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LABORATORY INVESTIGATION

MiR-212-3p inhibits glioblastoma cell proliferation by targeting SGK3

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Abstract Glioblastoma multiforme (GBM) is the most malignant brain tumor in humans. Previous studies have demonstrated that microRNA plays important roles in the development and proliferation of GBM cells. Here we defined the mechanism by which miR-212-3p regulated the proliferation of GBM. In this study, we showed that miR-212-3p expression was significantly down-regulated and negatively correlated with serum and glucocorticoid-inducible kinase 3 (SGK3) in GBM. Either over-expression of miR-212-3p or silence of SGK3 decreased viability of GBM cells. Moreover, miR-212-3p directly bound to 3′UTR of SGK3 and inhibited its mRNA and protein expression. And over-expression of SGK3 rescued the decreased proliferation of GBM cells induced by miR-212-3p. Importantly, miR-212-3p also suppressed tumor growth in vivo. Collectively, our results demonstrated that miR-212-3p inhibited proliferation of GBM cells by directly targeting SGK3, and could potentially serve as a new therapeutic target for GBM.

Keywords MiR-212-3p · Glioblastoma · Serum and glucocorticoid-inducible kinase 3 (SGK3)

Introduction

Malignant gliomas are the most common and aggressive type of primary adult brain tumors. Among gliomas, glioblastoma multiforme (GBM) is the most common and deadliest form of malignant glioma. In spite of aggressive therapies including surgical resection, radiotherapy, and chemotherapy, the prognosis of GBM patients remains incurable, with a mean survival of 12–15 months after diagnosis [1–4]. Due to the limitations of current treatment modalities, novel treatments based on the therapeutic genes are being actively studied.

Serum and glucocorticoid-inducible kinase 3 (SGK3) is a member of SGK family of serine/threonine kinase, which

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shares many common features with AKT [5–7]. AKT activation involves in many cellular processes contributing to GBM malignancy [8]. Thus we speculated that SGK3 might also contribute to GBM development. Previous studies have demonstrated that SGK3 can regulate cell growth, proliferation and migration of cancers [9, 10]. Moreover, SGK3 can be activated by IGF-1 and EGF which play important roles in GBM development [11–13]. Additionally, SGK3 can also regulate cell survival through regulation of some molecules, such as FOXO3a and GSK3β, indicating that SGK3 may provide another avenue for targeted therapy [9, 14, 15]. However, the biological function and the precise regulatory mechanism of SGK3 in GBM are unknown.

MicroRNAs (miRNAs) are a class of small non-coding endogenous RNAs that often inhibit gene expression post-transcriptionally through translational inhibition and/or mRNA degradation [16–19]. MiRNAs have been shown to involve in various physiological and pathological processes in GBM, such as cell proliferation, apoptosis and differentiation [20, 21]. After searching miRNA predicting database, we found that miR-212-3p might target SGK3. Recent studies revealed that miR-212-3p might be a tumor suppressor in non-small cell lung cancer, gastric cancer, and colorectal cancer, and its repressive effect was correlated with cell cycle arrest, epithelial-mesenchymal transition process and apoptosis [22–24]. However, little is understood about the function of miR-212-3p in GBM. Based on this background, we investigated the relationship between miR-212-3p and SGK3 in GBM. Here, we provided an evidence that the role of miR-212-3p as one of the tumor-suppressive miRNA that was strongly down-regulated and negatively correlated with SGK3 in GBM tissues. We showed that over-expression of miR-212-3p suppressed GBM proliferation by directly targeting SGK3. In all, these findings demonstrated a previously unknown mechanistic link between miR-212-3p and SGK3, which may offer a novel therapeutic target for GBM.

Materials and methods

Patient samples collection

Normal adjacent tissues (NATs) (n = 6) and human glioma tissues (WHO, Grade III and Grade IV, n = 27) were collected from the Department of Neurosurgery, the First Affiliated Hospital of Harbin Medical University (Table S1). NATs were obtained from 6 GBM patients. Tissues were frozen in liquid nitrogen immediately and stored at −80 °C after excision. Primary GBM-1, GBM-2 and GBM-3 cells were separated from three GBM tissues respectively as previously reported [3, 25]. This study was approved by the Ethical Committee of the First Affiliated Hospital of Harbin Medical University, China. All the participants gave informed consent.

Cell culture and transfection

Glioblastoma cell lines (U87, U251 and T98) were obtained from RIKEN Cell Bank (Tsukuba Science City, Japan). Human embryonic kidney (HEK) 293T cells were obtained from the American Type Culture Collection (ATCC). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, USA) with 10 % fetal bovine serum (FBS, Invitrogen), 1 % Streptomycin and penicillin (Beyotime, China) in a humidified 37 °C, 5 % CO_2 incubator.

