confirmed using Western blot (Fig. 2a). CCK8 assays indicated that cell proliferation was enhanced in RIT1-overexpressing LN229 cells relative to controls and instructively, more cells were found in the S and G2 phases of the cell cycle (Fig. 2b and c). RIT1-overexpressing LN229 cells also displayed more colonies and invasive ability compared with the control experimental groups (Fig. 2d and e).

RIT1 promotes glioma proliferation and invasion through NF- κ B/p65 signaling

We subsequently investigated the potential molecular mechanisms whereby RIT1 promotes the proliferation and invasion of glioma cells. P65 phosphorylation and nuclear translocation are key factors in NF- κ B signaling activation [11]. Our analysis of glioma cells subjected to RIT1 silencing confirmed that P-p65 and c-Myc protein expression were significantly downregulated in both U87 and GBM12 cells (Fig. 3a and b). In contrast, RIT1 overexpression upregulated the expression of P-p65 and c-Myc in LN229 cells (Fig. 3c). Besides, we also detected the protein expression of p65 in the nucleus following nucleocytoplasmic separation. Consistently, a lower p65 expression was observed in RIT1-knockdown U87 cells, whereas a higher p65 expression was shown in RIT1-overexpressing LN229 cells compared with the control group (Fig. 3d and e). In concert with the RIT1 manipulation experiments, we also employed the p65 siRNA siRELA to determine whether NF- κ B/p65 signaling participated in RIT1-mediated proliferation and invasion. As shown in Fig. 3f-3h, cell proliferation and invasion was partially inhibited in RIT1-overexpressing LN229 after transfected with siRELA, which was also reflected in the downregulation of P65 protein expression. Together these data suggest that RIT1 is engaged in the regulation of proliferation and invasion of glioma cells through the NF- κ B/p65 signaling pathway.

RIT1 silence suppresses glioma tumorigenesis in vivo

As a logical extension of the previous experiments, we next determined if RIT1 also affected glioma tumorigenesis in vivo. For these experiments, stable Lv-shRIT1 or control U87 cells were prepared and the relevant transfection efficiency was validated using immunofluorescence staining (Fig. 4a). These cells were then inoculated subcutaneously into nude mice and the growth of tumor xenografts measured over time. Notably, tumors formed by the LV-shRIT1 U87 cells grew more slowly and were smaller than those formed by the control Lv-shNC U87 cells (Fig. 4b-d). Moreover, consistent with the siRNA-based experiments, Western blot analysis confirmed that the protein expression of p-p65 and c-Myc in RIT1-knockdown tumor xenografts were reduced compared with the control tumors (Fig. 4e). Thus, these findings are consistent with our in vitro results and indicative that RIT1 accelerates glioma progress in vivo.

Discussion

RIT1 appears to be a critical driver in some malignancies while in others such as ESCC it appears to fulfil an opposite role [6-10]. However, there have been few studies reported concerning the role of RIT1 in glioma. Assessing the clinical expression of RIT1 via the GlioVis platform established RIT1 was highly expressed in glioma and positively related to tumor grade together with worse prognosis for patients where RIT1 was most highly expressed. Moreover, we confirmed RIT1 overexpression in glioma tissues and cell lines. Therefore, RIT1 appears to be widely upregulated in glioma, especially in late stage malignancies where it is prognostic for outcome, proposing that RIT1 functions as an oncogenic regulator of glioma tumorigenicity. On this basis, we further investigated the functional role of RIT1 in glioma using a series of in vitro assays together with investigations in xenograft experiments. Significantly we observed that knockdown of RIT1 significantly suppressed the proliferation and invasive ability of glioma cells while RIT1 overexpression accelerated cell proliferation. This indicates that RIT1

functions to drive glioma cell proliferation and importantly, the growth promoting characteristic of RIT1 was maintained in vivo when U87 cells were grown as xenografts. For the latter experiment, the volume and weight of tumor xenografts were particularly smaller in the LV-U87 shRIT1 group compared with the controls. Thus, consistently, the oncogenic effects of RIT1 suggested from the patient data could be readily reproduced in cell line models.

