Matrix stiffness mediates glioma cells stemness through an integrin/JAK/STAT3 signaling pathway

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Abstract

Background Matrix stiffness in tumor microenvironment is tightly correlated with the tumor progression in solid cancer. However, the potential role of matrix stiffness in glioma progression remains controversial. Our study aimed to explore the underlying mechanism of glioma progression induced by matrix stiffness.

Methods: Atomic force microscopy analysis was performed to examine the stiffness of tumor tissues. tunable polyacrylamide (PA) hydrogels were used for cells culture with different matrix stiffness. CCK-8 analysis was used to examine the cells proliferation. Colony formation analysis and subcutaneous tumor-bearing mice model were used to detect the capability of tumorigenesis. Western blotting and immunofluorescent staining were performed to examine the signals activation in glioma cells.

Results: In our study, we observed a higher matrix stiffness in high degree glioma tissues from patients. We demonstrated that a higher stiffness culture system could mediate the upregulation of stemness in glioma cells, resulting in the strengthen capability of tumor growth and relapse in glioma. In mechanism, we observed activation of integrin $\alpha 5\beta 1$ /JAK1/STAT3/c-Myc signaling pathway in glioma cells under the simulation of a higher matrix stiffness. Combination of STAT3 inhibitor STAT3-IN-1 and chemotherapeutic temozolomide (TMZ) obviously suppressed the xenogenous glioma mice model, which revealed strengthen anticancer effects and improved outcome.

Conclusion: We demonstrated that a higher matrix stiffness could promote glioma progression through an integrin $\alpha 5\beta 1$ /JAK1/STAT3/c-Myc signaling pathway. Blockade of STAT3 signals improved the anticancer effects of chemotherapy, providing new insights for glioma therapy in clinic.

1 Introduction

Glioma is the most common primary malignant brain tumors worldwide, with a high risk of peripheral tissue infiltration and tumor relapse. Currently, many glioma patients suffer intensive tissue aggression and tumor recurrence despite maximal surgery or adjuvant radiation/chemotherapy(1–3). More importantly, the median survival of glioma patients remained at a poor stage (12–16 months) and the 5-year survival rate is less than 10%(4). However, the underlying mechanism of sustained glioma development remains unclear, which limited the clinical benefit improvement. Thus, exploring the molecular mechanism of glioma to develop innovative approaches for tumor eradication is urgently needed in clinic.

Cancer stem cells (CSCs), also named as cancer initiating cells or tumor repopulating cells, are a small population cells among tumor tissues, which reveal capability of self-renewal and differentiation(5). Increasing evidence implicates that the presence of CSCs is highly correlated to the tumor progression, including tumor sustained growth, cancer relapse and so on(6). Compelling findings have described the crucial role of glioma stem cells in glioma development. Compared to the bulk tumor cells, those glioma cells with stem-like phenotype usually reveal high potential of proliferation and invasion, resulting in the

tumor relapse or development(7). Traditional studies declared that CSCs typically have plasticity and are impacted by the stimulation from microenvironment(8). Several crucial factors in microenvironment are considered to determine the maintain of CSCs, including hypoxia, cytokines secreted from stromal cells and extracellular matrix induced pro-survival signals(9). However, the specific mechanism of CSCs induced tumor progression remains elusive.

Current findings implicated that the matrix stiffness from the extracellular matrix participates in the regulation of tumor stemness in several tumor types(10–12). Song and his colleagues demonstrated that soft matrix culture using tunable PA hydrogels could strengthen the stem-like phenotype of hepatoma cells(13). Meanwhile, Zhu and his colleagues also implicated that a higher matrix stiffness might mediate the stemness characteristics through the Yes-associated protein in colorectal cancer(14). Notably, the relevant clinical experiments found that carcinoma tissues might possess mechanical heterogeneity with a different stiffness, and glioma stem cells are prone to aggregate in those invasive front tissues with a higher stiffness instead of the core of glioma tissues(15). This reminds us whether the matrix stiffness might participate in the glioma stemness regulation.

