Abstract. Glioblastoma is the most common primary malignancy of the adult central nervous system (CNS) and is associated with an exceptionally poor prognosis. Elucidation of the pathogenesis and molecular changes will help us to further understand the pathogenesis and progression of the disease and offer new therapeutic targets. FUS1 (TUSC2, tumor suppressor candidate 2) is a tumor-suppressor gene located on human chromosome 3p21. Restoration of FUS1 function in human non-small cell lung cancer (NSCLC) cells was found to significantly inhibit tumor cell growth and modulate the chemosensitivity of lung cancer cells. Yet, its role in human glioblastoma has rarely been addressed. In the present study, we demonstrated that low expression of FUS1 was detected in high-grade human glioma, implying that FUS1 expression is negatively associated with progression of the disease. Subsequent studies confirmed that FUS1 overexpression inhibited the proliferation, migration and invasion of human glioblastoma cells. In addition, we found that FUS1 overexpression significantly upregulated miR-197 expression in the glioblastoma cells. We also revealed that miR-197 suppressed the proliferation, migration and invasion of the cells as well as the silencing of miR-197 attenuated the biological functions of FUS1. Using human glioblastoma tissue samples, we demonstrated that miR-197 is negatively associated with metastasis. All the results demonstrated that FUS1 acts as a tumor-suppressor gene by upregulating miR-197 in human glioblastoma and implied that restoration of FUS1 and miR-197 could be new therapeutic strategies for glioblastoma.

Introduction

FUS1 (TUSC2, tumor suppressor candidate 2) is a tumor-suppressor gene identified in the human chromosome 3p21.3 region in which allele losses and genetic alterations occur early and frequently in many human cancers, including breast and lung (1-10). Loss or reduction of FUS1 expression has been detected in 100% of small-cell lung cancer (SCLC) and 82% of non-small cell lung cancer (NSCLC) cases. Meanwhile, its loss or reduction is associated with significantly worse overall patient survival in NSCLCs (11). FUS1 deficiency causes increased susceptibility to certain types of tumors and results in defects in natural killer (NK) cell maturation coupled with IL-15 insufficiency (12). NK cells can rapidly recognize and destroy infected or malignant cells and play a decisive role in the regulation of adaptive immunity by stimulating other components of the antitumor immune response (13). Numerous studies have emphasized the major role of IL-15 in NK cell development (14-16). Moreover, overexpression of FUS1 was found to significantly inhibit tumor growth and progression in mouse models (17). However, it remains unknown whether FUS1 regulates glioblastoma cell proliferation, invasion and metastasis, and the role of FUS1 in microRNA (miRNA) regulation remains undetermined.

MicroRNAs (miRs) are small non-coding RNAs that control mRNA stability and the translation of target mRNAs by binding to regulatory sites which are mostly located in the 3'-untranslated region (3'UTR) of the transcript (18). Recently, the involvement of miRNAs in phenotypic modulation of human glioma has been reported. For example, miRNA-21 knockdown was found to disrupt glioma growth in vivo and to display synergistic cytotoxicity with neural precursor cell delivered S-TRAIL in human gliomas (19); miRNA-34a is tumor suppressive in brain tumors and glioma stem cells (20); and miRNA-181a was found to sensitize human malignant glioma U87MG cells to radiation by targeting Bel-2 (21).

In the present study, we firstly showed that high expression of FUS1 was detected in low-grade human glioma and that FUS1 inhibited the proliferation, migration and invasion of human glioblastoma cells. Moreover, FUS1 overexpression upregulated miR-197 expression in the glioblastoma cells. Subsequent studies showed that miR-197 suppressed the proliferation, migration and invasion in the cells. Silencing
of miR-197 partly reduced the effects of FUS1. Finally, using human glioblastoma tissue samples, we demonstrated that miR-197 expression was negatively associated with metastasis. All the results demonstrated that FUS1 acts as a tumor-suppressor gene by at least partly upregulating miR-197 in human glioblastoma and implied that restoration of FUS1 and miR-197 could be new therapeutic targets for glioblastoma.

