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Original Article

CHST11-modified chondroitin 4-sulfate as a potential therapeutic target for glioblastoma

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Abstract: Aberrant chondroitin sulfate (CS) accumulation in glioblastoma (GBM) tissue has been documented, but the role of excessive CS in GBM progression and whether it can be a druggable target are largely unknown. The aim of this study is to clarify the biological functions of CHST11 in GBM cells, and evaluate therapeutic effects of blocking CHST11-derived chondroitin 4-sulfate (C4S). We investigated the expression of CHST11 in glioma tissue by immunohistochemistry, and analyzed CHST11 associated genes using public RNA sequencing datasets. The effects of CHST11 on aggressive cell behaviors have been studied *in vitro* and *in vivo*. We demonstrated that CHST11 is frequently overexpressed in GBM tissue, promoting GBM cell mobility and modulating C4S on GBM cells. We further discovered that CSPG4 is positively correlated with CHST11, and CSPG4 involved in CHST11-mediated cell invasiveness. In addition, GBM patients with high expression of CHST11 and CSPG4 have a significantly shorter survival time. We examined the effects of treating C4S-specific binding peptide (C4Sp) as a therapeutic agent *in vitro* and *in vivo*. C4Sp treatment attenuated GBM cell invasiveness and, notably, improved survival rate of orthotopic glioma cell transplant mice. Our results propose a possible mechanism of CHST11 in regulating GBM malignancy and highlight a novel strategy for targeting aberrant chondroitin sulfate in GBM cells.

Keywords: CHST11, C4S, glioma, glioblastoma, CSPG4, therapeutic peptide

Introduction

Glioblastoma (GBM) is the most aggressive type of brain tumor in humans, with a median survival time less than two years for those affected. The high mortality rate of GBM is largely due to its inevitable recurrence after surgical removal of the primary tumor and a lack of understanding about its targetable genetic changes [1, 2]. Furthermore, the spread of tumor cells into the surrounding brain tissue makes it impossible to remove all of the cells through surgery. Therefore, strategies aimed at reducing the invasiveness of glioma cells hold promise for improving the success of surgical removal and reducing the risk of recurrence.

Accumulated evidence indicates that extracellular and membrane-associated proteoglycans, which often have chondroitin sulfate (CS) chains (also known as CSPG), have either direct or indirect effects on the invasiveness of glioma cells [3]. For instance, Brevican (BCAN), a CSPG specific in CNS tissue, is greatly up-regulated in gliomas [4, 5] and has been shown to promote cell invasiveness by modulating fibronectin and activating EGFR signaling [6]. Versican (VCAN), a large aggregating CSPG found in glial scars of CNS lesions, is also frequently up-regulated in gliomas and associated with tumor metastasis and expansion [7, 8]. Despite the connection between CSPGs and aggressive glioma behavior, the molecular mechanisms involved in abnormal CSPGs and methods for targeting these CSPGs to treat glioma remain largely unexplored.

These CSPGs contain one or more CS side chains that contribute to a variety of biological functions regulating cell behavior. One major

function is CS displays various affinities to bind many growth factors, proteases, cytokines, chemokines [9, 10], which is considered as important signal regulators in cancer microenvironment. Additionally, CS chains interact directly with adhesion molecules and other extracellular matrix (ECM) proteins, determining tissue biophysical properties and morphological integration. In GBM, for example, enzyme digestion of excessive CS in tumor tissue has been demonstrated to enhance temozolomide antitumor effects [11], and promote spreading of oncolytic virus in tumor tissue of murine GBM models [12].

In humans, the biosynthesis of CS chains is initiated by the linkage of N-acetylgalactosamine (GalNAc) to a tetrasaccharide structure by CS GalNAc-transferases (CSGALNACT1 and CSGALNACT2). Next, the polymerization (elongation) step is catalyzed by a group of bifunctional enzymes (CHSY1, CHPF, CHPF2, and CHSY3), which have both β1-3 glucuronosyltransferase and β1-4 N-acetylgalactosaminyltransferase activities. One single CS chain can consist of up to 50 disaccharide repeats of D-glucuronic acid (GlcA)-GalNAc. Furthermore, the CS subunits are further classified according to their modifications [9, 13, 14]. Through sulfotransferase (CHSTs) catalysis, CS subunits are occasionally 6-O-sulfated at GalNAc residue (C6S) by CHST3 and/or 4-O-sulfated at GalNAc (C4S) mainly by CHST11. Depending on the spectrotemporal expression of these enzymes, a single CS chain usually consists of a series of variably sulfated units.

In this study, we focused on exploring the role of the C4S sulfotransferase, CHST11, in glioma progression. Additionally, we attempted to block C4S-derived cellular functions using a C4S specific binding peptide. The obtained results indicated C4S could be a potential target for suppressing GBM invasions and increasing survival rates.

Materials and methods

Cell culture and reagents

Human glioblastoma cell lines Ln18, U118, U251 and A172 cells, and mouse glioblastoma cell line GL261 were purchased from the American Type Culture Collection (Manassas, VA), and GBM8901 was purchased from Bioresources Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan). Cells were cultured in DMEM (Life Technologies, Grand Island, NY, USA) with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell cultures were maintained at 37°C in 5% CO_2 in a humidified incubator. pCMV3 vector was used to construct overexpressed cell clones. CHST11-pCMV3 plasmids were transfected to Ln18 and GL261 cells by TOOLstrong Transfection Reagent (BIOTOOLs, TW) and selected with 600 μg/mL of hygromycin. Chondroitinase ABC was purchased from Sigma-Aldrich (C2905). Gene silence assay were used *CHST11* ON-TARGETplus SMARTpool siRNA against (Dharmacon; Table S2) and transfected by Lipofectamine RNAiMAX (Invitrogen). Sequence of C4S blocking peptide was referring to the references [15]. This N-terminal biotinylated C4S specific binding peptide and scrambled peptides (Table S2) were synthesized by Kelowna International Scientific Inc., New Taipei City, Taiwan. The peptides were purified by HPLC (>99% in purity), and confirmed by mass spectrometry.

Tissue array and immunohistochemistry

To analyze CHST11 expression in human glioblastoma, human glioblastoma tissue microarrays were purchased from Shanghai Outdo Biotech. Tissue slide was blocked with 10% bovine serum albumin containing 0.1% Triton X-100 (Sigma) for 2 hours. After blocking, the CHST11 antibody (Santa Cruz Biotechnology, Inc.; sc-100868; 1:150) incubated at 4°C for 16 hours. The specific immunostaining was using UltraVision Quanto Detection System (Thermo Fisher Scientific Inc.) to amplify primary antibody signals. Using 3,3-diaminobenzidine (DAB) and hematoxylin (Sigma) for visualized staining. The staining intensity was scored (0, negative; +1, <30%; +2, 30-60%; +3, >60%) and compared to clinical parameters.

Western blot

Western blot assay was used to determine the protein expressions; cell lysates were collected with sodium dodecyl sulfate-solubilization buffer 5 mM EDTA, 1 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5 and 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 1 mM N-ethylmaleimide for 30 minutes on ice. Then, cell

lysates were centrifuged at 12000 g at 4°C and the protein concentrations determined with Bradford reagent (Bio-RAD) using bovine serum albumin as standard. For C4S antibody blotting, cells lysate were incubated with chondroitinase ABC (0.01 U/ml) for 1 hour in 37°C. Equivalent amounts of total protein per sample of cell extracts were used on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and overnight with following antibodies: CHST11, CSPG4 (A3592; ABclonal, Inc.), C4S (MAB2030l; Merck), phospho-FAK (Tyr397) antibody (#3283, Cell Signaling) and FAK (#3285, Cell Signaling), GAPDH and beta-actin. Detected protein expression by chemiluminescence using an ECL detection kit (Amersham Biosciences UK Limited, Buckinghamshire, England). Chemiluminescence images were captured and quantified by the MultiGel-21 image system (TOP BIO CO., Taiwan).