Negative control (NC), miR-212-3p mimics, NC inhibitor and miR-212-3p inhibitor oligonucleotides were purchased from GenePharma company (Shanghai, China). The SGK3 siRNAs and NC were synthesized by Invitrogen. GBM cells were transfected with miRNAs and siRNAs using x-treme GENE siRNA transfection reagent (Roche, Switzerland). Total proteins and mRNAs were collected for western-blot and qRT-PCR 48 h after transfection, respectively.

MTT assay

Glioblastoma cells were seeded on 96-well plates at a density of 5×10^4/ml. Cells were transfected with x-treme GENE siRNA transfection reagent or Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's instructions. After 96 h, 10 μl MTT dye (5 mg/ml, Sigma, USA) was added to each well for 4 h at 37 °C in the dark. Then the supernatants were removed and 150 μl dimethyl sulfoxide (Sigma, USA) was added to stop the reaction. Optical density was measured on a spectrophotometric (Tecan, Switzerland) at a wavelength of 490 nm.

Western blot analysis

Total proteins were extracted from cultured cells. The cells were lysed in RIPA buffer. The lysates were separated by 10–15 % SDS-PAGE gels, and then transferred to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5 % skim milk with tween for 1 h and incubated with primary antibodies overnight at 4 °C, and thereafter were incubated with fluorescence-conjugated secondary antibodies (1:5000, Invitrogen, USA). Immunoreactive complexes were visualized using BeyoECL Plus (Beyotime, China). To perform western blot analysis, the following antibodies were used: rabbit anti-SGK3 (1:1000; Cell Signaling Technology, USA), mouse anti-β-Actin (1:2000; Santa Cruz, CA, USA).

Quantitative real-time PCR (qRT-PCR)

Total RNAs were isolated from samples with Trizol reagent (Invitrogen, USA) according to manufacturer's instructions. Expression level of miR-212 and SGK3 were analyzed using SYBR Green PCR Master Mix kit (Applied Biosystems, USA). U6 and Actin were used as internal control for miR-212-3p and SGK3 respectively. qRT-PCR was run on thermocycler ABI Prism® 7500 fast (Applied Biosystems, CA). All the data were normalized to the internal control. The primers used in the experiments were listed in Table 1.

Plasmid construction

SGK3 CDS (1488 bp) was successfully amplified using the human hybrid cDNA. SGK3 CDS sequence was cloned into p-EGFP-N3 vector (BD, Biosciences Clontech) after enzyme digestion and collection. The sequence of p-EGFP-N3-SGK3 vector was confirmed, and then blast analysis with known SGK3 CDS sequence on NCBI, the sequencing data showed that no mutation sites were found. p-EGFP-N3-SGK3 vector can be used for subsequent over-expression of SGK3 in GBM cells. The primers used in these procedures were listed in the Table 1.

Luciferase assay

To verify directly binding relationship between SGK3 and miR-212-3p, wild type (WT) and mutant (mut) luciferase reporter plasmids were constructed respectively as following: psi-CHECK2-WT-SGK3-3′-UTR, psi-CHECK2-mut-1-SGK3-3′-UTR, psi-CHECK2-mut-2-SGK3-3′-UTR and psi-CHECK2-mut-1&2-SGK3-3′-UTR. Mut-1, mut-2 and mut-1&2 referred to mutation of SGK3′s UTR at 287–293, 332–338 and two positions together respectively. All constructed plasmids were completely confirmed by sequencing. To perform the luciferase reporter assay, HEK293T cells were transfected with 0.5 μg either psi-CHECK2-WT-SGK3-3′-UTR or psi-CHECK2-mut-1/2/1&2-SGK3-3′-UTR with NC, miR-212-3p mimics, NC inhibitor, miR-212-3p inhibitor together using Lipofectamine 2000. The luciferase activity was measured using Dual-Luciferase Reporter Assay System (E1910, Promega) at 48 h post transfection. The primers used for constructing WT or mutant plasmid of SGK3′s 3′ UTR were listed in the Table 1.