Materials and methods

Immunohistochemistry. Tissues of human glioma were obtained from the Yishui Central Hospital, Linyi People’s Hospital, Hubei Cancer Center and Tianyou Hospital (29, WHO grade I; 27, WHO grade II; 32, WHO grade III; and 27, WHO grade IV); 46 patients were females. The mean age was 55 years (range, 30-74). Immunohistochemistry was performed using standard techniques. Antigen retrieval was performed by autoclaving. Incubation with 10% normal goat serum in phosphate-buffered saline was performed for 18 min to eliminate non-specific staining. Incubation with the anti-FUS1 antibody (Abcam, Cambridge, MA, USA) was carried out. Finally, sections were lightly counterstained with 10% Mayer's hematoxylin, dehydrated, mounted and observed. Staining was evaluated by a neuropathologist and an investigator blinded to the diagnosis. Sections were classified: - (negative), + (focal and weak immunoreactivity), ++ (diffuse and weak or focal and intense immunoreactivity) and +++ (diffuse and intense immunoreactivity). The data were analyzed by SPSS 19.0 statistical package. Quantitative image analysis was performed as previously described (22). The comparison of high expression rates was by Chi-square test. The use of the human tissue samples followed internationally recognized guidelines as well as local and national regulations. Research carried out on humans followed international and national regulations. The medical ethics committees of the participating institutions approved the experiments undertaken.

Cell line and transfection. Human glioblastoma U87MG cells were obtained from MD Anderson Cancer Center (Houston, TX, USA). The cells were cultured in a complete medium [RPMI-1640 supplement with 10% fetal calf serum (FCS); Gibco, Grand Island, NY, USA]. Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. FUS1-expressing plasmids, empty vector (pcDNA3.1), FUS1-sh-RNA and the scramble were purchased from Tiangene (Tianjin, China). Pre-miR-197 and control-miR were purchased from Ambion (Austin, TX, USA). A final concentration of 50 nM of pre-miR-197/anti-miR-197 and its respective negative control (control-miR/scramble) were used for each transfection. For transfection experiments, the cells were cultured in serum-free medium without antibiotics at 60% confluency for 24 h, and then transfected with transfection reagent (Lipofectamine 2000; Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. After incubation for 6 h, the medium was removed and replaced with normal culture medium for 48 h, unless otherwise specified.

Examination of cell proliferation with MTT assay. Examination of cell proliferation with the MTT assay was performed as previously described (23). The effect on the cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (MTT assay; Sigma, St. Louis, MO, USA). Absorbance was directly proportional to the number of surviving cells.

BrdU proliferation analysis. Cell proliferation was assessed using a colorimetric BrdU proliferation kit according to the manufacturer’s instructions (Roche, Indianapolis, IN, USA). The transfected cells were labeled with BrdU for 4 h. The genomic DNA was fixed and denatured, and then incubated with peroxidase-conjugated anti-BrdU antibody for 100 min. A substrate for the conjugated peroxidase was then added, and the reaction product was quantified by measuring the absorbance. The results were then normalized by the number of total viable cells.

Migration and invasion assays. Migration and invasion assays were performed as previously described (24). For the Transwell migration assays, 5x10⁵ cells were plated in the top chamber with a non-coated membrane (24-well insert; pore size, 8-μm; BD Biosciences, Lincoln, NE, USA). For the invasion assays, 1.5x10⁵ cells were plated in the top chamber with a Matrigel-coated membrane (24-well insert; pore size, 8-μm; BD Biosciences). In both assays, the cells were plated in medium without serum, and medium supplemented with serum was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h, and cells that did not migrate or invade through the pores were removed by a cotton swab. Cells on the lower surface of the membrane were stained with the Diff-Quick Stain Set (Dade) and counted.

Western blot analysis. Western blot analysis was performed as previously described (23). Mainly, after incubation with the primary antibody anti-FUS1 (1:500), anti-c-myc (1:500), anti-PCNA (1:500), anti-Ki67 (1:500), anti-RB (1:500) or anti-β-actin (1:500) (all from Abcam) overnight at 4°C, IRDye™-800-conjugated anti-rabbit secondary antibodies (LI-COR Biosciences, Lincoln, NE, USA) were used for 30 min at room temperature. The specific proteins were visualized by Odyssey™ Infrared Imaging System (Gene Company, Lincoln, NE, USA).