In our study, we found a higher matrix stiffness in those high degree glioma tissues compared to low degree tissues from patients. More importantly, we further demonstrated that glioma cells cultured in a high PA gels system reveals strengthen stem-like phenotypes, resulting in the sustained tumor progression and high potential of tumorigenesis. Mechanically, we found that a higher matrix stiffness could mediate the integrin/JAK/STAT3 signal activation, resulting in the cells stemness upregulation in glioma. Thus, we further took advantages of the combination of STAT3 inhibitor and chemotherapeutic agent temozolomide (TMZ) to treat glioma, which revealed improved tumor suppression effects and provided a feasible approach for clinical glioma treatment.

2 Materials And Methods

2.1 Cell lines culture and reagents

The human glioma cancer cells line LN229 and U87 were purchased from American Type Culture Collection (ATCC), and cultured with RMPI-1640 complete medium (Gibco, MA, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, MA, USA) at 37°C with 5% CO₂. ATN-161 (Ac-PHSCN-NH₂) was purchased from Selleck Chemicals (MA, USA). JAK1-IN-3 and STAT3-IN-1 were obtained from MCM (MA, USA). TMZ was purchased from Sangon (Shanghai, China).

2.2 Patients' tumor tissues samples

Formalin-fixed, paraffin-embedded human glioma tumor tissue sections, lacking any patient-identifying information, were obtained from the Affiliated Hospital of Southwest Medical University, and divided into high degree group (HD, WHO level 3 ~ 4) and low degree group (LD, WHO level 1 ~ 2). The protocols were approved by Regional Scientific Ethics Committee of the Affiliated Hospital of Southwest Medical

University. Written informed consent was attained from all subjects, and all methods were performed in accordance with the Declaration of Helsinki.

2.3 Cell proliferation and colony formation assay

The cell proliferation was determined by Cell Counting Kit-8 (CCK-8, Solarbio, Beijing, China). Briefly, 2000 human glioma cancer cells were seeded into 96 well plate and cultured for 72 h at 37°C. Then, 10 µL CCK8 solution was added into each well and incubated for 90 min at 37°C. The cells proliferation was analyzed through the absorbance at 450 nm, measured by a microplate reader (Bio-Rad, Hercules, USA). For cell colony formation assay, LN229 or U87 cells (~ 500) were seeded in a 6-well plate. After 14 days, colonies were fixed with methanol for 10 min and stained with 1% crystal violet (Sigma, MA, USA) for 1 min. Each group was measured in triplicate.

2.3 Preparation of different stiffness PA gel

Different stiffness PA gel used in cell culture were prepared as indicated in previous studies(13). Briefly, we coated the coverslips with a thin layer of gel containing a mixture of 3–10% acrylamide and 0.01%-0.3% bis-acrylamide, which produced gels of 2, 8 and 20 kPa stiffness. PA gel polymerization was promoted by the addition of 10% APS (1/100 volume) and TEMED (3/1000 volume). Then the coverslips were washed with PBS twice for 20 minutes, followed by sterilizing in PBS solution for 1 hour with ultraviolet light. Next, we added 50 µL heterobifunctional sulphosuccinimidyl 6-(4'-azido-2'-nitrophenylamino) hexanoate and photo-activated for 5 minutes with ultraviolet light. Finally, the coverslips were coated with 10 µL/mL fibronectin for 1.5 hours and rinsed before cell seeding. Gels were soaked in serum-free culture media for 24 hours for usage. All regents of PA gel were purchased from Solarbio (Beijing, China).

2.4 Atomic force microscopy (AFM) analysis

Atomic force microscopy and analysis were performed as previously described(16). Frozen tissue blocks were cut into 20 µm thick sections. Each section was immersed in PBS and thawed at room temperature before AFM measurement. The samples were maintained in proteinase inhibitor in PBS (protease inhibitor cocktail, Roche 14 Diagnostics, 11836170001) supplemented with Propidium Iodide (SIGMA P4170, 20 µg/ml) during the AFM session. AFM indentations were performed using an MFP3D-BIO inverted optical AFM (Asylum Research) mounted on a Nikon TE2000-U inverted fluorescent microscope, as previously described(17).