Immunoprecipitation

Calculated total 0.8 mg cell lysates incubated with 4 μl of CSPG4 primary antibody and 20 μl of protein-A beads at 4°C for 16 hours. The pull-down beads were spin washed for three times and incubated with chondroitinase ABC (0.01 U/ml) for 1 hour in 37°C. Samples were analyzed by western blot.

Immunofluorescence

Cells were cultured and fixed on cover slide, and incubated with chondroitinase ABC (0.01 U/ml) for 1 hour before C4S and CSPG4 double staining. For immunofluorescence staining of peptides, streptavidin conjugated with Alexa Fluor 594 (Invitrogen; REF S11227) was used for anti-biotin staining. Hoechst 33342 (AAT Bioquest, Cat: 17533) was used to label DNA in immunofluorescence.

Cell viability assay

Cell viability was using CCK8 assay. 2×10^3 cells were seeded into 96-well culture plates and incubated for times. At 0, 24, 48, and 72-hour time points following CCK8 manufacturer's protocol (Sigma-Aldrich) and incubated with CCK8 reagent for four hours. Measures the absorbance of each well by OD 450 nm, experiments were repeated for three times independently and data were shown by relative fold changes.

Cell migration and invasion assay

Cell migration assay were used transwell inserts (Coring). Cell transwell assay were used with transwell inserts (Coring), with or without Matrigel (BD Biosciences) on the inserts filters (pore size 8 μm), and the inserts were set into a 24-well plate for experiments. 2×10^4 cells were seeded into each insert with serum-free DMEM culture medium and the lower part of the well filled with 0.6 ml DMEM culture medium containing 10% FBS. Incubated for hours, took out the filters and fixed the cells by methanol for five minutes. The filters were stained by crystal violet dye and counting cell numbers. The data showed the average cell numbers under microscopy, each filter was randomly selected three fields and independent experiments were repeated at least three times. $P<0.05$ as statistically significant.

Reverse-trancription-PCR

Total RNA was isolated from cultured cells using TOOLSmart RNA Extractor (DPT-BD24) following the manufacturer's instructions. For reverse transcription, first-strand cDNA synthesis was performed with random primers (hexamers; Promega, Madison, WI) and 100 U of moloney murine leukemia virus reverse transcriptase and performed at 42°C for 60 minutes and terminated at 90°C for 10 minutes. The quantitative real-time PCR analysis was performed using SYBR green one-step PCR Master Mix (Applied Biosystems, Carlsbad, CA) with primer *CHST11* and *CSPG4, GAPDH* and *18S* used as internal control (primer sequence were listed in Table S2).

Flow cytometry

Cells were detached by 10 mM EDTA and spin washed two times for CSPG4 antibody staining. For C4S antibody staining, cells were fixed by 4% paraformaldehyde (PFA) for 30 minutes and incubated with chondroitinase ABC for 1 hour in 37°C. Cells were incubated with C4S antibody for 30 minutes FITC anti-mouse IgG was used for the secondary antibody and incubated for 30 minutes. All results were analyzed using the FlowJo 10.7 software program.

Confocal microscopy

Confocal images were captured by Leica TCS SP8 confocal microscope. Each confocal image

from cell slides was stacked five scans images merged into one graphic. The total thickness in one capture was 1.5 μm.

Animal model

The GL261 injection mouse model was performed according to previous study [16]. In brief, GL261 mock transfectant and CHST11 overexpressed cells were intracranial injected to the 8 week old male C57BL/6J mouse, $2*10^5$ cells were injected to the right cerebral from bregma (0, 0, 0) to the right 2 mm and backward 1 mm and depth 3 mm (2, -1, -3) position. The longest length of tumor (L) and the shortest diameters (S) of the tumors were measured. Tumor volume was estimated using the formula $L \times S^2 \times 0.4$. For peptide treatment experiments, 20 μg/g peptide in PBS was intraperitoneal injection 7 days after tumor cell transplanted. The peptides were given twice a week. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of National Chung Hsing University Experimental Animal Center.

Statistical analysis

Data was performed by using GraphPad Prism 6. CHST11 expression and clinic pathologic variables of glioblastoma tissue array were analyzed by two-tailed Fisher exact test. Mann-Whitney *U* test was used to analyze scores of immunohistochemistry. Survival curves were analyzed by Kaplan-Meier analysis and the log-rank test. When p value <0.05 is defined as statistically significant.

Results

CHST11 is upregulated in human glioma tissue and associated with poor prognosis

Searching on the GEPIA website (http://gepia2.cancer-pku.cn/#index) revealed the crucial C4S sulfotransferase, *CHST11*, is significantly upregulated several types of cancer tissue, including low grade glioma (LGG) and GBM in The Cancer Genome Atlas Program (TCGA) dataset (Figure S1A). The protein expression of CHST11 was further analyzed in another independent cohort of glioma patients by immunohistochemistry on tissue array containing 166 primary glioma tissue and 10 adjacent non-tumor brain tissue. Results indicated that CHST11 was frequently expressed in the cytoplasm of glioma cells and certain vessel structures in a subset of patients (**Figure 1A**), while the staining in non-tumor tissue is weak to undetectable under our experimental conditions (**Figure 1B**). The intensity of CHST11 staining was scored according to the percentage of positive cells in each sample (0, negative; +1, <30%; +2, 30-60%; +3, >60%). Our data indicated that staining intensity of CHST-11 is significantly increased in LGG tissue compared to non-tumor tissue (Mann-Whitney *U* Test, $P = 0.0005$), and that is further increased in GBM cases ($P = 0.0192$, **Figure 1C** and Table S1). Analyzing the TCGA GBM patient dataset (n = 152), it revealed that the progression-free survival (PFS) rate of patients with high expression of *CHST11* was significantly lower than those with low *CHST11* expression, and patients with high expression of *CHST11* revealed a short overall survival (OS) trend (**Figure 1D**). Similar result was further obtained using Chinese Glioma Genome Atlas (CGGA) dataset with 183 GBM tissues (Figure S1B) [17]. Examining CHST11 protein levels in normal human brain tissue, cultured normal human astrocyte, and in GBM cell lines, we found that the tested human GBM cell lines expressed relatively higher levels of CHST11 than normal brain tissue and cells (**Figure 1E**). Together, these data suggest that CHST11 is frequently upregulated in glioma cells, and that its expression correlates with the high tumor grade and poor prognosis.

CHST11 regulates C4S formation and enhances malignant phenotypes of GBM cells

CHST11 is one of chondroitin 4-sulfotransferases. To explore the influence of CHST11 on C4S formation in glioma cells, we have overexpressed CHST11 in a mouse GBM cell line GL261, and human GBM Ln18 cells, while silenced CHST11 in GBM8901 cells, which expressed relative high endogenous levels of CHST11 (**Figure 2A**). The CHST11-modulated C4S formation on the cell surface was quantified by flow cytometry using a C4S specific antibody. We found that C4S intensity was slightly increased in CHST11-overexpressing Ln18 cells, and was obviously decreased in CHST11-silenced GBM8901 cells (**Figure 2B**). The C4S antibody (clone BE-123) which reacts with chondroitinase ABC-digested CS chain

