Over-expression of Gαi3 in human glioma is required for Akt-mTOR activation and cell growth

Zheng-wei Li[1,*], Shang Cai[2,*], Yuanyuan Liu[3,*], Chuan-lai Yang[3,*], Ye Tian[2], Gang Chen[4], C. Cao[3]

[1]Surgical Department I, Xuzhou Children's Hospital, Xuzhou, China

[2]Department of Radiotherapy and Oncology, the Second Affiliated Hospital of Soochow University, Suzhou, China

[3]Institute of Neuroscience, Soochow University, Suzhou, China

[4]Department of Neurosurgery, the First Affiliated Hospital of Soochow University, Suzhou, Jiangsu, China

[*]Co-first authors

Correspondence to: Ye Tian, **email:** tianyesuzhoua163@163.com, dryetian@126.com
Gang Chen, **email:** nju_neurosurgery@163.com
C. Cao, **email:** caocong@suda.edu.cn

Keywords: *glioma, gαi3, receptor tyrosine kinase, Akt-mTOR, signalings*

Received: *June 13, 2016* ***Accepted:*** *July 19, 2016* ***Published:*** *August 01, 2016*

ABSTRACT

We have previously identified an unique function of G protein α inhibitory subunit (Gαi protein) in transducing Akt-mTOR signaling. Here, we examined the expression and biological functions of Gαi protein 3 (Gαi3) in human glioma. As compared to the normal brain tissues, mRNA and protein expressions of Gαi3 were significantly upregulated in multiple human glioma tissues. Its expression level was associated with receptor tyrosine kinases (RTKs, including EGFR, FGFR and PDGFRα) over-expression and Akt-mTOR hyperactivity. Gαi3 formed a complex with above RTKs and the adaptor protein Gab1 in glioma tissues and cells, which was required for downstream Akt-mTOR activation. Gαi3 shRNA knockdown or dominant negative mutation largely attenuated Akt-mTOR activation and glioma cell growth. Further, Gαi3-knockout (KO) mouse embryonic fibroblasts (MEFs) showed decreased Akt activation and cell growth. Reversely, introduction of a constitutively-active Gαi3 in glioma cells enhanced Akt-mTOR activation and cell growth. *In vivo*, Gαi3 shRNA-expressing U87MG tumors grew slower than the control shRNA-bearing U87MG tumors in nude mice. Akt-mTOR activation was also inhibited in U87MG tumors with Gαi3 shRNA. Collectively, these results indicate that over-expressed Gαi3 forms a complex with several RTKs in human glioma to transduce Akt-mTOR activation and tumor cell growth.

INTRODUCTION

Glioma is a main contributor of cancer-related death around the world [1–3]. The prognosis for high-grade glioma (grade III-IV) has been extremely poor [1–3]. Postoperative radiation and temozolomide (TMZ) chemotherapy have become the standard treatments for glioma [4–6]. Yet, the overall survival has not been significantly improved over the years [4–6].

Akt-mammalian target of rapamycin (mTOR) signaling is frequently dysregulated and hyper-activated in glioma, which promotes tumorigenesis, progression and chemoresistance [7–9]. Existing evidences have suggested that over-expressed and/or constitutively-active receptor tyrosine kinases (RTKs) cause Akt-mTOR over-activation in the glioma [10, 11]. These RTKs, including EGFR (epidermal growth factor receptor), FGFR (fibroblast growth factor receptor) and PDGFRα (platelet-derived growth factor receptor α), along with their downstream Akt-mTOR signalings are thus valuable oncotargets for glioma [11].

G protein α inhibitory subunit (Gαi protein) was originally identified by its ability to couple with GPCRs (G-protein coupled receptors) and to inhibit adenylate cyclase (AC) [12]. Intriguingly, our group [13, 14] and others [15] have identified an un-anticipated function of

Gαi protein in transducing Akt-mTOR signaling by several RTKs. For example, Gαi protein was required for the activation of Akt-mTOR signaling by EGFR and FGFR ligands [13, 14]. Following ligand binding, Gαi protein physically associates with RTK to activate the adaptor protein Gab1 (growth factor receptor binding 2 [Grb2]-associated binding protein 1) and downstream Akt-mTOR signaling [13, 14].

Having found the requirement of Gαi protein in RTK-activated Akt-mTORC1 signaling, we here examined the expression and biological functions of Gαi3 in human glioma cells and tissues. Our results indicate that over-expressed Gαi3 forms a complex with several RTKs in human glioma to transduce Akt-mTOR activation.

RESULTS

Gαi3 over-expression is associated with RTK upregulation and Akt-mTOR hyperactivity in human glioma tissues

First, we examined Gαi3 expression in human glioma tissues. As described, a total of twelve pairs of glioma tissues ("T") and surrounding normal brain tissues ("N") were collected. Real-time quantitative PCR assay was performed to examine Gαi3 mRNA expression. As demonstrated in Figure 1A and 1B, Gαi3 mRNA expression level in glioma tissues was 3–4 times higher than that in normal brain tissues. Intriguingly, we didn't detect a significant difference in Gαi3 mRNA level between low-grade (grade I–II, Figure 1A) and high grade (grade III–IV, Figure 1B) tumors. Western blotting assay was performed to test Gαi3 protein expression in above tissues. As compared to the surrounding brain tissues, Gαi3 protein in glioma tissues was again upregulated (see quantified results in Figure 1C and 1D). Once again, Gαi3 upregulation was equivalent between low-grade (Figure 1C) and high grade (Figure 1D) tumors. Remarkably, in these glioma tissues, over-expression of Gαi3 was positively correlated with upregulation of mentioned RTKs, including EGFR, FGFR and PDGFRα (see quantified results, Figure 1E).