Table 1 Primers and sequences used in experiment

Primer used for miR-212-3p and U6 detection	
Hsa-miR-212-3p-RT	5′ GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCA CTGGATACGACGGCCG 3′
Hsa-miR-212-3p-F	5′ GGTAACAGTCTCCAGTCA 3′
Hsa-miR-212-3p-R	5′ GCAATTGCACTGGATACG 3′
U6-RT	5′ CGCTTCACGAATTTGCGTGTCAT 3′
U6-F	5′ GCTTCGGCAGCACATATACTAAAAT 3′
U6-R	5′ CGCTTCACGAATTTGCGTGTCAT 3′
Primers used for SGK3 and actin detection	
SGK3 F	5′ CAGGCTGTAAGACTCACTCC 3′ R: 5′ TTGCTATTTCTGACACCACTA 3′
Actin F	5′ AGAGCTACGAGCTGCCTGAC 3′ R: 5′ AGCACTGTGTTGGCGTACAG 3′
Primers used for construct SGK3 plasmid	
SGK3 XhoIF	5′ CCGCTCGAGGCCACCATGCAAAGAGATCACACCATGGAC 3′
SGK3 BamHIR	5′ CGCGGATCCCAAAAATAAGTCTTCTGAAGGAGGTG 3′
NC or siRNA sequences of SGK3	
NC	5′ UUCUCCGAACGUGUCACGUTT 3′, 5′ ACGUGACACGUUCGGAGAATT 3′
SGK3 siRNA 1	5′ GCAUUGGGUUACUUACAUUTT 3′, 5′ AAUGUAAGUAACCCAAUGCTT 3′
SGK3 siRNA 2	5′ GCUAUUUCUGACACCACUATT 3′, 5′ UACUGGUGUCAGAAAUAGCTT 3′
Primer used for wild type or mutant SGK3 3′ UTR	
SGK3 XhoIF	5′ CCGCTCGAG GCAGTTTGCCATTCAGAAACCATTG 3′
SGK3 NotIR	5′ ATAAGAATGCGGCCGC TTGATCTTTTTGCTGATTTTATTATAC 3′
Mut-SGK3-1 F	5′ AAGAAACCTTTTTTGCTATTTCAGTGGTTTTCCCTCTAAGTTTACAC 3′
Mut-SGK3-1 R	5′ GTGTAAACTTAGAGGGAAAACCACTGAAATAGCAAAAAAGGTTTCTT 3′
Mut-SGK3-2 F	5′ CTAACATCTACCCAAGATATCAGTGGTTTTAACAGTCAATTTCAG 3′
Mut-SGK3-2 R	5′ CTGAAATTGACTGTTAAAACCACTGATATCTTGGGTAGATGTTAG 3′

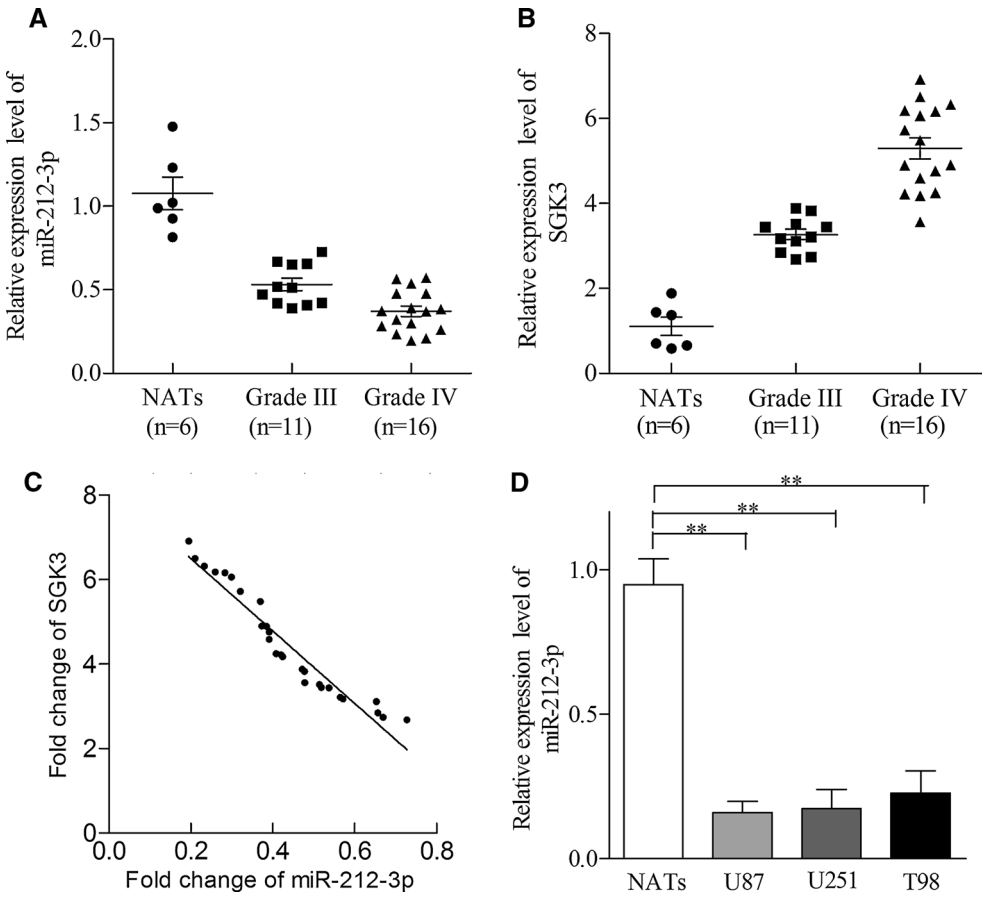

Fig. 1 Endogenous expression level of miR-212-3p and SGK3 is negatively correlated in glioma tissues. **a**, **b** qRT-PCR analyzed endogenous miR-212-3p and SGK3 expression level in normal adjacent tissues (NATs), human glioma tissues (WHO Grade III, n = 11, Grade IV, n = 16). **c** Negative linear correlation between miR-212-3p and SGK3 in human glioma tissues (WHO III-IV, n = 27) were determined by linear correlation analysis ($p < 0.05$, r = −0.931). **d** qRT-PCR analysis of endogenous miR-212-3p expression level in NATs, U87, U251 and T98 GBM cell lines. miR-212-3p and SGK3 expression level were normalized to U6 and Actin respectively. $^{**}p < 0.01$