miRNA microarray. Total RNA from cultured cells, with efficient recovery of small RNAs, was isolated using the mirVana miRNA Isolation kit (Ambion). cRNA for each sample was synthesized using the 3’ IVT Express kit (Affymetrix, Santa Clara, CA, USA) according to the manufacturer’s protocols. The purified cRNA was fragmented by incubation in fragmentation buffer (provided in the 3IVT Express kit) at 95°C for 35 min and chilled on ice. The fragmented labeled cRNA was applied to MicroRNA2.0 array and hybridized in the GeneChip Hybridization Oven 640 (both from Affymetrix) at 45°C for 18 h. After washing and staining in GeneChip Fluidics Station 450, the arrays were scanned using GeneChip Scanner 3000 (both from Affymetrix). The gene expressions levels of samples were normalized and compared using Partek GS 6.5 (Partek Inc., St. Louis, MO, USA). Average-linkage hierarchical clustering of the data was applied using Cluster (http://rana.lbl.gov) and the results were displayed...
using TreeView (http://rana.lbl.gov) (both from Eisen et al.,
Stanford University, Stanford, CA, USA).

Real-time PCR for miRNA. Total RNA from cultured cells,
with efficient recovery of small RNAs, was isolated using the
mirVana miRNA Isolation kit. Detection of the mature form of
miRNAs was performed using the mirVana qRT-PCR miRNA
detection kit, according to the manufacturer’s instructions
(Ambion). The U6 small nuclear RNA was used as an internal
control.

Results

Low-expression of FUS1 is detected in high-grade human
glioma. To show the general importance of FUS1 in the patho-
genesis of glioblastoma, we applied human glioma specimens
to detect FUS1 expression. Staining was evaluated by a neuro-
pathologist and an investigator blinded to the diagnosis. All
the tissue sections expressed FUS1 protein, thus there was no
tissue section that could be classified as - (negative). Positive
staining for FUS1 was observed in the tumor cells (data not
shown). Tissue sections of glioma for each grade were divided
into two groups (+ and +++/+++ (Table I). The expression rates
of the FUS1 protein in the +++/+++ group in glioblastoma of
grades I, II, III and IV were 83, 66, 34 and 22%, respectively,
and compared with the + group (Table I).

In order to further confirm that low expression of FUS1 is
associated with high-grade human glioma, quantitative image
analysis was performed to analyze FUS1 protein expression
in the tissue sections. We found that FUS1 expression was
negatively associated with the grade of glioma (Fig. 1).

Overexpression of FUS1 in human glioblastoma U87MG cells
inhibits cell proliferation, migration and invasion. In an attempt
to identify the role of FUS1 in regulating the proliferation
of U87MG cells, the cells were transfected with FUS1-expressing
plasmids. After stable transfection, FUS1 protein expression
was detected by western blotting. The results showed that
FUS1-expressing plasmids evidently increased FUS1 protein
expression in the U87MG cells (Fig. 2A). Moreover, the
expression rates of the FUS1 protein in the +++/+++ group in glioblastoma
of grades I, II, III and IV were 83, 66, 34 and 22%, respectively,
and compared with the + group (Table I).

To provide further evidence that low expression of FUS1 is
negatively associated with the grade of glioma (Fig. 1).

Knockdown of FUS1 promotes the proliferation, migration
and invasion of U87MG cells. To provide further evidence that
FUS1 is involved in the proliferation, migration and invasion
of U87MG cells, we studied the effects of an inhibitor of FUS1.
After transfection, FUS1 expression was detected by western
blotting, and the proliferation rate of the U87MG cells was
tested by MTT assay. The results showed that FUS1-sh-RNA
significantly decreased FUS1 expression in the U87 mg cells,
and proliferation of the cells transfected with FUS1-sh-RNA
was found to be higher than that of the cells transfected with the
scramble (Fig. 3A and B). Consistent with the MTT assay,
BrdU incorporation analysis demonstrated that DNA synthesis
in the cells was increased by FUS1-sh-RNA (Fig. 3C). In
addition, we also performed migration and invasion assays
and found that FUS1-sh-RNA significantly increased the
migration and invasion of the U87MG cells (Fig. 3D). FUS1-
sh-RNA played an opposite role when compared with FUS1
in regulating the proliferation, migration and invasion in the
U87MG cells.