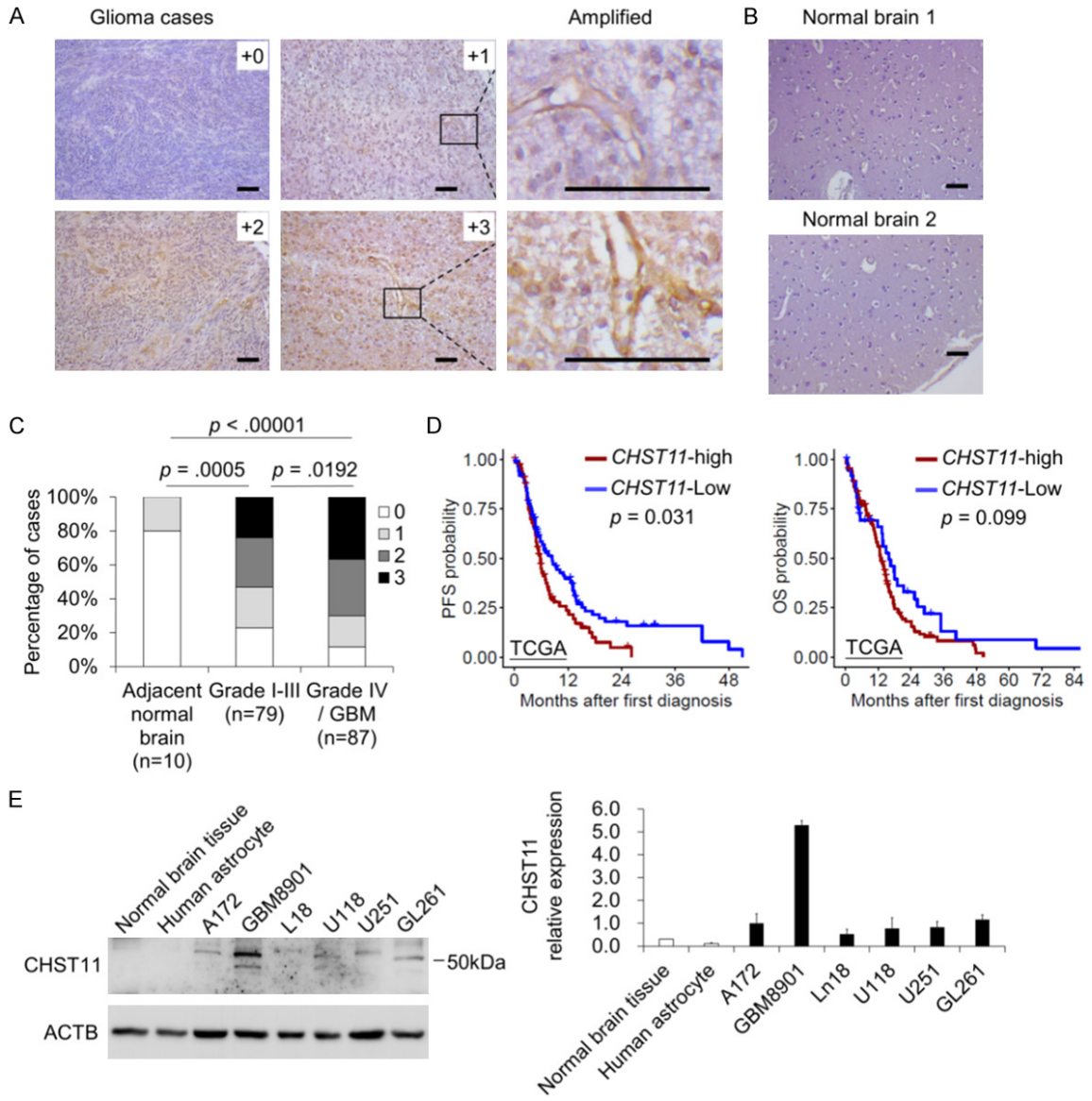

Figure 1. CHST11 is frequently upregulated in human glioma. A. Representative images of different intensities of immunohistochemistry on glioma tissue array using anti-CHST11 antibody. Tissues were counterstained with hematoxylin. Two amplified images were shown at right. Scale bar = 150 μm. B. Representative images of CHST11 staining on normal human brain tissue. Scale bar = 150 μm. C. Statistical analysis of immunohistochemistry in glioma tissue array with 10 normal brain tissue. The *p* value of Mann-Whitney *U* test was shown. D. Kaplan-Meier analysis of progression-free survival (PFS, left) and overall survival (OS, right) of TCGA GBM dataset. E. Western blots revealed expression levels of CHST11 in normal brain tissue, cultured human astrocytes, and GBM cell lines. Beta-actin (ACTB) was taken as loading control. The relative quantities (refer to the expression of A172) were shown at right.

was used to measure CHST11-modulated CS formation [18, 19]. Immunofluorescence staining was used to further examine the cellular distribution of C4S in glioma cells. The results revealed increase of C4S staining on plasma membrane and cytoplasm in CHST11 overexpressed cells, while decrease of C4S staining in CHST11-silenced cells (**Figure 2C**). Additionally, western blots confirmed that C4S was attenuated in several proteoglycans when CHST11 was silenced (**Figure 2D**). All of these data indicated that CHST11 is a crucial enzyme to modulate C4S formation in GBM cells.

Next, CHST11-mediated cancer phenotypes were evaluated. We found that CHST11 did not have significant effects on cell viability in monolayer culture conditions (**Figure 2E**). However,

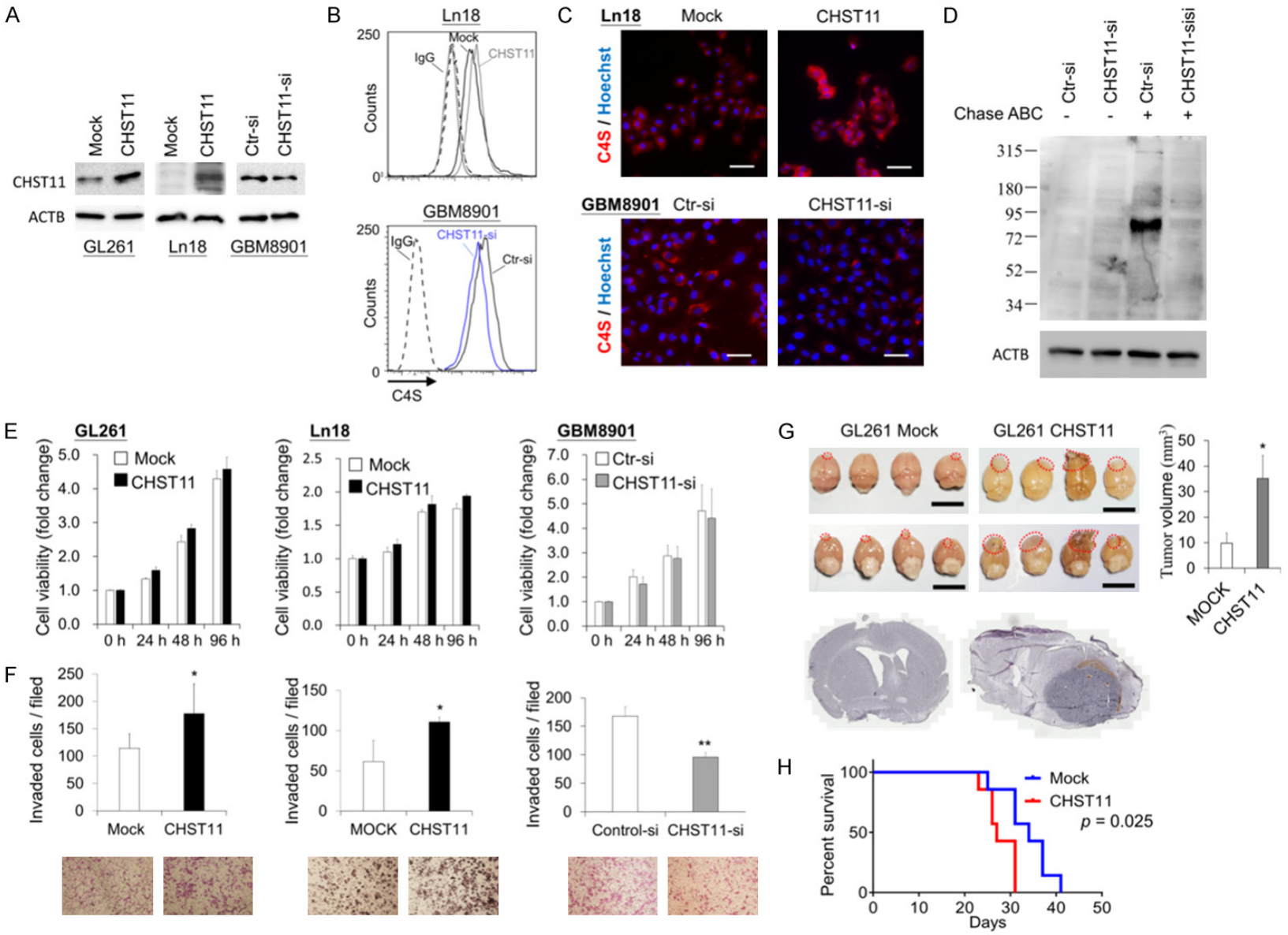
A
Mock
CHST11
Mock
CHST11
Ctr-si
CHST11-si
CHST11
ACTB
GL261
Ln18
GBM8901
B
Ln18
Mock
IgG
CHST11
Counts
GBM8901
IgG
CHST11-si
Ctr-si
C4S
C
Ln18
Mock
CHST11
C4S / Hoechst
GBM8901
Ctr-si
CHST11-si
D
Chase ABC
- Ctr-si
- CHST11-si
+ Ctr-si
+ CHST11-sisi
315
180
95
72
52
34
ACTB
E
GL261
Ln18
GBM8901
Mock
CHST11
Ctr-si
CHST11-si
Cell viability (fold change)
0 h 24 h 48 h 96 h
F
Invaded cells / filed
Mock CHST11
MOCK CHST11
Control-si CHST11-si
G
GL261 Mock
GL261 CHST11
Tumor volume (mm³)
MOCK
CHST11
H
Percent survival
Mock
CHST11
p = 0.025
Days