In vivo assay

All BALB/C nude mice were purchased from Shanghai slack laboratory animal limited liability company (Shanghai, China). 2×10^6 U87 or U251 cells were then transplanted into the axillary subcutaneous of 4 weeks BALB/C female nude mice (n = 4/group for U87, n = 5/group for U251). The implanted mice were randomly divided into two groups on the tenth day. Negative control (5′-UUCUCCGAACGUGUCACGUTT-3′) and chemical modified miR-212-3p mimics oligonucleotides (GenePharma company, Shanghai, China) were then suspended in 10 μl PBS and locally injected into the xenograft tumors at multiple sites of the control and experimental group mice respectively every 3 days for 30 days. Tumor volumes were measured every 10 days using the following formula: volume = length × width^2 × 0.5. The representative images of implanted nude mice were pictured on the fortieth day. Total protein were extracted from resected xenograft tumor tissues on the fortieth day. The expression of SGK3 in the xenograft tumor tissues was examined by western-blot. All the experiments were performed according to institutional guidelines and approved by Ethical Committee of the First Affiliated Hospital of Harbin Medical University.

Statistical analysis

The data from triplicate experiments were expressed as the mean ± SD. Statistical analysis was performed using Student's *t* test or ANOVA (prism version 5.0), where $p < 0.05$ was considered significant.

Results

miR-212-3p expression is inversely correlated with SGK3 expression in glioma specimens

To investigate the function of miR-212-3p and SGK3 in glioma cells, we first detected the expression levels of miR-212-3p and SGK3 in glioma tissues via qRT-PCR. We found that endogenous miR-212-3p expression level was significantly down-regulated in glioma tissues (WHO, Grade III-IV) compared with NATs (Fig. 1a). In contrast, the expression level of SGK3 was strongly up-regulated in glioma tissues (Fig. 1b). As SGK3 was predicted as a potential target gene of miR-212-3p by miRbase target database, we next analyzed whether there was any relationship between the expression level of miR-212-3p and SGK3 in gliomas. As expected, the results of linear