FUS1 significantly upregulates miR-197 expression in
U87MG cells. Tumor-suppressor genes exert their functions
by regulating miRNA expression in glioma (26) and miRNA
involvement in glioma pathogenesis and some function as
tumor-suppressor genes or oncogenes (20,27,28). Thus, we
reasoned that FUS1 functions as a tumor suppressor by
regulating relevant miRNAs. Thus, an miRNA microarray

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Table I. FUS1 expression in human glioma.

Figure 1. Low expression of FUS1 was detected in high-grade human glioma. Mean OD values for FUS1 protein expression of immunohistochemical images in glioma of grades I, II, III and IV, n=115.
Figure 2. FUS1 inhibits proliferation, migration and invasion in human glioblastoma U87MG cells. (A) Western blotting for FUS1 protein expression in the U87MG cells. Cells were transfected with FUS1-expressing plasmids or pcDNA3.1 (mock). β-actin was a loading control. n=3. (B) MTT assay of U87MG cells transfected with FUS1-expressing plasmids or pcDNA3.1 (mock). Cells were transfected with 0.1, 0.2, 0.3, 0.4 or 0.5 µg FUS1-expressing plasmids. n=3. (C) BrdU incorporation analysis of the U87MG cells transfected with FUS1-expressing plasmids or pcDNA3.1 (mock). n=3. (D) Western blotting for c-myc, PCNA, Ki67 and RB protein expression in the U87MG cells. Cells were transfected with FUS1-expressing plasmids or pcDNA3.1 (mock). β-actin was a loading control. n=3. (E) Invasion and migration assays using U87MG cells infected with FUS1-expressing plasmids or pcDNA3.1 (mock). n=3.

Figure 3. Knockdown of FUS1 promotes the proliferation, migration and invasion of U87MG cells. (A) Western blotting for FUS1 protein expression in the U87MG cells. Cells were transfected with FUS1-sh-RNA or scramble (mock). β-actin was a loading control. n=3. (B) MTT assay of the U87MG cells transfected with FUS1-sh-RNA or scramble. n=3. (C) BrdU incorporation analysis of the U87MG cells transfected with FUS1-sh-RNA or scramble. n=3. (D) Invasion and migration assays of the U87MG cells infected with FUS1-sh-RNA or scramble. n=3.
was performed. RNAs isolated from the U87MG cells were hybridized to a custom miRNA microarray platform. After three series of hybridization, quantification and normalization, a number of miRNAs were found to be altered in the cells. Yet, we were interested in miR-197, since it was downregulated in glioblastoma (29). The results of the microarray showed that miR-197 was increased >10-fold (Fig. 4A). To further confirm the regulation of FUS1, we performed real-time PCR to detect the expression of miR-197. Consistent with previous studies, the results of the real-time PCR showed that FUS1 significantly upregulated miR-197 expression (Fig. 4b) and FUS1-sh-RNA downregulated miR-197 expression (Fig. 4C) in the U87MG cells.

Overexpression of miR-197 inhibits the proliferation, migration and invasion of U87MG cells. miR-197 expression was found to be downregulated in glioblastoma (29). Having demonstrated that FUS1 functions as a tumor-suppressor gene as well as that miR-197 expression is upregulated by FUS1 in human glioblastoma U87MG cells, we reasoned that FUS1 suppresses the proliferation, migration and invasion in U87MG cells by upregulating miR-197 expression.

In an attempt to identify the role of miR-197 in regulating the proliferation of U87MG cells, the cells were transfected with pre-miR-197. After stable transfection, miR-197 expression was detected by real-time PCR and the proliferation rates of U87MG cells were tested by MTT assay. The results showed that exogenous miR-197 stably increased its expression in the U87MG cells (Fig. 5A). Overexpression of miR-197 significantly reduced the proliferation rate of the U87MG cells after 48 and 72 h of transfection, and the inhibition of cell proliferation was time-dependent (Fig. 5B). This was further revealed by BrdU incorporation analysis showing that transfection of U87MG cells with pre-miR-197 resulted in reduced DNA synthesis activity/viable cells (Fig. 5C).

We next sought to determine whether miR-197 has any impact on migration and invasion in the U87MG cells. The cell migration and invasion assays of the U87MG cells showed that overexpression of miR-197 not only inhibited the migration of the U87MG cells, yet also suppressed cell invasion (Fig. 5D).