2.5 Western blotting

The protein lysates were separated by SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membrane was incubated with the primary antibodies against to JAK1, JAK2, STAT3, p-STAT3, p-JAK1, p-JAK2, c-Myc, integrin $\alpha 5$, integrin $\beta 1$ (1:500, Abcam, Cambridge, UK), followed by incubation with an HRP-conjugated secondary antibody (1:1000, Abcam, Cambridge, UK). Beta-actin (1:1000, Abcam, Cambridge, UK) was set as a control.

2.6 Flow cytometry

The LN229 and U87 cells were fixed with 4% PFA (Affymetrix, 19943), and stained with anti-human CD133 (Biolegend, MA, USA) at 4°C for 30 mins, according to the manufacture instructions. All the dates were acquired on an AccuriC6 (BD, MA, USA) and analyzed with FlowJo software.

2.7 Immunofluorescence Staining

The pathological sections of glioma cancer patients' tumor tissues were retrieved by microwave antigen retrieval (Citrate-EDTA Antigen Retrieval Solution, Beyotime, Beijing, China). After blocked by 5% BSA, the sections were incubated primary antibodies: anti-p-JAK1, anti-p-STAT3, anti-c-Myc (1:200, Abcam, Cambridge, UK) overnight at 4 °C and followed by signal amplification using the ABC HRP Kit (Thermo, MA, USA), for 2 hours at room temperature, and the nucleus was stained with DAPI. All immunofluorescence images were captured from FV1000 laser scanning confocal microscope (Leica, Barnack, Germany).

2.8 siRNA silence

For c-Myc silenced tumor cells, LN229 and U87 cells were infected with Lipofectamine 8000 (Beyotime, Beijing, China) according to the manufacturer's protocol. The relevant c-Myc siRNA sequence as followed: siRNA#1: 5'-CTATGACCTCGACTACGACTTCAAGACCGTCCTAGTCGAGGTCATAG-3' and siRNA#2:5'-CCACAGCATACATCCTGTTTCAAGACGACAGGATGTATGCTGTGGC-3'

2.9 Animal experiments

All the animal care and experimental procedures were approved by the Animal Care and Use Committee of the Affiliated Hospital of Southwest Medical University Committee Guidelines on the Use of Live Animals in research, which is according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. LN229 and U87 glioma subcutaneous models were established on female NSG mice at 4 weeks of age, which purchased from Huafukang company (Beijing, China) and raised in SPF level. For tumor analysis, 1×10^6 LN229 and U87 cells in 100 μ L PBS were subcutaneously injected into the right side of NSG mice. All mice were randomly assigned to 2–4 groups (n = 6 per group), and the tumor volume was measured every day for 4 weeks. For tumorigenesis analysis, LN229 or U87 cells

(1×10^5) were subcutaneously injected into the right side of mice. After 2 weeks, tumor numbers on each mouse was measured.

For drugs treatment, 14 days later, mice were treated with 2.5 mg/kg Temozolomide

(TMZ, Sangon, Shanghai, China), 10 mg/kg STAT3-IN-1 by oral administration for two weeks (twice a week) respectively. Tumor volume was measured using the formula: volume = length $\times$ width²/2. The survival time of mice was recorded.

2.10 Statistical analysis

GraphPad Software (La Jolla, CA) and SPSS 16.0 (SPSS, Chicago, IL, USA) was used for ANOVA, or Student's t-test. Each experiment was performed in triplicated. Data are expressed as the mean $\pm$ standard deviation (SD). Survival time was analyzed by a Log-Rank. $P < 0.05$ was defined as a statistically significant difference. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, n.s no significant difference.