Our previous studies have confirmed that Gαi3 is required for Akt-mTOR activation by EGFR [13] and FGFR [14]. Next, we wanted to know if Gαi3 expression was associated with Akt-mTOR activation level in these tissues. Indeed, glioma tumors with Gαi3 over-expression also showed high level of p-Akt (Ser-473) and p-S6K1 (Thr-389) (see quantified results, Figure 1F). Yet, p-Akt and p-S6K1 levels were much lower in normal

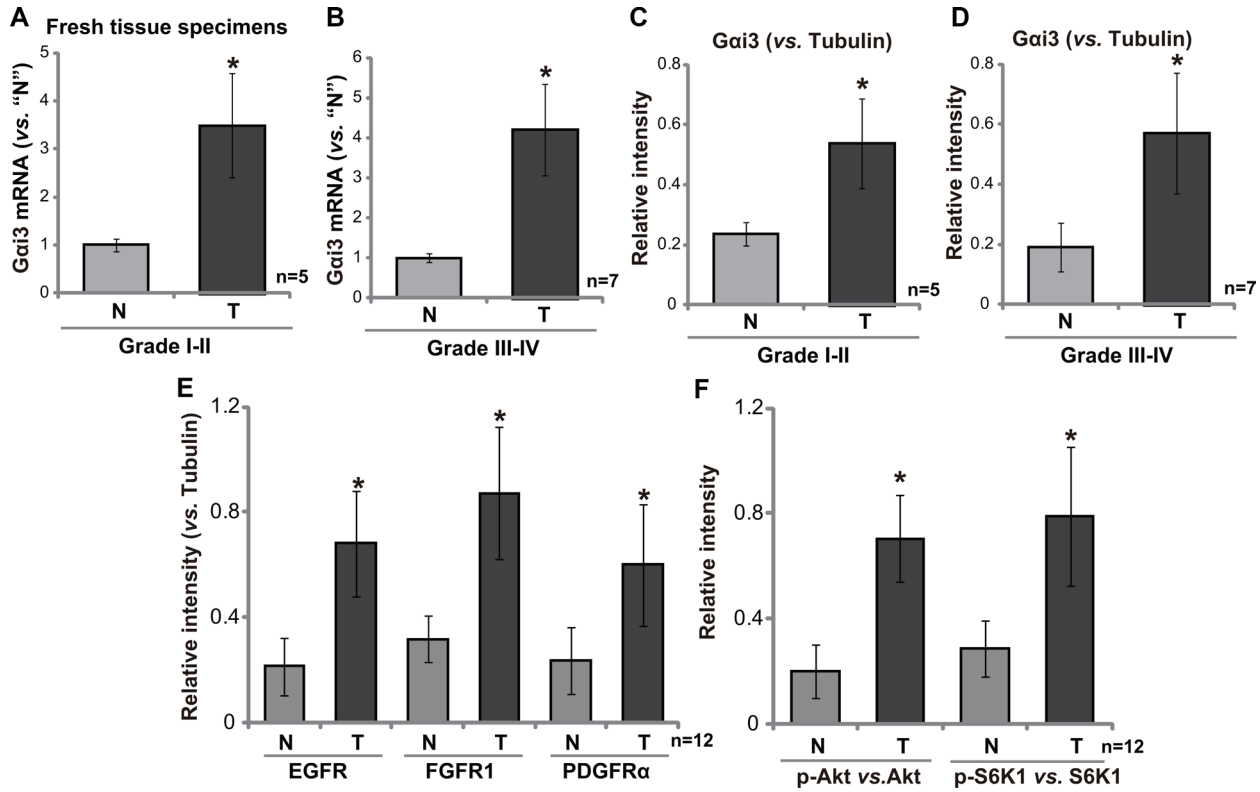

Figure 1: Gαi3 over-expression is associated with RTK upregulation and Akt-mTOR hyperactivity in human glioma tissues. Fresh human glioma tissues ("T", $n = 12$) and the surrounding normal brain tissues ("N", $n = 12$) were homogenized and dissolved in the tissue lysis buffer, relative Gαi3 mRNA expression (vs. GAPDH) was examined by real-time PCR assay (**A** and **B**); Quantified western blotting assay results showed expression of Gαi3 and other listed proteins in above patients' tissues (**C**–**F**). "MW" stands for molecular weight (Same for all figures). Bars stand for mean ± SD. *$p < 0.05$ vs. "N" group.

brain tissues (Figure 1F). Collectively, these results demonstrated that Gαi3 was over-expressed in multiple human glioma tissues, and its level was associated with RTKs upregulation and Akt-mTOR hyperactivity.

Over-expressed Gαi3 associates with several RTKs and the adaptor protein Gab1 in human glioma cells and tissues

Next, we tested Gαi3 expression in cultured human glioma cells. As compared to the primary human astrocytes ("Astrocytes"), Gαi3 protein (Figure 2A) and mRNA (Figure 2B) were up-regulated in U87MG glioma cells and primary human glioma cells (Patient-4-derived, "Glioma cells"). Further, Gαi3 over-expression was again correlated with high level of p-Akt/p-S6K1 in the glioma cells (Figure 2A). Similar results were also obtained in other patient-derived primary glioma cells (Data not shown). Importantly, co-immunoprecipitation (Co-IP) assay results showed that Gαi3 formed a complex with the mentioned RTKs (EGFR, FGFR and PDGFRα) and the adaptor protein Gab1 in U87MG cells (Figure 2C). Notably, the Gαi3-RTK-Gab1 association required FBS stimulation, and was not observed in phosphate-buffered solution (PBS)-starved cells ("starvation", Figure 2C).