Knockdown of miR-197 promotes the proliferation, migration and invasion of U87MG cells. To provide further confirm that miR-197 is involved in U87MG cell proliferation, migration and invasion, we studied the effects of an inhibitor of miR-197. After transfection, miR-197 expression was detected by real-time PCR, and the proliferation rate of the U87MG cells was tested by MTT assay. The results showed that miR-197 inhibitor (anti-miR-197) decreased miR-197 expression in the U87MG cells (Fig. 6A), and the proliferation rate of the U87MG cells transfected with miR-197 inhibitors was found to be higher than that of the cells transfected with the scramble (Fig. 6B). Consistent with the MTT assay, BrdU incorporation analysis

Figure 4. FUS1 significantly upregulates miR-197 expression in the U87MG cells. (A) Partial heat map of miRNA microarray analysis of U87MG cells infected with FUS1-expressing plasmids or pcDNA3.1 (mock), n=3. (B) Real-time PCR for miR-197 in the U87MG cells infected with FUS1-expressing plasmids or pcDNA3.1 (mock), n=3. (C) Real-time PCR for miR-197 in the U87MG cells. U87MG cells infected with FUS1-sh-RNA or scramble, n=3.
Figure 5. miR-197 inhibits proliferation, migration and invasion of U87MG cells. (A) Real-time PCR for miR-197 in the U87MG cells infected with pre-miR-197 or control miR (mock). n=3. (B) U87MG cells were transfected with pre-miR-197 or control miR (mock) and then cell viability was measured at the indicated time points by MTT assay. n=3. (C) U87MG cells were transfected with pre-miR-197 or control miR (mock) and then BrdU incorporation analysis was performed at the indicated time points. n=3. (D) Invasion and migration assays in the U87MG cells transfected with the pre-miR-197 or control miR (mock). Forty-eight hours after transfection, the cells were plated for invasion and migration assays. n=3.

Figure 6. Knockdown of miR-197 promotes proliferation, migration and invasion of U87MG cells. (A) Real-time PCR for miR-197 in the U87MG cells transfected with scramble or anti-miR-197. U6 was a loading control. n=3. (B) MTT assay for U87MG cells transfected with anti-miR-197 or scramble. n=3. (C) BrdU incorporation analysis of U87MG cells transfected with anti-miR-197 or scramble. n=3. (D) Invasion and migration assays of the U87MG cells transfected with the anti-miR-197 or scramble. Forty-eight hours after transfection, cells were plated for invasion and migration assays. n=3.
demonstrated that DNA synthesis was increased by the miR-197 inhibitor in the cells (Fig. 6C). Finally, we found that miR-197 inhibitors also significantly increased the migration and invasion of the U87MG cells (Fig. 6D). miR-197 inhibitors play an opposite role when compared with miR-197 in regulating the proliferation, migration and invasion of the U87MG cells.

miR-197 knockdown attenuates the biological functions of FUS1. Having demonstrated that FUS1 significantly promotes miR-197 expression as well as both FUS1 and miR-197 inhibit the proliferation, migration and invasion, we reasoned that FUS1 functions as a tumor-suppressor gene by regulating miR-197 in the U87MG cells. Thus, miR-197 knockdown attenuated the biological functions of FUS1. Anti-miR-197 effectively inhibited FUS1-mediated miR-197 regulation (Fig. 7A). Importantly, miR-197 knockdown attenuated the effects of FUS1 in the U87MG cells not totally but partially (Fig. 7B-D). All the results further illustrated that FUS1 inhibited the proliferation, migration and invasion by partly upregulating miR-197 in the U87MG cells.

Figure 7. miR-197 knockdown attenuates the effects of FUS1. (A) Real-time PCR for miR-197 in FUS1-expressing-plasmid or pcDNA3.1 (mock) treated U87MG cells transfected with scramble or anti-miR-197. U6 was a loading control. n=3. (B) MTT assay for FUS1-expressing plasmid or pcDNA3.1 (mock) treated U87MG cells transfected with scramble or anti-miR-197. n=3. (C) BrdU incorporation analysis for FUS1-expressing plasmid or pcDNA3.1 (mock) treated U87MG cells transfected with scramble or anti-miR-197. n=3. (D) Invasion and migration assays in FUS1-expressing plasmid or pcDNA3.1 (mock) treated U87MG cells transfected with scramble or anti-miR-197. n=3.