We then turned our attention to more focused mechanistic investigations of RIT1 function in glioma. NF- κ B is a transcription factor, mainly composed of p65, p50 and c-Rel, has been widely accepted as the critical regulator in diverse processes including cell survival, cell adhesion, differentiation, inflammation and growth [12-13]. Indeed, NF- κ B signaling is among one of the most frequently dysregulated pathways encountered in cancer with strong evidence linking its activation to both tumorigenesis and tumor progression [14-15]. For example, activated NF- κ B signaling enhances metastasis in both gastric and colorectal cancers [16-17]. Other studies in lung cancer have indicated NF- κ B signaling facilitates not only oncogenic growth but also participates in chemotherapy and radiotherapy resistance [18-19]. In addition, NF- κ B signaling is found to be frequently dysregulated in breast carcinoma [20-21]. Moreover, while NF- κ B signaling appears highly activated during the initiation and progression of glioma [22], the relationship between NF- κ B signaling and RIT1 still remains unclear. Our study demonstrated that genetically manipulating RIT1 levels in glioma by knockdown and overexpression modulated the expression of P-p65 and c-Myc, key proteins involved in NF- κ B signaling. In support, treatment of cells with the p65 siRNA siRELA partially reduced the enhanced cell proliferation and invasion induced by RIT1 overexpression. Together these results suggest that RIT1 promotes glioma proliferation and invasiveness via activation of the NF- κ B signaling pathway.

In summary, we found RIT1 to be overexpressed in glioma with a significant association with the prognosis of glioma patients. RIT1 promotes the proliferation and invasion of glioma via activation of the NF- κ B/p65 pathway. Our findings suggest that targeting RIT1 may be an effective strategy for the treatment of glioma.

Declarations

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Conflicts of interest

The authors declare no conflict of interest.

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Availability of data and material

The analyzed data used to support this study are available from the corresponding author on reasonable request.

Authors' contributions

QL made study concept and manuscript review; MGC collected data; MGC and QL analyzed the statistics; MGC prepared and edited manuscript; QL revised the manuscript; MGC and QL approved the final manuscript.

Ethics approval

Our study was approved by the Ethics Review Board of Second Affiliated Hospital of Soochow University.

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Figures

Figure 1

RIT1 is upregulated in glioma and is associated with poor prognosis. a mRNA expression of RIT1 in normal brain tissues (n=28) and glioma (n=444) from the Rembrandt dataset. b mRNA expression of RIT1 in normal brain tissues (n=8) and glioma (n=276) from the Gravendeel dataset. c-d The mRNA levels of RIT1 in grade II, grade III, and grade IV glioma tissues from the CGGA (c) and TCGA (d) databases analyzed

via GlioVis (d). e-f Kaplan-Meir survival curves from the CGGA (e) and TCGA (f) datasets. Clinical data were analysed via GlioVis. g Western blot detection of RIT1 expression in glioma and normal brain tissues. β -actin was used as a loading control.

Figure 2

RIT1 promotes the proliferation and invasion in glioma cells. U87 and GBM12 cells were transfected with siRIT1 or siNC for 48 hours. LN229 cells was transfected with Lv-RIT1 or Lv-NC. a Expression of RIT1 detected using Western blot. GAPDH was used as a loading control. b CCK-8 assays measuring

LN229 cells and RIT1-overexpression LN229 cells transfected with siRELA or siNC. g Transwell assays evaluating glioma cells (from f) invasion. Scar bar, 100μm. h Western blot analysis of p65 level in the indicated cells (from f). GAPDH was used as a loading control.

Figure 4

RIT1 silence inhibits tumor growth in vivo. a Immunofluorescence microscopy verifying the transfection efficiency in U87 cells treated with Lv-NC and Lv-RIT1. b-d The size (b), growth (c) and weight (d) of tumor

xenografts. e Protein expression of p-p65, p65, c-Myc and RIT1 detected using Western blot. GAPDH was used as a loading control.