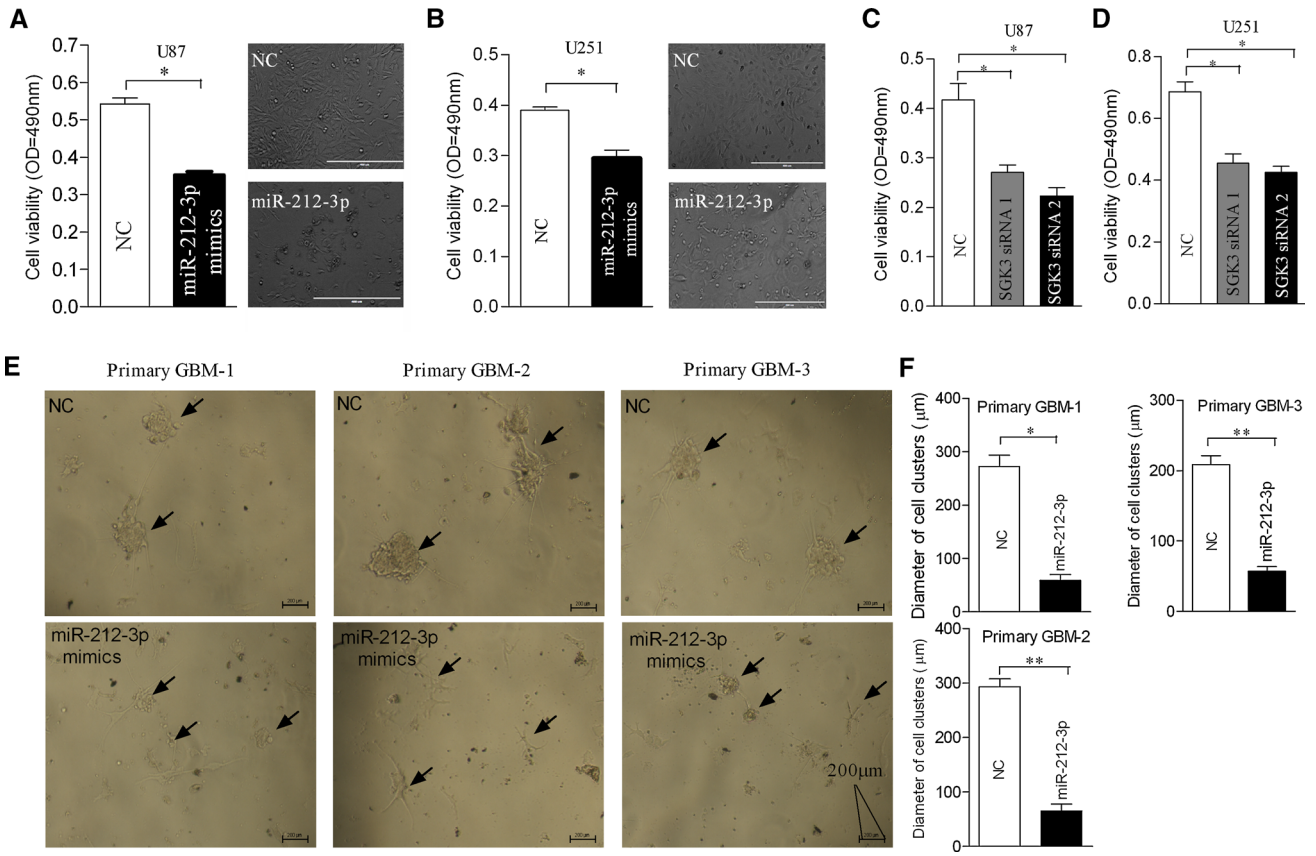

Fig. 2 MiR-212-3p inhibits proliferation of GBM cells. **a–d** U87 and U251 cells were transiently transfected with miR-212-3p mimics, or SGK3 siRNA, or negative control (NC). Then cell viability was detected by MTT assay at 96 h. Photomicrographs showing morphology of U87 and U251 cells after transfected with miR-212-3p or NC for 96 h. *Bar* represents 400 μm. **e**, **f** Three primary human GBM cell lines were dissociated from GBM tissues and were transiently transfected with miR-212-3p mimics or NC for 7 days, cell cluster were pictured and their diameters were quantified. *Bar* represents 200 μm. Every experiment repeated three times. *$p < 0.05$; ** $p < 0.01$

correlation analysis indicated that the levels of miR-212-3p were inversely correlated with SGK3 expression in gliomas (Fig. 1c, $p < 0.05$, $r = -0.931$). Moreover, miR-212-3p was also significantly down-regulated in U87, U251 and T98 GBM cells lines when compared with NATs (Fig. 1d). Collectively, these results demonstrated that miR-212-3p was negatively correlated with SGK3 in glioma tissues.

miR-212-3p over-expression and SGK3 suppression both inhibit proliferation of GBM cells

Having demonstrated the expression levels of miR-212-3p and SGK3 in GBM, we next tested whether miR-212-3p and SGK3 affected the viability of GBM cells. We transfected U87 MG cells and U251 MG cells with miR-212-3p mimics or SGK3 siRNAs. As shown in Fig. 2a–d, miR-212-3p over-expression or SGK3 suppression resulted in decreased cell viability in both cell lines. To further confirm the anti-tumor effect of miR-212-3p, miR-212-3p mimics were transfected into three different primary GBM cells dissociated from three different GBM patients. We found that the diameters of GBM cell clusters decreased markedly compared with negative control groups, supporting that miR-212-3p can suppress the formation of GBM cell cluster (Fig. 2e, f). Therefore, these findings collectively suggested that over-expressed miR-212-3p or SGK3 suppression inhibited proliferation of GBM cells in vitro.

SGK3 is a direct target of miR-212-3p in GBM cells

Previous results have indicated that expression levels of miR-212-3p and SGK3 were negatively correlated in gliomas. Supporting this, miR-212-3p was predicted to directly bind to 3′ UTR of SGK3 at the position 287–293 and 332–338 by target prediction algorithm in microRNA database (Fig. 3a). Therefore, in order to further validate their regulatory relationship, miR-212-3p mimics were transfected into U87 and U251 cells. Protein and mRNA expression level of SGK3 were examined by western-blot and qRT-PCR respectively. The results showed that miR-212-3p significantly decreased protein and mRNA