3 Results

3.1 Matrix stiffness up-regulates glioma cells stemness

To investigate the potential role of matrix stiffness in glioma development, we collected glioma tissues from patients, which have been divided into HD group (WHO level 3 ~ 4) and LD group (WHO level 3 ~ 4). Intriguingly, a higher matrix stiffness was observed in HD group compared to the LD group using our tissue stiffness analysis by AFM (Fig. 1A), which reminded us the potential correlation between matrix stiffness and glioma progression. To further explore the matrix stiffness involved tumor progression in glioma, we used tunable PA hydrogels for glioma cells culture with a matrix stiffness ranging from 2 kPa to 20 kPa. Notably, glioma LN229 and U87 cells seeded on 20 kPa gel are prone to possess strengthened capability of proliferation in vitro (Fig. 1B and S1A) and in vivo (Fig. 1C and S1B). Additionally, enhanced capability of colony formation (Fig. 1D and S1C) and tumorigenesis (Fig. 1E and S1D) was observed in glioma cells seeded on tunable PA hydrogels with a higher matrix stiffness. Cancer stem cells, a small subpopulation of tumor cells with capabilities of self-renewal and differentiation, have been demonstrated to be tightly involved in tumor formation and sustained growth. More importantly, compelling study has implicated that glioma stem cells are prone to aggregate in peripheral tumor tissues, which possess a higher matrix stiffness. Herein, we further detected the expression of CD133, a marker of glioma stem cells, in LN229 and U87 cells seeded on tunable PA hydrogels. As anticipated, a higher percentage of CD133 was observed in 20 kPa group (Fig. 1F and S1E), indicating that matrix with a higher stiffness up-regulates tumor stemness to mediate tumorigenesis and sustained growth in glioma. Consistently, the 3D fibrin gel culture for cancer stem cells/tumor repopulating cells selection also implicated the higher percentage of cancer stem cells/tumor repopulating cells in 20 kPa cultured cells

(Fig. 1G and S1F)(18). Taken together, those results suggested that matrix with a higher stiffness could mediate up-regulation of stemness in osteosarcoma, resulting in malignant glioma development.

3.2 Matrix stiffness medicates glioma stemness through integrin $\alpha 5\beta 1$

Integrins are tightly involved in the adhesion of cells to cells or to extracellular material, and mediate various chemical/biodynamic signals from extracellular material(19). Thus, we speculated that matrix stiffness from extracellular material might mediate the pro-survival signaling pathways in glioma cells through integrin associated biodynamic signals. Several types of integrins, including integrin $\alpha v\beta 3$, $\alpha 5\beta 1$, $\alpha v\beta 1$ have been demonstrated to be found in the cell surfaces of glioma and increasing evidence implicates that the elevated expression of integrins prone to facilitate tumor sustained growth and distant metastasis, as well as tumor stemness up-regulation(20). Intriguingly, we found strengthen expression of integrin $\alpha 5\beta 1$ in glioma tissues (HD group with higher stiffness) compared to those glioma tissues from LD group (Fig. 2A). Consistently, glioma cells cultured on 20kPa gels also revealed enhanced integrin $\alpha 5\beta 1$ expression (Fig. 2B and S2A), indicating that matrix stiffness might mediate the activation of integrin $\alpha 5\beta 1$ in glioma cells. To further explore the role of integrin $\alpha 5\beta 1$ in glioma, we used ATN-161 (Ac-PHSCN-NH₂), an integrin $\alpha 5\beta 1$ inhibitor, to treat glioma cells seeded on 20 kPa hydrogels. Notably, inhibition of integrin $\alpha 5\beta 1$ significantly suppressed the cells proliferation (Fig. 2C and S2B) and tumor growth (Fig. 2D and S2C) induced by matrix stiffness, as well as colony formation (Fig. 2E and S2D). More importantly, ATN-161 (Ac-PHSCN-NH₂) treatment decreased the CD133 positive glioma cells (Fig. 2F and S2E), suggesting that blockade of integrin $\alpha 5\beta 1$ could suppress the glioma stemness and tumor development induced by matrix stiffness. Together, those results reminded us that matrix stiffness could medicates glioma stemness through integrin $\alpha 5\beta 1$.