Significantly, as shown in Figure 2D, the Gαi3-RTK-Gab1 association was also observed in multiple human glioma tissues. "T1/T4/T8" stand patient-1/-4/-8's glioma tissues (Figure 2D). Similar Gαi3-RTK-Gab1 association was also observed in other glioma tissues (Data not shown). Therefore, these results demonstrated that over-expressed Gαi3 associated with several RTKs

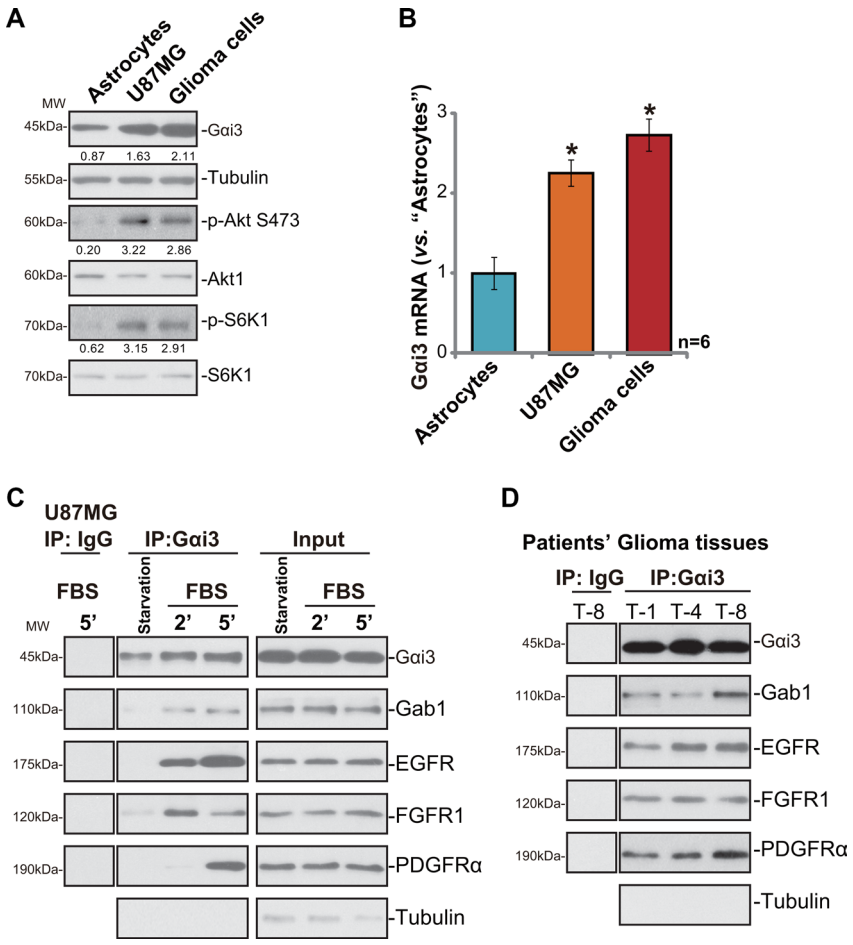

Figure 2: Over-expressed Gαi3 associates with several RTKs and the adaptor protein Gab1 in human glioma cells and tissues. Western blotting analysis showed expression of Gαi3 and listed kinases in primary human astrocytes ("Astrocytes"), U87MG glioma cells ("U87MG") and primary human glioma cells ("Glioma cells", Patient-4-derived) (**A**); Gαi3 mRNA expression (vs. GAPDH) in above cells was examined by real-time PCR assay (**B**). U87MG cells were phosphate-buffered solution (PBS)-starved (15 min, "starvation") or stimulated with 10% FBS for applied time, Gαi3 association with several RTKs (EGFR, FGFR and PDGFRα) and Gab1 was tested by co-immunoprecipitation (Co-IP) assay (**C**); "Input" showed expression of above proteins (C). Fresh glioma tissue lysates from patient-1/-4/-8 (named T-1/-4/-8) were subjected to same Co-IP assay of Gαi3-RTK-Gab1 association (**D**). Gαi3 protein expression (normalized to tubulin), p-Akt (normalized to Akt1) and p-S6K1 (normalized to S6K1) were quantified (A). For Western blotting assay, same set of lysate samples were run in sister gels. Experiments in this figure were repeated three times, with similar results obtained. Bars stand for mean ± SD. *$p < 0.05$ vs. "Astrocytes" group (B, $n = 6$).

(EGFR, FGFR and PDGFRα) and the adaptor protein Gab1 in human glioma cells and tissues.

In glioma cells, Gαi3 shRNA knockdown inhibits Akt activation and cell growth

To study the potential involvement of Gαi3 on glioma cell functions, lentiviral shRNA strategy was applied to stably knockdown Gαi3 in glioma cells. As shown in Figure 3A, the two non-overlapping Gαi3 shRNAs (–a/–b) downregulated Gαi3 expression in U87MG cells. As a result, p-Akt was inhibited (Figure 3A), indicating the requirement of Gαi3 in Akt-mTOR activation. Further, U87MG cell growth, tested by the MTT assay (Figure 3B) and clonogenicity assay (Figure 3C), was also inhibited by Gαi3 shRNAs. On the other hand, histone-DNA apoptosis ELISA assay results suggested a high basal apoptosis level in the Gαi3 knockdown cells (Figure 3D). Intriguingly, in U87MG cells, Gαi3 shRNA-b was more potent than Gαi3 shRNA-a in downregulating Gαi3 and pAkt (Figure 3A). Consequently, growth inhibition and apoptosis activation were also more significant in Gαi3 shRNA-b-expressing cells (Figure 3B–3D). To further study the function of Gαi3 in cell growth, Gαi3 knockout (KO) MEFs [13, 14] were applied. As compared to the wild-type (WT) MEFs, Akt activation (Figure 3G) and cell growth (Figure 3H) were significantly downregulated in Gαi3 KO MEFs. These results together suggest that Gαi3 is required for Akt activation and glioma cell growth.