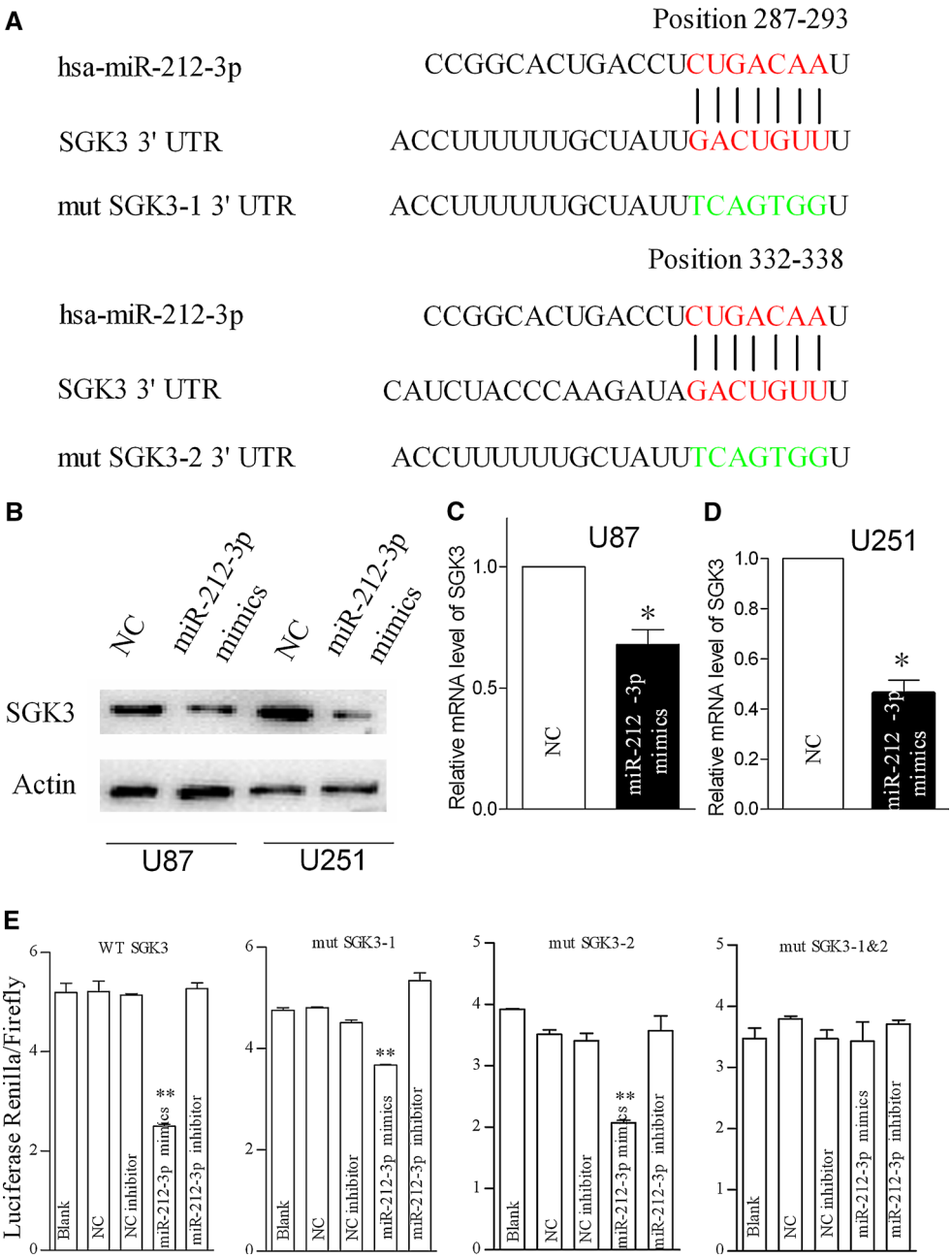

Fig. 3 SGK3 is a direct target of miR-212-3p in GBM cells. **a** Two direct binding sites of miR-212-3p with SGK3 were predicted by miRbase database. Two seed and mutated sequence at the position 287–293 and 332–338 of SGK3 were highlighted with *red* color. The mutated sequence was marked with *green* color and showed at the *bottom*. **b–d** U87 and U251 cells were transiently transfected with NC or miR-212-3p mimics for 48 h, and then protein and mRNA expression level of SGK3 were respectively analyzed by Western-blot and qRT-PCR. Actin was set as the internal control. **e** Luciferase reporter gene assay were used to detect interaction between miR-212-3p and direct binding and mutation sites of SGK3 in HEK293T cells. Mut-SGK3-1 and mut-SGK3-2 represented that seed sequence at the position 287–293 and 332–338 of SGK3 were respectively mutated. Mut-SGK3-1&2 represented that the two seed match sequence were all mutated. Wild type or mutant human SGK3 3′UTR Luciferase reporter were co-transfected with miR-212-3p mimics, miR-212-3p inhibitor, NC or NC inhibitor into HEK293T cells using Lipofectamine 2000. Every experiment repeated three times. $*p < 0.05$, $**p < 0.01$

expression level of SGK3 in both cell lines (Fig. 3b, c). Moreover, luciferase assay demonstrated that miR-212-3p inhibited luciferase activity of SGK3 compared with negative controls. Though mut-SGK3-1 or mut-SGK3-2 alone did not significantly reverse decreased luciferase activity of SGK3, mut-SGK3-1&2 in which both binding sites of SGK3 were mutated did rescued decreased luciferase activity induced by miR-212-3p in HEK293T cells (Fig. 3d). Hence, these results confirmed that miR-212-3p could directly bind to the two predicted sites of SGK3 3′UTR and regulate SGK3 expression.