3.3 Matrix stiffness mediates JAK1/STAT3/c-Myc signal activation through integrin $\alpha 5\beta 1$

Compelling researches have demonstrated that extracellular cytokines could mediate the STAT3 signals activation through integrins interaction in glioma, resulting in tumor growth and cells migration(21, 22). Thus, we examined the activation of JAK/STAT3 signal in glioma cells on hydrogels. Intriguingly, up-regulation of phosphorylated STAT3 and JAK1 was observed in glioma cells cultured on 20 kPa gels (no difference in JAK2 expression), implicating that activation of JAK1/STAT3 signal. Notably, suppression of integrin $\alpha 5\beta 1$ efficiently retarded the activation of JAK1/STAT3 signal (Fig. 3A and S3A), suggesting that matrix stiffness mediates JAK1/STAT3 signal activation through integrin $\alpha 5\beta 1$. To further investigate the role of JAK1/STAT3 in glioma stemness induced by matrix stiffness, we used JAK1 inhibitor JAK1-IN-3 and STAT3 inhibitor STAT3-IN-1 to treat glioma cells on 20 kPa gels. Of note, blockade of JAK1/STAT3 signal significantly suppressed proliferation (Fig. 3B and S3B) and colony formation (Fig. 3C and S3C) of LN229 and U87 on 20 kPa gels. Consistently, the percentage of CD133 positive

glioma cells was obviously reduced after JAK1 or STAT3 inhibition (Fig. 3D and S3D), indicating that matrix stiffness mediates glioma stemness through JAK1/STAT3 signal. JAK/STAT serves as pro-survival signals, which might participate in the regulation of tumor progression through downstream transcription factors, such as SOX2 and Slug(23–25). Here, we observed glioma cells with a higher stiffness culture system revealed elevated expression of c-Myc (Fig. 3E and S3E) and we speculated that a higher matrix stiffness might mediate the JAK1/STAT3 downstream c-Myc activation, resulting in the stemness up-regulation. Thus, we further suppressed the integrin $\alpha 5\beta 1$ or JAK1/STAT3 signal, as a result, blockade of integrin $\alpha 5\beta 1$ /JAK1/STAT3 signal suppressed the up-regulation of c-Myc in glioma cells on 20 kPa gels (Fig. 3F and S3F), indicating that matrix stiffness mediates c-Myc activation through integrin $\alpha 5\beta 1$ /JAK1/STAT3 signal. More importantly, c-Myc suppression by siRNA also weakened the capability of proliferation and colony formation in LN229 (Fig. 3G and H) and U87 cells (Fig. S3G and H) on 20 kPa gels, suggesting that matrix stiffness promotes glioma stemness through c-Myc. In consistently, the immunofluorescence analysis implicated that activation of JAK1/STAT3/c-Myc signal in HD glioma tissues from patients (Fig. 3I and J). Together, those results suggested that Matrix stiffness mediate integrin $\alpha 5\beta 1$ /JAK1/STAT3/c-Myc signal activation to promote glioma stemness.

3.4 Blockade of integrin $\alpha 5\beta 1$ /JAK1/STAT3/c-Myc signal improved anticancer effects in glioma

Given the crucial role of integrin $\alpha 5\beta 1$ /JAK1/STAT3/c-Myc in glioma stemness regulation, it might be feasible to block integrin $\alpha 5\beta 1$ /JAK1/STAT3/c-Myc signal for an improved anticancer effect. Here, we used STAT3 inhibitor STAT3-IN-1 to combine with chemotherapeutic agent TMZ. Notably, suppression of STAT3 signal efficiently retarded the tumor growth (Fig. 4A) and prolonged survival time of LN229 bearing mice compared to the TMZ group (Fig. 4B). Simultaneously, the same results were duplicated in U87 bearing mice (Fig. 4C and D), indicating that blockade of integrin $\alpha 5\beta 1$ /JAK1/STAT3/c-Myc signal could improve the anticancer effects in glioma treatment. To further simulate the high malignant glioma development in vivo, we used LN229 pre-cultured on 20 kPa gels to established subcutaneous tumor bearing mice model. Of note, TMZ treatment showed limited tumor suppressive effects in those LN229 bearing mice, which might be due to the intensive tumor growth and drug resistance caused by cancer stem cells. However, the STAT3-IN-1 and TMZ combination group revealed an enhanced tumor suppressive effect (Fig. 4E), as well as prolonged survival time in tumor bearing mice (Fig. 4F). Those results implicated that blockade of STAT3 signal efficiently improves the anticancer effects in glioma treatment, which provides a feasible approach in clinical glioma therapy.