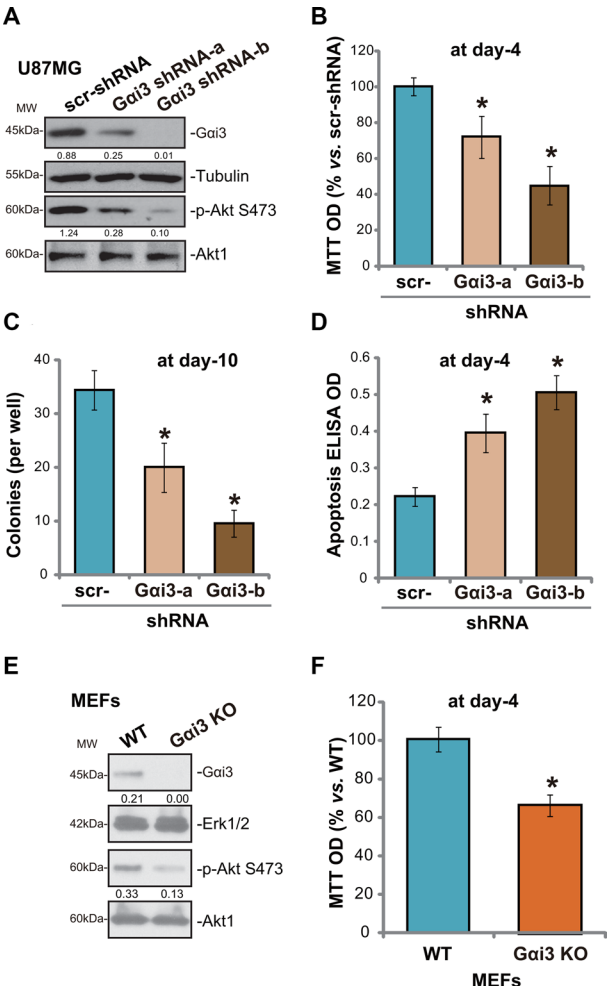

Figure 3: In glioma cells, Gαi3 shRNA knockdown inhibits Akt activation and cell growth. Western blotting analysis showed expression of Gαi3 and other listed proteins in stable U87MG cells (**A**) with Gαi3 shRNA ("–a/–b") or scramble control shRNA ("scr-shRNA"); Growth of these cells was tested by MTT assay (**B**, MTT OD at day-4) or colony formation assay (**C**, colony number at day-10). Basal apoptosis activation in the U87MG cells was tested by Histone DNA ELISA assay (**D**, apoptosis OD at day-4). Expression of Gαi3 and other listed proteins in wild-type (WT) and Gαi3 knockout (KO) MEFs was shown (**E**); Growth of the MEFs was tested by MTT assay (**F**, MTT OD at day-4). For all these assays, the exact same number of cells of different background were initially plated into each well. Same set of lysate samples were run in sister gels (A and E). Erk1/2 was tested as equal loading in (E). Experiments in this figure were repeated three times, with similar results obtained. Bars stand for mean ± SD. *$p < 0.05$ vs. "scr-shRNA" group (B–D, $n = 6$). *$p < 0.05$ vs. "WT MEFs" (F, $n = 6$).

Gαi3 mutation affects Akt-mTOR activation and glioma cell growth

To further confirm the requirement of Gαi3 in Akt-mTOR activation and glioma cell growth, a dominant negative interference strategy was applied. We replaced the conserved Gly (G) residue with Thr (T) in the G3 box of Gαi3 [13, 14]. The dominant negative Gαi3 (G202T, DN-Gαi3) will compete with WT Gαi3 for binding to other proteins [16, 17]. In stable U87MG cells expressing DN-Gαi3, we noticed substantially decreased phosphorylations of Akt and S6K1, as compared to that in vector control cells (Figure 4A). U87MG cell growth, tested by MTT assay and clonogenicity assay, was also inhibited in DN-Gαi3 expressing cells (Figure 4B and 4C). While the cell apoptosis level was increased (Figure 4D).

On the hand, a constitutively active Gαi3 (Q204L, CA-Gαi3) [13] was introduced into U87MG cells. We showed that overexpression of CA-Gαi3 in U87 MG cells led to increased phosphorylations of Akt and S6K1 (vs. vector control U87 cells) (Figure 4A). As a result, U87MG cell growth was increased (Figure 4B and 4C). Yet apoptosis of the CA-Gαi3 cells was inhibited (Figure 4D). Collectively, these results provided more evidence to support that Gαi3 is required for Akt-mTOR activation and glioma cell growth.

Gαi3 shRNA knockdown inhibits U87MG tumor growth in nude mice

At last, we evaluated the potential requirement of Gαi3 on U87MG cell growth *in vivo*. As described, same amount of stable U87MG cells expressing scramble control shRNA ("scr-shRNA") or Gαi3 shRNA (–b) were *s.c.* inoculated into the nude mice. After 10–15 days, the U87MG tumors were established. Tumor growth curve in Figure 5A demonstrated that Gαi3 shRNA-expressing U87MG tumors grew significantly slower than the scr-shRNA-bearing U87MG tumors. The estimated daily tumor growth results further confirmed the inhibitory effect of Gαi3 shRNA on U87MG tumor growth (Figure 5B). Notably, the mice body weights were not significantly different between the two groups (Figure 5C).

We also analyzed Gαi3 expression and Akt-mTOR activation in the U87MG tumors. As expected, Gαi3 was dramatically downregulated in U87MG tumors bearing Gαi3 shRNA (Figure 5D). In line with the *in vitro* findings, p-Akt and p-S6K1 were both inhibited in Gαi3 shRNA-expressing U87MG tumors (Figure 5D). IHC staining results (Figure 5E) further confirmed p-Akt inhibition in the U87MG tumor with Gαi3 shRNA. Together, these results demonstrated that Gαi3 shRNA inhibited Akt-mTOR activation and U87MG tumor growth *in vivo*.