Figure 2. CHST11 modulates C4S formation and cancer malignancy of GBM cells. (A) Western blots revealed expression of CHST11 in GL261 cells and Ln18 cells that transfected with empty plasmid (Mock) or CHST11-pCMV3 plasmid (CHST11), and GBM8901 cells transfected with control (Ctr) siRNA or *CHST11* siRNA (CHST11-si). (B) Flow cytometry revealed cell surface C4S formation after overexpression or knockdown of CHST11 in GBM cells. (C) Immunofluorescence of C4S in CHST11 overexpressed or silenced cells. Scale bar = 50 μm. (D) Western blotting of C4S in GBM8901 cell lysate treated with or without chondroitinase ABC (Chase ABC) treatments. ACTB was used as loading control. (E) Cell viability and (F) Cell transwell invasion of CHST11 overexpressed GL261 and Ln18 cells, and CHST11 silenced GBM8901 cells. Representative images was shown at bottom. **P*<0.05; ***P*<0.01. (G) Overexpression of CHST11 enhances tumor growth *in vivo*. GL261 transfectants were orthotopically injected into right cerebral hemisphere of mice (n = 4 for each group). Mouse brain was exercised 3 weeks after injection. **P*<0.05. Red dash lines indicate location of tumors. Representative images of H-E stains of the brain section are shown. (H) Survival analysis of orthotopic injection GL261 model. Seven mice were used for each group. Kaplan-Meier analysis was used.

overexpression of CHST11 significantly enhanced colony formation in GL261 cells (Figure S2A). Additionally, transwell assays were performed to determine glioma cell mobility and invasiveness. The overexpression of CHST11 significantly increased the cell invasion through matrix gel and cell migration, whereas the knockdown of CHST11 evidently suppressed the invasion and migration of GBM cells (**Figures 2F** and S2B). To analyze the effects of CHST11 on tumor growth *in vivo*, mock and CHST11-overexpressing GL261 cells were transplanted orthotopically into mouse cerebrum (n = 4 for each group). Results revealed that overexpression of CHST11 significantly increased tumor volume in the 3 weeks after transplantation (**Figure 2G**). The survival analysis indicated that overexpression of CHST11 in GBM cells significantly shortened the median survival time (**Figure 2H**; n = 7 for each group).

CHST11 positively associated with CSPG4 expression in tumor cells

Previous studies indicated that CS biosynthesis genes are usually positively associated with specific clustering CSPGs in cancer tissue [20]. To explore the CHST11-regulated cellular mechanisms in human glioma, we examined the list of *CHST11* co-expressed genes in the TCGA glioma dataset (GBM + LGG) using the GEPIA2 on line tool [21]. *CHST3* is positively associated with *CHST11*, which is in consistency with a previous report [20], and *CSPG4* is the most significant CSPG that is positively associated with *CHST11* expression (**Figure 3A**). Similar gene associations were also observed in CGGA GBM dataset (Figure S3A). In contrast, *CSPG4* is not co-expressed with *CHST11* in normal brain tissue by analyzing The Genotype-Tissue Expression (GTEx) dataset (data not shown) [22]. We have previously reported that *ITGB1* (integrin β1) is associated with *CHSY1* (CS synthase 1) [23], but which is not in the case of *CHST11*. The TCGA and CGGA data represent bulk tumor RNA which mix all cell types in the tumor. To verify the association of *CHST11* and *CSPG4* in specific cell types of GBM tumor tissue, we leveraged a public single cell RNA sequence (scRNA-seq) dataset of 8 GBM patients (**Figure 3B**) [24]. Four cell clusters, glioma-associated macrophage/microglia (GAM), tumor cells, oligodendrocyte, and T cells, were displayed using the UMAP method. *CHST11* was expressed in both tumor cells and GAM, while *CSPG4* mainly expressed in tumor cells (**Figure 3C**). Accordingly, the positive association of *CHST11* and *CSPG4* is more significant in tumor cells. Moreover, there was no significant difference in the PFS and OS of GBM patients with *CSPG4* high expression or *CSPG4* low expressions in TCGA dataset, but the subset with high expression of both *CSPG4* and *CHST11* was significantly associated with poor PFS and OS (**Figure 3D**). The poor OS of GBM patients with *CSPG4/CHST11* high expression was also observed in the CGGA dataset (Figure S3B).

CHST11 regulates CSPG4 expression and C4S formation of CSPG4

To investigate the relationship between CHST11 and CSPG4, we measured the expression of CSPG4 when CHST11 was overexpressed or silenced in GBM cell lines. The results indicated that overexpression of CHST11 slightly increased CSPG4 expression, while silence of CHST11 attenuated CSPG4 expression in mRNA levels (**Figure 4A**). In contrast, silencing CSPG4 in GBM cell lines has no significant effect on the expression of CHST11 (Figure S4). Additionally, the cell surface expression of CSPG4 was increased on CHST11-overexpresing