4 Discussion

In our study, we described the essential role of matrix stiffness in glioma cells stemness regulation. A higher matrix stiffness could mediate glioma cells stemness upregulation, eventually resulting in the cells proliferation and tumor relapse. In mechanism, matrix stiffness facilitates tumor stemness through the activation of integrin $\alpha 5\beta 1$ /JAK1/STAT3/c-Myc signaling pathway. Suppression of STAT3 signal by

STAT3-IN-1 is proved to efficiently improve the anticancer effects of chemotherapeutics, which provides a feasible approach for clinical glioma treatment (Fig. 5).

Alteration of extracellular matrix have been reported to be strictly correlated to the tumor stages development(26–28). Traditional opinions implicated that the compounds in extracellular matrix, such as type I collagen, fibronectin and laminin could directly promote tumor growth, facilitate cancer metastasis and mediate the drugs resistance development through activation of pro-survival signaling pathways in tumor cells, eventually leading to the tumor malignancy(11, 29, 30). Current researches provide evidence that biomechanical force associated signals from tumor microenvironment might serve as novel regulators of tumor stemness maintenance(31, 32). For example, increasing matrix stiffness is capable of facilitate proliferation of cancer stem cells and mediate the tumor stemness modification in breast cancer and hepatocellular carcinoma(13, 33–35). More importantly, enrichment of some elements in extracellular matrix, such as collagen deposition, could mediate the formation of matrix stiffness directly, which is likely to participate in the tumor progression induced by collagen(36, 37). Here, we further demonstrated that increase of matrix stiffness could promote the glioma stemness and mediate the tumor sustained growth. This study explains the heterogeneous distribution of glioma stem cells in tumor tissues, in which those cancer stem cells are prone to aggregate in marginal area with a higher matrix stiffness. This study was further confirmed by our clinical glioma tissues, in which higher matrix stiffness of tumor tissues is highly correlated to the tumor malignancy. However, the underlying mechanism of matrix stiffness associated tumor stemness remodel remains unclear, despite of the crucial role of matrix stiffness in several tumor types.

Preliminary findings have shown that JAK/STAT serve as key molecular signal in regulating tumor growth and migration in various tumor types(24, 38). JAK-STAT signaling pathway is a chain of interactions between proteins in somatic or tumor cells, which participates in various processes such as immunity regulation, cell division and tumor development(39, 40). Phosphorylation of STAT3 is a key step in the tumor behavior regulation by extracellular matrix (ECM) stiffness. For example, type IV collagen could mediate the colorectal cancer stemness upregulation through the STAT5 signals. Some cytokines such as IL-6 and IL-12 from microenvironment are tightly involved in the STAT3 associated cancer migration(40). Here, we confirmed the role of JAK1/STAT3 in matrix stiffness induced tumor stemness modification, which depends on the signal transduction of biomechanical integrin receptors. Given the crucial role of STAT3 signal in matrix stiffness associated tumor progression, it might be a feasible strategy to block STAT3 for improved anticancer effects. Our study further combined STAT3 inhibitor STAT3-IN-1 and chemotherapeutic TMZ for glioma treatment, which was proved to efficiently suppress the glioma development and prolong survival time of tumor bearing mice. Given previous studies implicating that STAT3-IN-1 could suppress the tumor cells proliferation by oral administration, it might be superior to attack those tumor cells depending on STAT3 associated pro-survival signaling pathway. Meanwhile, oral administration ensures the feasibility for glioma treatment and avoid the potential obstruction from blood-brain barrier. Thus, application of STAT3 inhibitor to strengthen chemotherapeutic TMZ anticancer effects provides a potential strategy for glioma therapy.