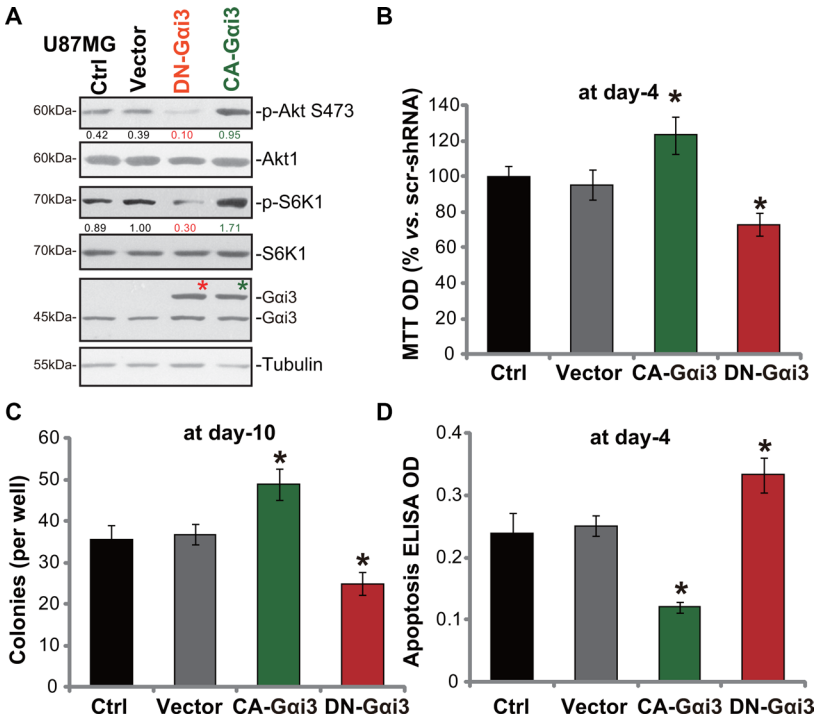

Figure 4: Gαi3 mutation affects Akt-mTOR activation and glioma cell growth. Western blotting analysis tested expression of Gαi3 and other listed proteins in stable U87MG cells with the dominant negative Gαi3 (G202T, DN-Gαi3), the constitutively active Gαi3 (Q204L, CA-Gαi3) or the empty vector (pGCL-GFP-puro) (**A**). Growth of these cells was tested by MTT assay (**B**, MTT OD at day-4) or colony formation assay (**C**, colony number at day-10). Basal apoptosis activation was tested by Histone DNA ELISA assay (**D**, apoptosis OD at day-4). For all these assays, the exact same number of cells of different background were initially plated into each well. For Western blotting assay, same set of lysate samples were run in sister gels (A). "Ctrl" stands for the un-transfected parental cells. Experiments in this figure were repeated three times, with similar results obtained. Bars stand for mean ± SD. *$p < 0.05$ vs. "Vector" cells (B–D, $n = 6$).

DISCUSSIONS

Our previous studies [13, 14] have revealed an unique mechanism of Gαi protein in transducing Akt-mTOR signaling by several RTKs. Upon RTK ligand (i.e. EGF) stimulation, Gαi protein was shown to associate with the RTKs and to transduce signaling to the adaptor protein Gab1, causing Gab1 and downstream Akt-mTOR activation [13, 14]. shRNA knockdown, dominant negative mutation or complete knockout of Gαi protein thus inhibited RTK signaling to Gab1, and significantly attenuated downstream Akt-mTOR activation [13, 14].

Here, we showed that Gαi3 was over-expressed in multiple human glioma tissues and cells, which was associated with RTK (EGFR, FGFR and PDGFRα) upregulation and Akt-mTOR hyperactivity. Intriguingly, over-expressed Gαi3 formed a complex with above RTKs and the adaptor protein Gab1 in glioma tissues and cells, which was also required for downstream Akt-mTOR activation. shRNA knockdown or dominant negative mutation of Gαi3 potently inhibited Akt-mTOR activation and glioma cell growth. Meanwhile, Gαi3-depleted MEFs demonstrated reduced Akt-mTOR activity and cell growth. On the other hand, introduction of a constitutively active