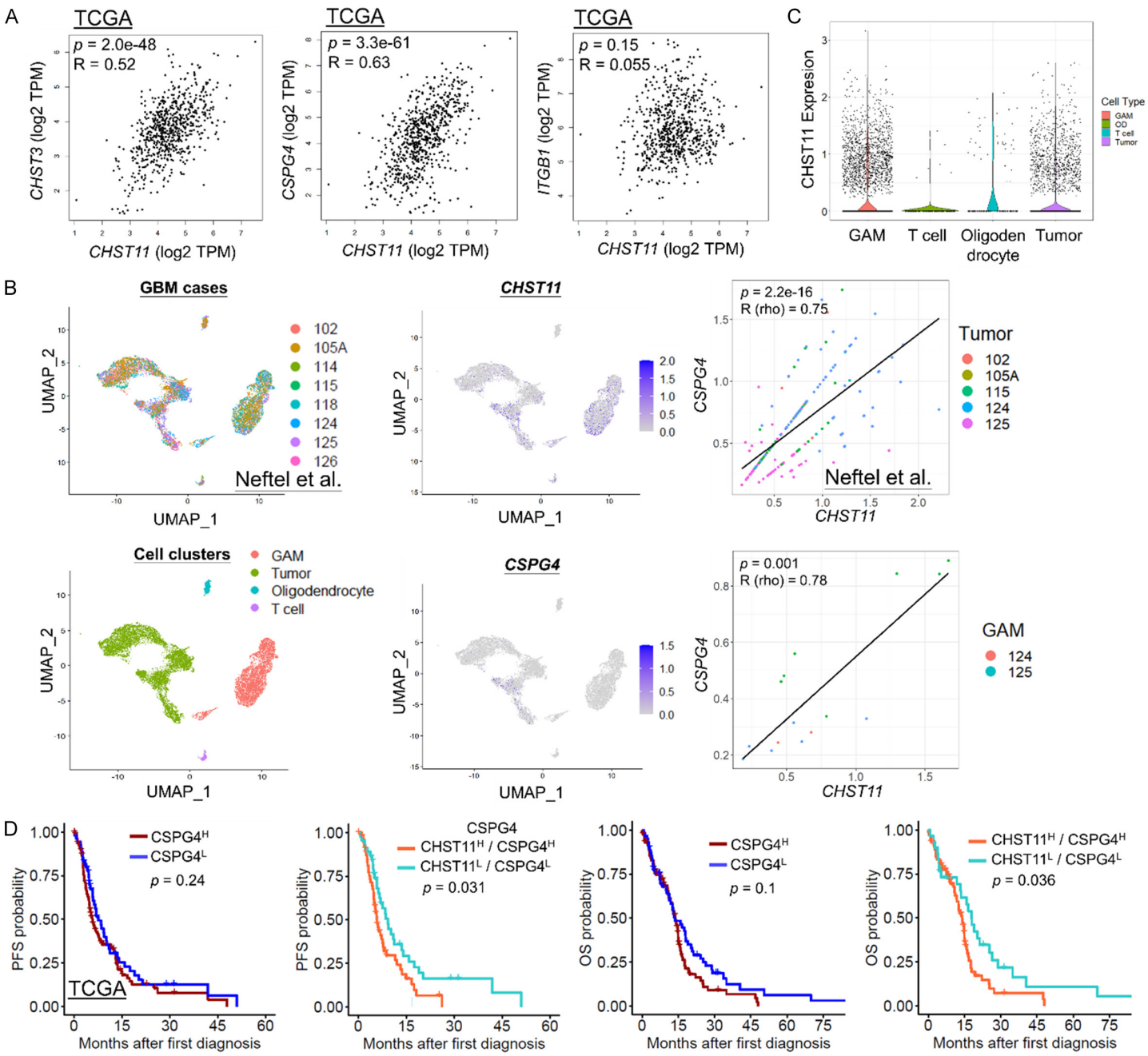
A
TCGA
p = 2.0e-48
R = 0.52
CHST3 (log2 TPM)
CHST11 (log2 TPM)
TCGA
p = 3.3e-61
R = 0.63
CSPG4 (log2 TPM)
CHST11 (log2 TPM)
TCGA
p = 0.15
R = 0.055
ITGB1 (log2 TPM)
CHST11 (log2 TPM)
C
CHST11 Expresion
Cell Type
GAM
OD
T cell
Tumor
GAM
T cell
Oligoden drocyte
Tumor
B
GBM cases
102
105A
114
115
118
124
125
126
Neftel et al.
UMAP_2
UMAP_1
CHST11
UMAP_2
UMAP_1
p = 2.2e-16
R (rho) = 0.75
CSPG4
CHST11
Neftel et al.
Tumor
102
105A
115
124
125
Cell clusters
GAM
Tumor
Oligodendrocyte
T cell
UMAP_2
UMAP_1
CSPG4
UMAP_2
UMAP_1
p = 0.001
R (rho) = 0.78
CSPG4
CHST11
GAM
124
125
D
CSPG4H
CSPG4L
p = 0.24
TCGA
PFS probability
Months after first diagnosis
CSPG4
CHST11H / CSPG4H
CHST11L / CSPG4L
p = 0.031
PFS probability
Months after first diagnosis
CSPG4H
CSPG4L
p = 0.1
OS probability
Months after first diagnosis
CHST11H / CSPG4H
CHST11L / CSPG4L
p = 0.036
OS probability
Months after first diagnosis

Figure 3. CSPG4 is positively associated with CHST11 gene in glioma cells. A. The co-expression of *CHST11* with *CHST3*, *CSPG4*, and *ITGB1* in TCGA LGG and GBM cases. B. Analysis of cellular expression of *CHST11* and *CSPG4* using a single cell RNA-sequencing dataset (scRNA-seq) (Neftel et al., *Cell*, 2019) [24] with 8 GBM patients. Smart-seq2 data were downloaded with cell type annotations (Left). Expression of *CHST11* and *CSPG4* is shown (middle). Correlation analysis for CSPG4 and CHST11 mRNA level in tumor cells and glioma-associated macrophage/microglia is shown at right. C. Violin plot of CHST11 expression in different cell types from scRNA-seq dataset. D. Kaplan-Meier analysis of progression-free survival (PFS, left) and overall survival (OS, right) of TCGA GBM dataset, comparing CSPG4 high ($CSPG4^{H}$) expression and CSPG4 low ($CSPG4^{L}$) expression subsets and CHST11/CSPG4 double high and double low subsets.