Based on the limitations of relevant findings, our study further describes the crucial role of matrix stiffness in glioma progression. Firstly, we demonstrated that a higher matrix stiffness could mediate the glioma cells stemness upregulation, resulting in the glioma development in clinic. Secondly, we clarified the specific mechanism of glioma stemness regulation induced by matrix stiffness, which depends on an integrin $\alpha 5\beta 1$ /JAK1/STAT3/c-Myc associated signaling pathway. Thirdly, we further demonstrated that combination of STAT3 inhibitor and TMZ could efficiently suppress glioma stemness induced by matrix stiffness and improve the anticancer effects in vivo. Meanwhile, STAT3-IN-1 could be taken by oral administration, which takes advantage for clinical glioma treatment and provides new sight for glioma therapy. Finally, the matrix stiffness of the tumor tissues might serve as potential biomarker for tumor progression analysis in more tumor types.

In conclusion, our study provides evidence that a higher matrix stiffness could mediate glioma cells stemness upregulation through an integrin $\alpha 5\beta 1$ /JAK1/STAT3/c-Myc signaling pathway. Blockade of STAT3 by oral administration of STAT3-IN-1 efficiently strengthens tumor suppressive effects, which might serve as an innovative approach for clinic glioma treatment.

Abbreviations

Cancer stem cells (CSCs)

Temozolomide (TMZ)

Polyacrylamide (PA)

Atomic force microscopy (AFM)

Extracellular matrix (ECM)

High degree (HD)

Low degree (LD)

Declarations

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Availability of data and materials:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CRedit authorship contribution statement :

Wu Yao: Conceptualization, Software, Investigation, Formal analysis, Writing - original draft. Tao Bei: Methodology, Software, Formal analysis, Writing - review & editing. Peng Tangming: Methodology. Yang Xiaobo: Validation, Resources. Peng Lilei: Resources. Xia Xiangguo: Software, Funding acquisition. Chen Ligang: Methodology. Zhong Chuanhong: Conceptualization, Supervision, Funding acquisition.

Ethics approval and consent to participate:

All animal procedures were approved by the Medical Ethics Committee of The Affiliated Hospital of Southwest Medical University, and were in accordance with the Guide for the Care and Use of Laboratory Animals.

Patient consent for publication

Investigators have to obtain informed consent before enrolling participants in clinical trials.

Declaration of competing interest:

The authors declare that they have no competing interests.

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Figures

Figure 1

Matrix stiffness up-regulates glioma cells stemness. A, the matrix stiffness of tumor tissues from high degree (HD) and low degree (LD) patients (n=10 per group). B, the relative cells proliferation of glioma LN229 cells cultured on dish or tunable PA hydrogels with a matrix stiffness of 2 kPa, 8 kPa and 20 kPa. C, the tumor growth of LN229 cells, pre-cultured with dish or a matrix stiffness of 2 kPa, 8 kPa and 20 kPa, in NSG mice established as indicated, tumor volume was measured at the indicated time points (n = 6 mice per group). D, the relative colony number of LN229 cells cultured with dish or tunable PA hydrogels with a matrix stiffness of 2 kPa, 8 kPa and 20 kPa. E, the tumorigenesis of LN229, pre-cultured with dish or a matrix stiffness of 2 kPa, 8 kPa and 20 kPa, in NSG mice as indicated (n = 6 mice per group). F, the CD133 expression of LN229 cells cultured with dish or tunable PA hydrogels with a matrix stiffness of 2 kPa, 8 kPa and 20 kPa. G, the relative colony number of LN229 cells in 3D fibrin gel, pre-cultured with dish or tunable PA hydrogels with a matrix stiffness of 2 kPa, 8 kPa and 20 kPa relatively. Data represent mean $\pm$ SEM, *P < 0.05, **P < 0.01 or as indicated, Student's t-test.