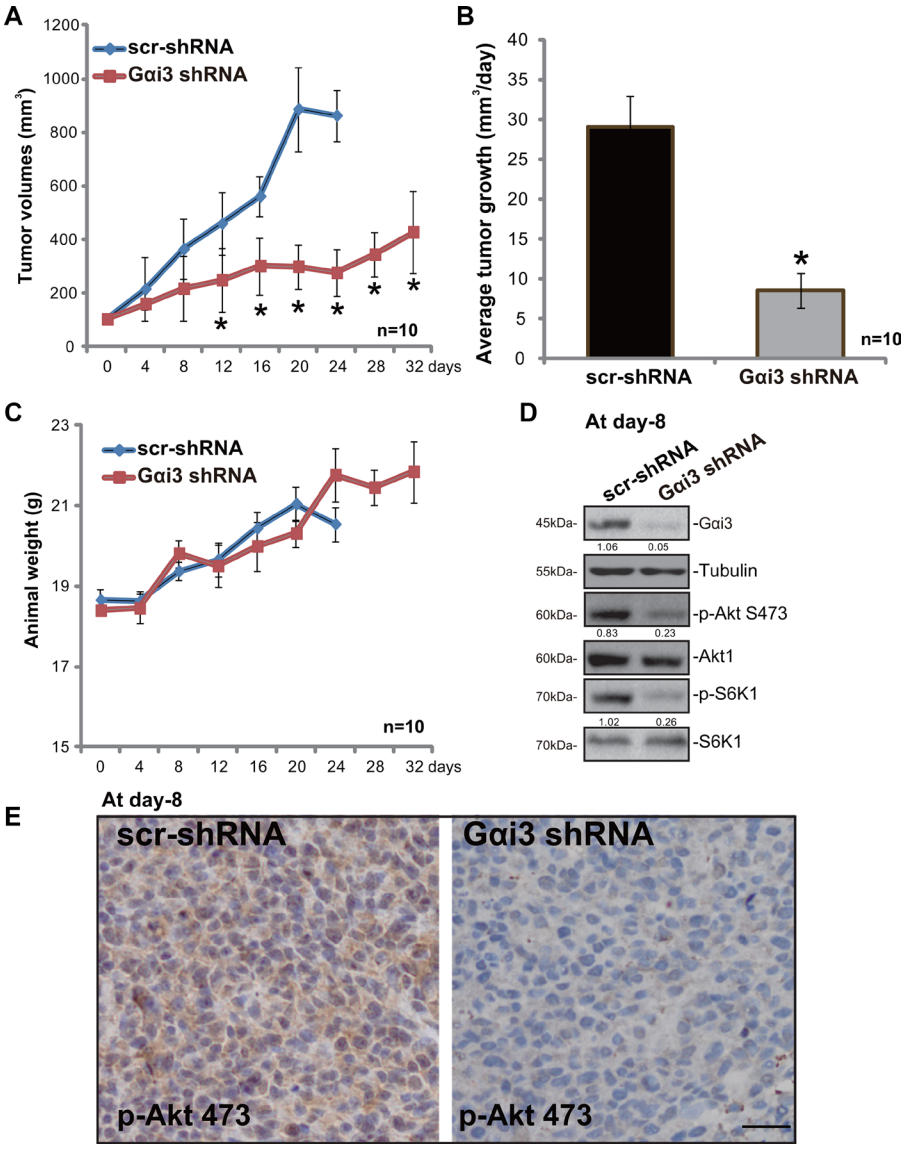

Figure 5: Gαi3 shRNA knockdown inhibits U87MG tumor growth in nude mice. Same amount (five millions cells per mouse) of Gαi3 shRNA-expressing stable U87MG cells ("Gαi3 shRNA") or scramble control shRNA-expressing stable U87MG cells ("scr-shRNA") were inoculated *s.c.* into the nude mice (10 mice per group), tumor volumes (**A**) and mice body weights (**C**) were recorded every four days; Estimated average daily tumor growth was calculated (**B**); At day-8, one U87MG tumor per group was isolated, expression of listed proteins in the fresh tumor tissue lysates was tested by Western blotting assay (**D**). p-Akt Ser473 (**E**) was also tested by IHC staining, representative images were presented. For Western blotting assay, same set of lysate samples were run in sister gels (D). Bars stand for mean ± SD (A–C). *$p < 0.05$ vs. "scr-shRNA" tumor group (A and B). Bar = 50 μm (E).

Gαi3 enhanced Akt-mTOR activation and glioma cell growth. These results suggest that over-expressed Gαi3 is required for Akt-mTOR activation and glioma cell growth.

It is known that multiple RTKs are amplified and/or hyper-activated simultaneously in human glioma, which have important clinical implications for tumor progress and resistance to targeted therapies [18, 19]. The efficiency of a single targeted therapy (*i.e.* EGFR inhibitors) could therefore be compromised due to co-existence other RTKs activation [10, 11]. Indeed, recent studies have shown that glioma patients often respond better to a combination of inhibitors of different RTKs [18, 19]. Our previous and current findings implied that Gαi protein could be the common adaptor protein for several RTKs, which participates in downstream Akt-mTOR activation and tumor cell progression. Significantly, our *in vivo* studies showed that Gαi3 shRNA inhibited Akt-mTOR activation and U87MG tumor growth in nude mice. These results, together with the findings of Gαi3 over-expression in human gliomas, imply that Gαi3 might be a valuable oncotarget for glioma.

MATERIALS AND METHODS

Reagents

The antibodies utilized in this study were described previously [13, 14, 20, 21]. Cell culture reagents were obtained from Gibco (Shanghai, China).

Cell lines

Wild-type (WT) and Gαi3 knockout (KO) mouse embryonic fibroblasts (MEFs) were described early [13, 14]. Human U87MG glioma cell line was purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China). Cells were maintained in DMEM medium, supplemented with 8% fetal bovine serum (FBS) and necessary antibiotics, and in the CO_2 incubator at 37°C.

Human glioma tissues

All studies involving human specimens were conducted according to the principles expressed in the Declaration of Helsinki, and to the national and international guidelines. The protocol was approved by the Ethics Review Board (ERB) of Soochow University. A total of twelve informed-consent glioma patients were enrolled (All hospitalized in the Affiliated Hospitals of Soochow University). The patients' basic parameters were as following: Male: 8/Female: 4; 43–62 years old; Grade I–II: 5 and Grade III–IV: 7. Fresh human glioma tissues along with the surrounding normal brain tissues were obtained at the time of surgery. Fresh tissues were minced, homogenized and dissolved in the tissue lysis buffer (Biyuntian, Wuxi, China). Samples were then subjected to real-time PCR assay or Western blotting assay.

Primary culture of human glioma cells

Similar to our previous protocol [17], fresh glioma tissues were thoroughly washed, and were minced into small pieces. Single-cell suspensions were achieved by re-suspending tissues in 0.10% (w/v) collagenase I (Sigma, Shanghai, China). The resulting cell suspensions were then washed, filtered through a 70-μm cell strainer. Afterwards, the primary glioma cells were pelleted and rinsed twice with DMEM. Cells were then cultured in primary cell culture medium as previously described [21].