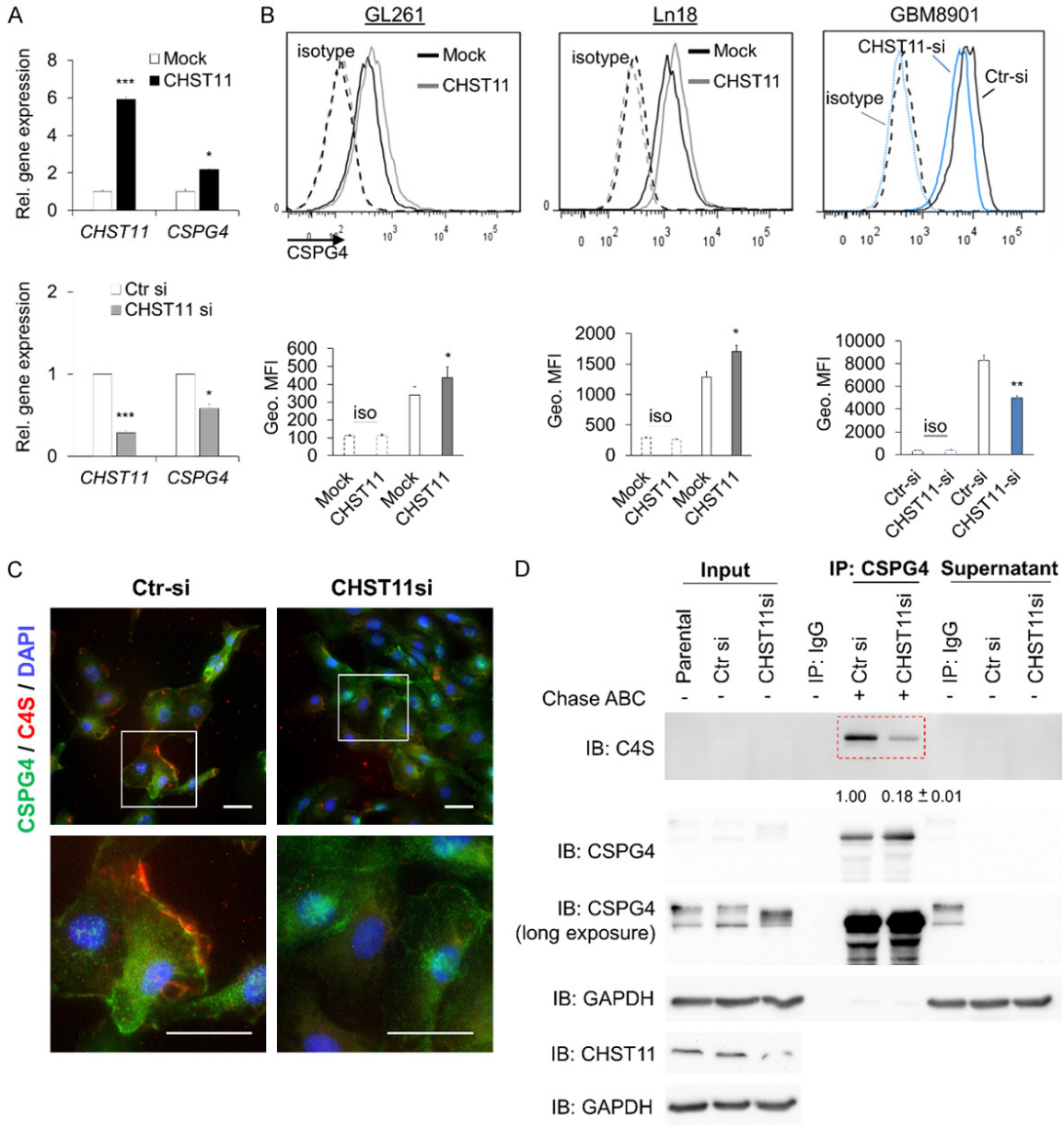

Figure 4. CHST11 modulates expression and glycosylation of CSPG4. A. q-PCR of CHST11 and CSPG4 in CHST11 overexpressed GL261 cells (upper) or CHST11-silenced GBM8901 cells. **P*<0.05; ****P*<0.001. B. Flow cytometry analysis of surface expression of CSPG4 on CHST11 overexpressed GL261 and Ln18 cells, and CHST11-silenced GBM8901 cells. Isotype IgG (iso) was used as nonspecific background staining control. **P*<0.05; ***P*<0.01. C. Immunofluorescence staining of CSPG4 (green) and C4S (red) in control and CHST11-silenced GBM8901 cells. Scale bar = 30 μm. D. Western blots of immunoprecipitation (IP) of control (Ctr si) and CHST11-silenced (CHST11si) GBM8901 protein lysate using CSPG4 antibody. The IP protein samples were digested with chondroitinase ABC (Chase ABC). IP samples, input protein (30 μg), and supernatant protein after IP were blotted with anti-C4S, anti-CSPG4, and anti-GAPDH antibodies. IP of non-specific IgG was used as control. Note that after IP, the CSPG4 depleted in the supernatant of protein lysate. The red dashed square indicates the relative quantitative bands.

GBM cells, while it decreased on CHST11-silenced GBM8901 cells (**Figure 4B**). We further investigated whether CHST11 modified CS chains on CSPG4. Immunofluorescence staining revealed that C4S was colocalized with CSPG4 on the surface of control cells, while that was diminished on CHST11 silenced cells (**Figure 4C**). Immunoprecipitation assay of CSPG4 confirmed that C4S was decreased on CSPG4 when CHST11 was silenced (**Figure 4D**), and silencing CHST11 also decreased the molecular weight of CSPG4. These data suggested that CHST11 slightly modulates CSPG4 gene expression and directly modifies CS chains on CSPG4 on glioma cells.

CSPG4 involved in the CHST11-mediated malignant phenotypes of glioma cells

To investigate the possible effects of CHST11 and CSPG4 co-expression on GBM cells, we analyzed the differential expressed genes between *CSPG4/CHST11* double high expression and double low expression GBM patients using the TCGA database (**Figure 5A**). These differential expressed genes were analyzed using gene set enrichment analysis (GSEA). The results indicated these genes were positively associated with chondroitin sulfate biosynthesis gene set and extracellular matrix (ECM) interaction gene set (**Figure 5B**), which were in consistency with the known biological functions of CHST11 and CSPG4. To confirm whether CSPG4 is involved in CHST11-induced ECM interaction, CSPG4 was silenced in GBM cells. Transwell ECM invasion assay revealed that silencing CSPG4 suppressed CHST11-enhanced cell invasion (**Figure 5C**). In addition, CHST11 significantly enhanced the cell adhesion-induced FAK activation when cell attached to collagen I (the major component of ECM protein), while silencing CSPG4 inhibited CHST11-enhanced FAK activation (**Figure 5D**).

C4S binding peptide suppressed CHST11-induced malignant phenotypes in vitro and in vivo

As all our evidence indicates that CHST11-mediated C4S exacerbated GBM cells invasiveness, we suppose the method that can block C4S biological functions may reduce malignancy of GBM cells. A recent study discovered a 4-O-sulfated CS binding short peptide (C4Sp) by a phage display peptide library, and proven it can modulate neurite outgrowth [15]. We found that the C4Sp (biotinylated) can bind to GBM8901, but the binding was diminished when CHST11 was silenced by siRNA (**Figure 6A**). Treating GBM cells with C4Sp or corresponding scrambled peptides have no significant influence on cell viability *in vitro* (Figure S5), suggesting low cytotoxicity of these peptides. Importantly, we found the C4Sp treatment markedly suppressed CHST11-enhanced invasion of GBM cells (**Figure 6B**). To further develop a C4Sp treatment for GBM animal model, we first examined whether the biotinylated C4Sp can target the orthotopic GL261 tumor tissue. The tissues from tumor bearing animals were prepared 6 hours after the third injection of peptides or solvent. The C4Sp was observed in tumor tissue of brain sections, while no obvious signals were observed in solvent (PBS) and scrambled peptides treating groups (**Figure 6C**). The biotinylated C4Sp also accumulated in kidney and liver tissues (data not shown), but it did not cause adverse tissue damage in liver, lung, kidney and intestine by histological examination (Figure S6). To evaluate the therapeutic effects of C4Sp, mice were orthotopic transplanted with CHST11 overexpressed GL261 cells and treated with C4Sp or scrambled peptide twice a week. Results indicated that the overall survival was significantly prolonged in the C4Sp treatment group, and 15.4% (2/13) of C4Sp treating mice survived over 50 days (**Figure 6D**).

Discussion

Here, we provide evidence that the C4S sulfotransferase, CHST11, upregulated in glioma tissue may serve as a risk factor of cancer progression. The expression level of CHST11 directly modulates C4S formation on GBM cells, and consequently regulates malignant behavior of GBM cells, particularly cell invasiveness. Mechanically, CHST11 is not only positively associated with CSPG4 expression in GBM cells in primary cancer tissue, and CS chains on CSPG4 are also modified by CHST11. Silencing CSPG4 in GBM cells reverses CHST11-promoted cell invasion, providing evidence that CSPG4 is one of crucial CSPGs involved in CHST11-regulated phenotype. Importantly, using a C4S binding peptide successively suppressed CHST11-induced invasiveness of GBM cells and prolonged the survival of GBM animal model.