Figure 2

Matrix stiffness mediates glioma stemness through integrin $\alpha 5 \beta 1$. A, the relative integrin $\alpha 5$ and integrin $\beta 1$ expression of tumor tissues from HD and LD patients, measured by western blot and calculated by image J (n=10 per group). B, the integrin $\alpha 5$ and integrin $\beta 1$ expression of LN229 cells cultured with dish or tunable PA hydrogels with a matrix stiffness of 2 kPa, 8 kPa and 20 kPa, measured by western blot. C, the relative cells proliferation of LN229 cells seeded on 20 kPa tunable PA hydrogels, treated with PBS or ATN-161(50 $\mu\text{mol/L}$). D, the tumor growth of LN229 cells pre-cultured on 20 kPa tunable PA hydrogels, in NSG mice as indicated, and two weeks later, intravenously treated with PBS or ATN-161(0.5 mg/kg), twice a week (n = 6 mice per group). E, the relative colony number of LN229 cells cultured on 20 kPa tunable PA hydrogels, and treated with PBS or ATN-161(50 $\mu\text{mol/L}$). F, the CD133 expression of LN229 cells cultured on 20 kPa tunable PA hydrogels, and treated with PBS or ATN-161(50 $\mu\text{mol/L}$), measured by flow cytometry. Data represent mean $\pm$ SEM, *P < 0.05, **P < 0.01 or as indicated, Student's t-test.

Figure 3

Matrix stiffness mediate JAK1/STAT3/c-Myc signal activation through integrin $\alpha 5 \beta 1$. A, the protein expression of p-STAT3, total-STAT3, p-JAK1, total-JAK1, p-JAK2, total-JAK2 and actin in LN229 cells, cultured on dish or tunable PA hydrogels with a matrix stiffness of 2 kPa and 20 kPa treated with ATN-161 (50 μ mol/L) or not, measured by western blot. B, C, the relative cells proliferation (B) and relative colony number (C) of LN229 cells cultured on 20kPa tunable PA hydrogels, treated with PBS, JAK1-IN-3 (5 nM) or STAT3-IN-1 (5 μ mol). D, the CD133 expression of LN229 cells cultured on 20kPa tunable PA hydrogels, treated with PBS, JAK1-IN-3 (5 nM) or STAT3-IN-1 (5 μ mol), measured by flow cytometry. E, the protein expression of c-Myc and actin of LN229 cells cultured on dish or matrix stiffness of 2kPa or 20 kPa, measured by western blot. F, the protein expression of c-Myc and actin in LN229 cells cultured on 20kPa tunable PA hydrogels, and treated with PBS, JAK1-IN-3 (5 nM) or STAT3-IN-1 (5 μ mol), measured by western blot. G, H, the relative cells proliferation (G) and relative colony number (H) of LN229 cells, with c-Myc gene inhibiting by siRNA or not. I, J, the immunofluorescent analysis of phosphorylated JAK1,

phosphorylated STAT3(I) and c-Myc (J) in tumor tissues from glioma patients in LD or HD groups; scale bar, 20 μ m. Data represent mean $\pm$ SEM, * P < 0.05, ** P < 0.01 or as indicated, Student's t-test.

Figure 4

Blockade of integrin $\alpha 5 \beta 1$ /JAK1/STAT3/c-Myc signal improved anticancer effects in glioma. A, B, the LN229 glioma tumor bearing NSG mice, established as indicated, and treated with PBS, TMZ, STAT3-IN-1 or STAT3-IN-1 combined with TMZ on day 14, twice a week, and tumor volume (A) and survival time (B) were followed as indicated (n = 6 mice per group). C, D, the U87 glioma tumor bearing NSG mice, established as indicated, and treated with PBS, TMZ, STAT3-IN-1 or STAT3-IN-1 combined with TMZ on day 14, twice a week, and tumor volume (C) and survival time (D) were followed as indicated (n = 6 mice per group). E, F, the LN229 cells pre-cultured on tunable PA hydrogels with a matrix stiffness of 20 kPa, were subcutaneously injected into NSG mice as indicated, then treated with PBS, TMZ, STAT3-IN-1, or STAT3-IN-1 combined with TMZ on day 14, twice a week, and tumor volume (E) and survival time (F) were followed as indicated (n = 6 mice per group). Data represent mean $\pm$ SEM, * P < 0.05, ** P < 0.01 or as indicated, Student's t-test.

Figure 5

The schematic diagram of matrix stiffness mediates glioma cells stemness.

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