Primary culture of human astrocytes

Human primary astrocyte cultures were purchased from the Cell Bank of Fudan University (Shanghai, China). The astrocytes were derived from the cerebral cortices of a single trauma patient. Virtually all the astrocytes were positive of glial fibrillary acidic protein (GFAP). The astrocytes were maintained in astrocyte media (Science Cell, Carlsbad, CA) containing 10% FBS, 1% astrocyte growth supplement and 1% Penicillin/Streptomycin.

Real-time reverse transcriptase polymerase chain reaction

As described in our previous study [18], total RNA was prepared via the standard TRIzol reagents (Invitrogen, Shanghai, China). The PCR reaction mixture contained 1 × SYBR Master Mix (Applied Biosystem, Foster City, CA), 500 ng RNA and 200 nM primers. An ABI Prism 7300 Fast Real-Time PCR system (Foster City, CA) was applied for PCR reactions. mRNA expression was quantified via the $^{\Delta\Delta}$Ct method. GAPDH served as the internal control. The following primers were applied: Gαi3, Forward: 5′-AAACGGTTATGGCGAGATGGT-3′. Reverse: 5′-TG GTCTTCACTCTCGTCCGAA-3′. The GAPDH primers were described previously [22]. The primers were purchased from Genechem (Shanghai, China).

Western blotting analysis

Cells or glioma tissues were lysed with lysis buffer described previously [13, 14, 20]. Aliquots of 40 μg of protein from each treatment were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). The membranes were then incubated with specific primary antibody and corresponding second antibody. Antibody-antigen binding was detected with the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Piscataway, NJ). Each band was quantified through Image J software (NIH). For the Western blot assay, each lane was loaded with exact same amount of quantified protein lysates (30 μg per sample). Same set of lysate samples were run in sister gels to test different proteins if necessary.

Co-immunoprecipitation (Co-IP)

The detailed protocol was described in our previous studies [13, 20]. Briefly, aliquots of 600 μg of protein lysates from each sample were pre-cleared, followed by incubation with anti-Gαi3 antibody overnight. Thirty μl of protein A/G beads (Sigma) were then added, and the lysates were incubated for 2 hours at 4°C. The beads were washed, and Gαi3-assocaited proteins were then detected by Western blotting.

Generation of Gαi3 stable knockdown glioma cells

Two lentiviral Gαi3 shRNAs ("–a/–b", with non-overlapping sequences) were purchased from Genechem (Shanghai, China). The target sequence for Gαi3 shRNA-a and Gαi3 shRNA-b were 5′-TCAATCATTCTCTTCCTTA-3′ and 5′-CCTCAGTG ATTATGACCTT-3′, respectively. The detailed protocol was described in our previous studies [14, 21, 22]. Briefly, glioma cells were seeded onto six-well plate with 50% confluence. Twenty μL/mL of lentiviral Gαi3 shRNA was added to the cells for 24 hours. Afterward, shRNA-containing medium was replaced by the culture medium for additional 48 hours. The stable glioma cells expressing Gαi3 shRNA were selected by puromycin (0. 5 μg/mL, Sigma) for a total of 6–8 days. Gαi3 expression in stable cells was detected by Western blotting. Same amount of lentiviral scramble shRNA ("scr-shRNA", Santa Cruz, sc-108080) was added to the control cells.

Gαi3 mutation and stable cell selection

The plasmid (0.25 μg per well) encoding a constitutively-active-Gαi3 (CA-Gαi3-GFP-puro, Q204L) [13], a dominant negative Gαi3 (DN-Gαi3-GFP-puro, G202T) [13, 14], or the empty vector (pGCL-GFP-puro, GeneChem) was transfected to U87MG cells via the Lipofectamine 2000 protocol as described [14]. Twenty four hours after transfection, cells were cultured in complete medium for additional 48 hours. Puromycin (0.5 μg/mL, Sigma) was then applied to select stable clones expressing target construct. The target protein (Gαi-GFP) in stable cells was tested by Western blotting.

Cell growth and apoptosis assay

MTT assay of cell growth, clonogenicity assay of cell growth and histone DNA apoptosis ELISA assay were described in detail in our previous studies [13, 20, 21, 23, 24].

Nude mice xenograft assay

As described [25], Gαi3 shRNA(–b)-expressing stable U87MG cells or scr-shRNA-expressing stable U87MG cells (five millions cells in 200 μl of Matrigel gel per mouse) were subcutaneously (*s.c.*) injected into the right flanks of 4–5 week-old female nude mice (ten mice per group, at Soochow University Animal Facility, Soochow, China). When the tumor volume reached approximately 100 mm^3, the recording was started. The size of the U87MG tumors and mice body weights were measured every four days, and tumor volumes were calculated using the following formula: $\pi/6 \times \text{width}^2 \times \text{length}$ [25]. Estimated average daily tumor growth was also calculated [25]. All animal procedures were approved by Soochow University Ethics Review Board and IACUC.

Immunohistochemistry (IHC) staining

As described in our previous study [14], the IHC staining was performed on cryostat sections (3 μm) of U87 xenograft tumors according to standard methods. We incubated slides in the appropriate dilution of primary antibody (anti-Akt Ser 473, 1: 25, Cell Signaling Tech), which were subsequently stained with corresponding secondary antibodies (Santa Cruz). The peroxidase activity was visualized via the 3-amino-9-ethyl-carbazol (AEC) method (Merck) [14].

Statistical analysis

All experiments were performed in triplicate and were repeated at least three times, similar results were obtained in each case. The data presented were means ± standard deviation (SD). Statistical differences were analyzed by one-way *ANOVA* followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 18.0). Values of $p < 0.05$ were considered as statistically significant.

ACKNOWLEDGMENTS

This work was generously supported by grants from the National Natural Science Foundation of China (Nos. 81302195, 81372411 and 31371139); Grants from Natural Science Foundation of Jiangsu Province (BK20130301); Jiangsu Provincial Special Program of Clinical Medical Science (BL2014040); Suzhou Key Laboratory for Radiation Oncology (To Y.T.). Suzhou Science and Technology Development Program (SZS201509).