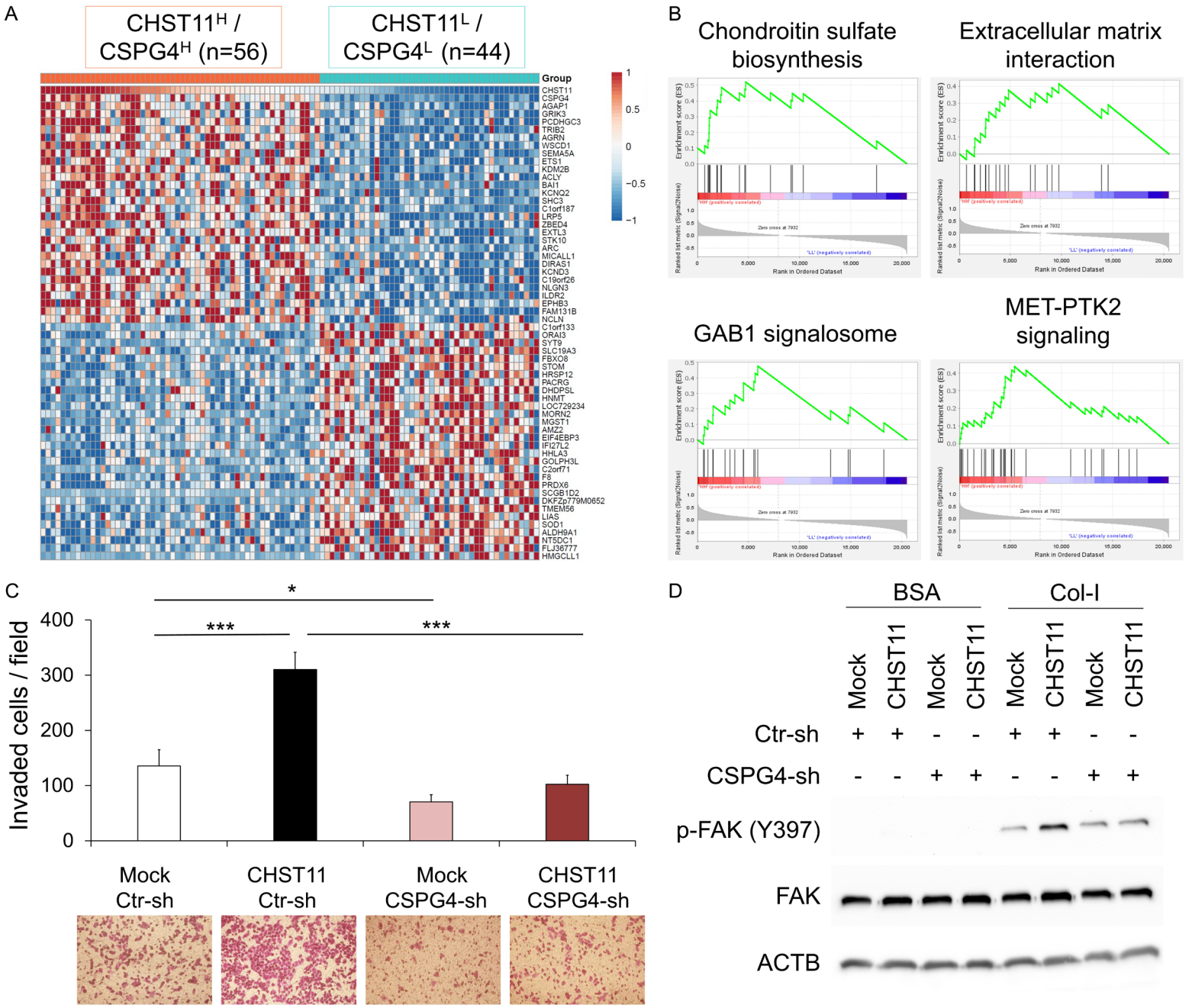
A
CHST11H / CSPG4H (n=56)
CHST11L / CSPG4L (n=44)
Group
CHST11
CSPG4
AGAP1
GRIK3
PCDHGC3
TRIB2
AGRN
WSCD1
SEMA5A
ETS1
KDM2B
ACLY
BAI1
KCNQ2
SHC3
C1orf187
LRP5
ZBED4
EXTL3
STK10
ARC
MICALL1
DIRAS1
KCND3
C19orf26
NLGN3
ILDR2
EPHB3
FAM131B
NCLN
C1orf133
ORAI3
SYT9
SLC19A3
FBXO8
STOM
HRSP12
PACRG
DHDPSL
HNMT
LOC729234
MORN2
MGST1
AMZ2
EIF4EBP3
IFI27L2
HHLA3
GOLPH3L
C2orf71
F8
PRDX6
SCGB1D2
DKFZp779M0652
TMEM56
LIAS
SOD1
ALDH9A1
NT5DC1
FLJ36777
HMGCLL1
1
0.5
0
−0.5
−1
B
Chondroitin sulfate biosynthesis
Extracellular matrix interaction
GAB1 signalosome
MET-PTK2 signaling
Enrichment score (ES)
Ranked list metric (Signal2Noise)
Rank in Ordered Dataset
Zero cross at 7932
'HH' (positively correlated)
'LL' (negatively correlated)
C
Invaded cells / field
400
300
200
100
0
*
***
***
Mock Ctr-sh
CHST11 Ctr-sh
Mock CSPG4-sh
CHST11 CSPG4-sh
D
BSA
Col-I
Mock
CHST11
Mock
CHST11
Mock
CHST11
Mock
CHST11
Ctr-sh + + - - + + - -
CSPG4-sh - - + + - - + +
p-FAK (Y397)
FAK
ACTB

Figure 5. CSPG4 involved in CHST11-mideated invasive phenotype of GBM cell. A. Heat map of differential expressed genes between CSPG4/CHST11 high expression subset and low expression subset using TCGA GBM database. B. Representative plots of GSEA revealed the associated pathways. C. Silencing CSPG4 suppressed CHST11-enhanced cell GL261 transwell invasion. *P<0.05; ***P<0.001. D. Western blot of cell adhesion signaling. GL261 transfectants were seeded on a BSA (negative control) or Collagen-I (Col-I) coated plate. Note that silencing CSPG4 suppressed CHST11-enhanced activation of focal adhesion kinase (p-FAK Y397).