Restoration of SGK3 expression rescues the reduced proliferation caused by miR-212-3p in GBM cells, and miR-212-3p prevents tumor formation of GBM cells in vivo

MiR-212-3p has been demonstrated to directly target 3′ UTR of SGK3 and inhibit its mRNA and protein expression level. To investigate whether miR-212-3p and SGK3 have regulatory effect on the survival of GBM cells, we constructed SGK3 over-expression plasmid named p-EGFP-N3-SGK3, and p-EGFP-N3 vector was used as negative

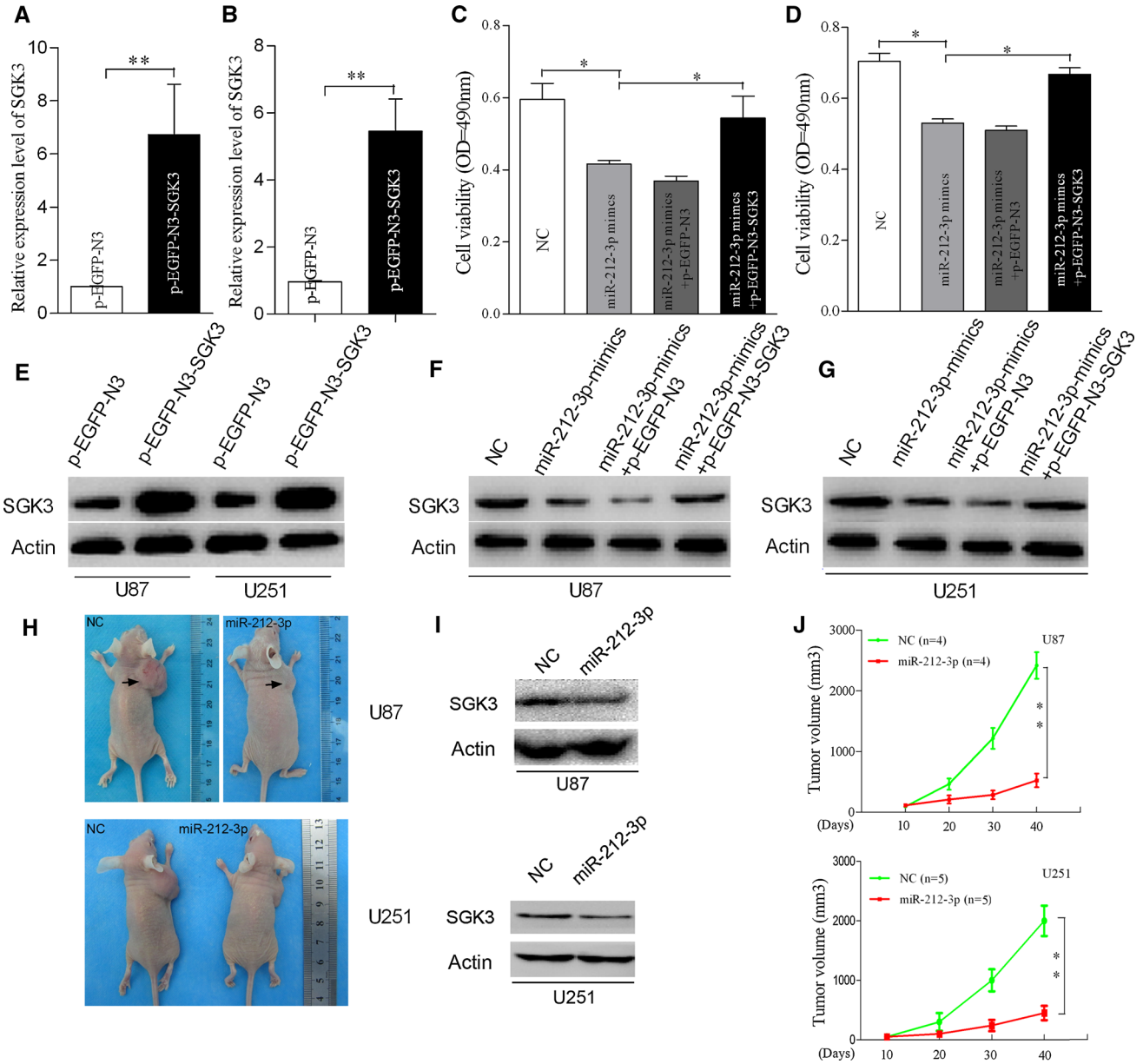

Fig. 4 Restoration of SGK3 expression reverses the role of miR-212-3p against glioma proliferation and miR-212-3p inhibits tumor formation of glioma cells in vivo. **a**, **b** qRT-PCR analyzed SGK3 mRNA expression level in p-EGFP-N3 and p-EGFP-N3-SGK3 transfected U87 (**a**) and U251 (**b**) GBM cells. Actin was set as internal control. **c**, **d** U87 (**c**) and U251 (**d**) cells were respectively transfected with NC, miR-212-3p mimcs, miR-212-3p mimcs+ p-EGFP-N3 or miR-212-3p mimcs+ p-EGFP-N3-SGK3 for 96 h, then cell viability was analyzed by MTT assay. **e–g** Western blot analyzed protein level of SGK3 in U87 and U251 cells treated as described in **a–d**. **h** Representative images of BALB/c mice with tumor on the fortieth day. **i** Western blot analyzed SGK3 level in xenograft GBM tissues. **j** Tumor volumes curves of mice with xenografts locally injected with miR-212-3p mimcs. $*p < 0.05$, $**p < 0.01$