CONFLICTS OF INTEREST

The authors declare no competing financial interests.

REFERENCES

1. Westphal M, Lamszus K. The neurobiology of gliomas: from cell biology to the development of therapeutic approaches. Nat Rev Neurosci. 2011; 12:495–508.

2. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2012. CA Cancer J Clin. 2012; 62:10–29.
3. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin. 2014; 64:9–29.
4. Khasraw M, Lassman AB. Neuro-oncology: late neurocognitive decline after radiotherapy for low-grade glioma. Nat Rev Neurol. 2009; 5:646–647.
5. Pollack IF. Neuro-oncology: Therapeutic benefits of reirradiation for recurrent brain tumors. Nat Rev Neurol. 2010; 6:533–535.
6. Wang Y, Jiang T. Understanding high grade glioma: molecular mechanism, therapy and comprehensive management. Cancer Lett. 2013; 331:139–146.
7. Huang TT, Sarkaria SM, Cloughesy TF, Mischel PS. Targeted therapy for malignant glioma patients: lessons learned and the road ahead. Neurotherapeutics. 2009; 6:500–512.
8. Lefranc F, Rynkowski M, DeWitte O, Kiss R. Present and potential future adjuvant issues in high-grade astrocytic glioma treatment. Adv Tech Stand Neurosurg. 2009; 34:3–35.
9. Li X, Wu C, Chen N, Gu H, Yen A, Cao L, Wang E, Wang L. PI3K/Akt/mTOR signaling pathway and targeted therapy for glioblastoma. Oncotarget. 2016.
10. Lo HW. EGFR-targeted therapy in malignant glioma: novel aspects and mechanisms of drug resistance. Curr Mol Pharmacol. 2010; 3:37–52.
11. Snuderl M, Fazlollahi L, Le LP, Nitta M, Zhelyazkova BH, Davidson CJ, Akhavanfard S, Cahill DP, Aldape KD, Betensky RA, Louis DN, Iafrate AJ. Mosaic amplification of multiple receptor tyrosine kinase genes in glioblastoma. Cancer Cell. 2011; 20:810–817.
12. Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol. 1997; 7:261–269.
13. Cao C, Huang X, Han Y, Wan Y, Birnbaumer L, Feng GS, Marshall J, Jiang M, Chu WM. Galpha(i1) and Galpha(i3) are required for epidermal growth factor-mediated activation of the Akt-mTORC1 pathway. Sci Signal. 2009; 2:ra17.
14. Zhang YM, Zhang ZQ, Liu YY, Zhou X, Shi XH, Jiang Q, Fan DL, Cao C. Requirement of Galphai1/3-Gab1 signaling complex for keratinocyte growth factor-induced PI3K-AKT-mTORC1 activation. J Invest Dermatol. 2015; 135:181–191.
15. Wang Z, Dela Cruz R, Ji F, Guo S, Zhang J, Wang Y, Feng GS, Birnbaumer L, Jiang M, Chu WM. G(i)alpha proteins exhibit functional differences in the activation of ERK1/2, Akt and mTORC1 by growth factors in normal and breast cancer cells. Cell Commun Signal. 2014; 12:10.
16. Hubbard KB, Hepler JR. Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. Cell Signal. 2006; 18:135–150.
17. Barren B, Artemyev NO. Mechanisms of dominant negative G-protein alpha subunits. J Neurosci Res. 2007; 85: 3505–3514.
18. Joensuu H, Puputti M, Sihto H, Tynninen O, Nupponen NN. Amplification of genes encoding KIT, PDGFRalpha and VEGFR2 receptor tyrosine kinases is frequent in glioblastoma multiforme. J Pathol. 2005; 207:224–231.
19. Kuhnhenn J, Kowalski T, Steenken S, Ostermann K, Schlegel U. Procarbazine, carmustine, and vincristine (PBV) for chemotherapy pre-treated patients with recurrent glioblastoma: a single-institution analysis. J Neurooncol. 2012; 109:433–438.
20. Cao C, Rioult-Pedotti MS, Migani P, Yu CJ, Tiwari R, Parang K, Spaller MR, Goebel DJ, Marshall J. Impairment of TrkB-PSD-95 signaling in Angelman syndrome. PLoS Biol. 2013; 11:e1001478.
21. Yang L, Zheng LY, Tian Y, Zhang ZQ, Dong WL, Wang XF, Zhang XY, Cao C. C6 ceramide dramatically enhances docetaxel-induced growth inhibition and apoptosis in cultured breast cancer cells: a mechanism study. Exp Cell Res. 2015; 332:47–59.
22. Zhang H, Liu YY, Jiang Q, Li KR, Zhao YX, Cao C, Yao J. Salvianolic acid A protects RPE cells against oxidative stress through activation of Nrf2/HO-1 signaling. Free Radic Biol Med. 2014; 69:219–228.
23. Chen MB, Wei MX, Han JY, Wu XY, Li C, Wang J, Shen W, Lu PH. MicroRNA-451 regulates AMPK/mTORC1 signaling and fascin1 expression in HT-29 colorectal cancer cells. Cell Signal. 2013.
24. Wu CH, Cao C, Kim JH, Hsu CH, Wanebo HJ, Bowen WD, Xu J, Marshall J. Trojan-horse nanotube on-command intracellular drug delivery. Nano Lett. 2012; 12:5475–5480.
25. Chen MB, Jiang Q, Liu YY, Zhang Y, He BS, Wei MX, Lu JW, Ji Y, Lu PH. C6 ceramide dramatically increases vincristine sensitivity both *in vivo* and *in vitro*, involving AMP-activated protein kinase-p53 signaling. Carcinogenesis. 2015; 36:1061–1070.