Figure 8. miR-197 expression is downregulated in metastatic glioblastoma tissues. Real-time RT-PCR of miR-197 in 24 glioma tumors from patients with the indicated status of metastasis. N, normal tissue. Error bars indicate SEM of triplicate experiments.
miR-197 expression is downregulated in metastatic glioblastoma tissues. We determined miR-197 expression levels in primary tumor samples from 23 patients with glioblastoma. When compared with the normal tissues, the miR-197 expression level was lower in all of the glioblastoma tissues from metastasis-positive patients (6/6). In contrast, 50% of the metastasis-free patients (9/18) had elevated miR-197 levels in their primary tumors (P<0.05, Fig. 8). These results are consistent with the expression pattern of miR-197 in the cultured human glioblastoma cells.

Discussion

FUS1 is a novel candidate tumor-suppressor gene frequently inactivated in lung cancer, and loss of FUS1 expression has been observed in almost all SCLC cell lines and tumor tissue specimens (11), suggesting that FUS1 functions as a tumor suppressor in SCLC. Although the importance of FUS1 in lung cancer development has been firmly established, its role in glioma has rarely been addressed. Consistent with previous studies in lung cancer, we found that FUS1 expression is negatively associated with the grade of glioblastoma. In vitro, our results for the first time demonstrated that FUS1, as an antitumor agent, inhibited the proliferation, migration and invasion of U87MG cells. In addition, consistent with the results of MTT and BrdU assays, western blotting of proliferation markers (PCNA, c-myc and Ki67) showed that the proliferation of U87MG cells was inhibited by FUS1.

The levels of miR-197 were found to be markedly upregulated in cancer cell lines, suggesting that miR-197 functions as an oncogene in lung cancer and miR-197 downregulated tumor-suppressor gene FUS1 expression through targeting its 3'UTR (30). Yet, in glioblastoma, miR-197 was downregulated compared with normal tissues (29). We demonstrated that miR-197 was significantly upregulated by FUS1 in the U87MG cells. Yet, miR-197 did not suppress FUS1 protein expression in U87MG and MDA-MB-468 cells (data not shown). We showed that miR-197 inhibited the proliferation, migration and invasion as well as was negatively associated with metastasis in glioblastoma. Thus, miR-197 plays a completely different role between lung cancer and glioblastoma.

miR-197 knockdown partly reduced FUS1-mediated proliferation, and partly eliminated the migration and invasion promoted by it. Thus, except for miR-197, we reasoned that there are other downstream effectors of FUS1 responsible for its tumor-suppressing function.

FUS1 deficiency results in increased susceptibility to a certain range of tumors and causes defects in NK maturation coupled with IL-15 insufficiency (12). In future studies we will aim to ascertain whether FUS1 overexpression upregulates IL-15 in glioblastoma, whether FUS1 is associated with NK cell maturation, how miR-197 is related to this process, and which downstream target genes mediate the roles of miR-197.

Consistent with previous studies showing that miR-197 expression is downregulated in glioblastoma compared with normal tissues (29), we demonstrated that FUS1 inhibited U87MG cell proliferation, invasion and migration, at least in part, by regulating miR-197 expression. The FUS1 functions in glioblastoma U87MG cells demonstrated in the present study have potential basic and clinical implications. FUS1 may be a powerful suppressor of proliferation/migration/invasion in human glioblastoma, and pharmacological restoration of FUS1 may represent a promising therapeutic strategy. Expression of FUS1 protein is regulated at various levels, leading to loss or greatly diminished tumor-suppressor function. miR-93, miR-98 and miR-197 negatively regulate the expression of tumor-suppressor gene FUS1 in SCLC cells (30). Yet, we did not detect any change in FUS1 in miR-197-transfected U87MG and MDA-MB-468 cells. Thus, miR-197 appears to negatively regulate the expression of FUS1 only in SCLC. Mechanisms involved in the regulation of FUS1 expression and targets of miR-197 continue to be identified in glioblastoma.

References