control. In the p-EGFP-N3-SGK3 transfected U87 and U251 cells, mRNA level of SGK3 was strongly up-regulated compared with p-EGFP-N3 vector transfected controls (Fig. 4a, b). Importantly, over-expression of SGK3 rescued miR-212-3p-induced cytotoxic effects (Fig. 4c, d). In addition, western blots results revealed that p-EGFP-N3-SGK3 transfection not only elevated the protein level of SGK3, but also reversed the decreased level of SGK3 induced by miR-212-3p mimics in GBM cell lines, suggesting that SGK3 was a crucial downstream target of miR-212-3p (Fig. 4e, f, g). To further investigate the role of miR-212-3p in regulation of tumor growth in vivo, we injected NC and miR-212-3p mimics into the xenograft tumors of mice. Consistent with in vitro results, miR-212-3p inhibited tumor formation, decreased tumor volumes and reduced protein level of SGK3 in xenograft tumor tissues (Fig. 4h, i,

j). Taken together, these results suggested that inhibition of SGK3 was essential for the restoration of decreased cell viability caused by miR-212-3p over-expression. Notably, miR-212-3p also demonstrated suppressive effect on GBM growth in vivo.

Discussion

In this study, we demonstrated that miR-212-3p was down-regulated in glioma specimens of WHO grade III and IV, associated with a higher level of SGK3. We further revealed that miR-212-3p inhibited the survival of GBM cells by directly targeting SGK3. In addition, the suppressive role of miR-212-3p in GBM cells occurred both in vitro and in vivo. We also observed that over-expression of miR-212-3p could not only induce certain toxic effect on GBM cells derived from GBM patients in vitro, but also inhibit GBM growth in vivo. Therefore, these data strongly suggested an evidence that miR-212-3p might be a crucial negative regulator of GBM development in patients.

Recently, down-regulation of miR-212-3p has been observed in various cancers, including lung cancer, gastric cancer, colorectal cancer, leukemia, and hepatocellular carcinoma [24, 26–30]. However, it has been reported that miR-212-3p was up-regulated in pancreatic cancer [31]. In addition, miR-212-3p has also been found to have oncogenic effect on the generation of non-small cell lung cancer through targeting tumor suppressor PTCH1 [32]. These previous studies suggested that the role of miR-212-3p in human cancers may be tumor-type specific. Our results showed that miR-212-3p was strongly down-regulated in glioma tissues compared with NATs. Moreover, the expression level of miR-212-3p was much lower in GBM than grade III gliomas, suggesting that the expression level of miR-212-3p may be correlated with grades of glioma malignancy.

In this study, we demonstrated that miR-212-3p directly targeted SGK3 and inhibited its expression. The SGK family share structural similarity with AKT, and also regulate some common cellular processes with AKT [6, 7, 9, 11, 33]. SGK3 mediates a series of cellular processes including membrane transport, cell proliferation, and survival [7, 34, 35]. SGK3 has also been identified as a crucial downstream of PI3-K signaling in hepatocellular carcinoma independent of AKT [6]. SGK3 is hyperactive in various cancers such as breast cancer, ovarian cancer, and hepatocellular carcinoma, particularly in those harboring PIK3CA mutations (activating mutations in the alpha catalytic subunit of PI3-K) [34]. Moreover, it has been confirmed that PIK3CA mutations occur in a significant number of human GBMs [36]. In our study, elevated level of SGK3 was also observed in glioma tissues. In addition, many PIK3CA oncogenic mutant cancer cell lines exhibit quite low AKT activation. Instead, SGK3 becomes an alternative signaling effector of PI3-K pathway [37]. Since AKT signaling pathway involves in various cellular processes including cell survival, cell cycle and DNA repair, which contribute to tumorigenesis of GBMs, SGK3 may have equally important functions in GBMs [8]. Therefore, SGK3 may offer a novel avenue for GBM therapy.

In conclusion, we have demonstrated that miR-212-3p exerted its tumor suppressive effect on GBM through direct repression of SGK3. Therefore, we predicted that miR-212-3p may provide a molecular basis for improved diagnosis and effective treatment of GBM, though further investigation is needed to verify this.

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Conflict of interest None of the authors has any conflict of interest associated with the present work.

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