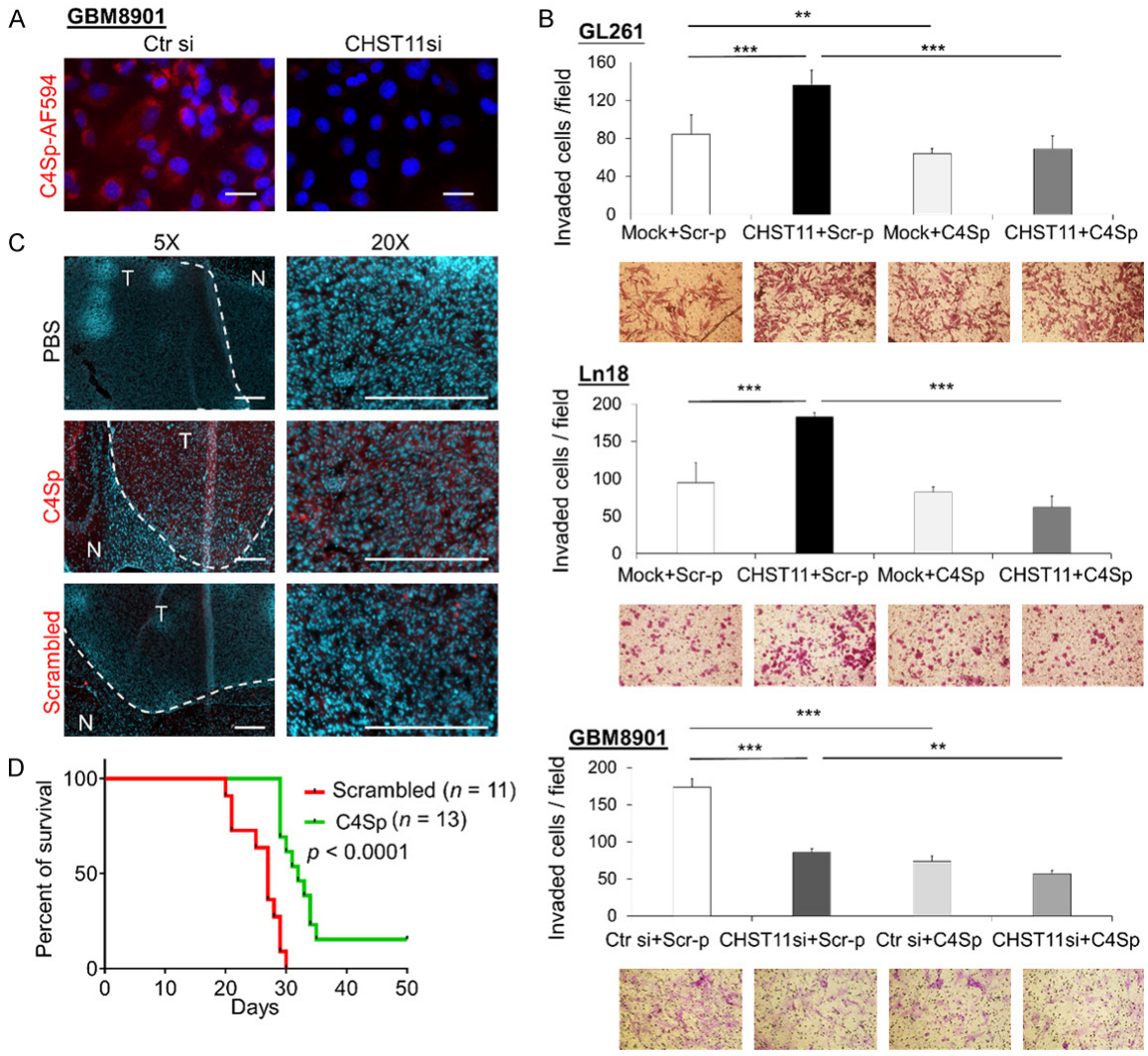

Figure 6. C4S binding peptide (C4Sp) suppressed CHST11-induced malignant phenotypes in GBM cells. A. The C4Sp binding to GBM8901 cells transfected with control siRNA (Ctr si) or CHST11 siRNA (CHST11si). Scale bar = 30 μm. B. Cell invasion assay of GBM transfectants treated with scrambled peptide (Scr-p) or C4Sp. Representative images are shown. *P<0.05; **P<0.01; ***P<0.001. C. Distribution of treated peptide in orthotopic GL261 brain tumor section. PBS was used as solvent control. Dash line indicated the boundary of tumor tissue (T) and non-tumor brain tissue (N). Scale bar = 300 μm. D. Survival analysis of peptide treatment. Orthotopic injection model of CHST11-overexpressed GL261 cells was used. Animals were treated with scrambled peptide or C4Sp twice a week. Kaplan-Meier analysis was used.

CSPG4 also known as neuron-glial antigen 2 (NG2) is a type I transmembrane core proteoglycan found in normal brain tissue, which have distinct functions in regulating CNS development [25, 26]. Several studies indicated that abnormal up regulation of CSPG4 is associated with the pathology of multiple types of cancers, including GBM, melanoma, breast cancer, and so on [27, 28]. It has been reported that over 60% of patients with GBM express CSPG4,

which is an important prognostic factor for patients with this type of tumor, and CSPG4 has been preclinical tested as a target for chimeric antigen receptor T (CAR-T) cell therapy in mouse models of GBM [29-32]. While the present survival analysis using TCGA and CGGA datasets revealed that CSPG4 expression has only slightly influence on OS of GBM patients, while the OS is significantly shorter when CHST11 is co-overexpressed. These results implied CHST11 may unleash oncogenic functions of CSPG4.

In fact, limited information documented the role of CS chains for CSPG4 bioactivity. Previous studies revealed that the CS chains on CSPG4 contribute to its interaction with integrins and activation of metalloprotease [33, 34]; both are crucial factors for cancer invasiveness. We found that CHST11 could be an upstream for modulating mRNA levels of *CSPG4* expression, and overexpression CHST11 resulted in slightly increased cell surface expression of CSPG4 protein. These changes may be through indirect or reciprocal mechanisms in GBM cells. Thus, further studies are required to determine whether CHST11-modified CS chains exert a profound influence on CSPG4 activities in promoting aggressive cancer phenotypes in GBM as well as other types of cancer cells.

Recently, series studies uncovered the aberrant expression of CS synthases and sulfotransferases associated with poor prognosis of GBM [16, 23, 35, 36]. Up-regulation of CHSY1 is associated with short survival and accompanied with excessive accumulation of CS in tumor tissue [16]. Direct treating purified C4S or C6S to cultured GMB cells could enhance malignant behavior of cancer cells, which suggested independent bioactivities of these carbohydrates chains [36]. However, only few studies examined the effects of blocking CS bioactivities for glioma treatment. Our previous study used C6S-specific binding peptide on cultured GBM cells, and the treatment suppressed glioma cell mobility accompanied with promoting CD44 degradation. However, the effects of C6S-specific binding peptide on the glioma animal model are still undefined [23]. Dr. Karumbaiah reported that Surfen could block extratumoral CS and inhibit the invasion of F98 (a rat GBM cell line) cells, but the therapeutic effects of Surfen are not significant in animal models [37]. The present report provided evidence that C4S binding peptide can target orthotopic transplant GL261 brain tumor tissue by simply intraperitoneal injection. This could be the result from the blood brain barrier (BBB) being leaky in this murine model [38]. In human glioma, the heterogeneity of BBB integrity may limit peptide permeability, and other methods to deliver this peptide drug to human glioma tissue should be taken into consideration [39, 40]. Besides, we have to note that using intravenous injection of peptide does not give more peptide target to brain tumor (data not shown), we assume this result may be caused by rapid clearance of peptides in kidney and liver, or degradation of peptide in blood serum [40]. Nevertheless, our proposed C4S peptide treatment revealed a mild but significant increase of survival rate in brain tumor bearing mice. It is worth further optimizing the treatment on other preclinical glioma models.

In summary, our work demonstrates a malignant function of excessive CHST11 in GBM cells, and its derived C4S accumulation could be a druggable target for suppressing GBM invasion.

Acknowledgements

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Disclosure of conflict of interest

None.

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Table S1. Correlation of CHST11 expression with clinicopathological features of glioma tissue array

Factor		CHST11 expression		*p* value (Two-sided Fisher's exact test)
		Low (0 and +1)	High (+2 and +3)	
Tissue types	Non-tumor	10	0	0.0002*
	Tumor	66	93	
Sex#	Male	33	48	1.0000
	Female	30	45	
Age#	<55 years	40	46	0.1016
	≥55 years	23	47	
Tumor stage	Grade I-III$	37	42	0.0264*
	Grade IV (GBM)	26	61	

*P<0.05 was considered as statistically significant. #Ten patients' sex and age were not provided. $Astrocytoma and Oligodendroglioma.

Table S2. List of PCR primers, siRNA, and peptide sequence

Symbol	Gene name	Used	Sequence (5'-3')
hCHST11	Human Carbohydrate sulfotransferase 11	qPCR	F: GTATGTTGCACCCAGTCATGC
			R: TGTAGAGTTCCTGCAGGGGG
mCHST11	Mouse Carbohydrate sulfotransferase 11	qPCR	F: TCCAAAGTATGTTGCACCCAG
			R: AGGGGACTTCTCGATCCCTT
hCSPG4	Human Chondroitin sulfate proteoglycan 4	qPCR	F: GTCCTG CCTGTCAATGACCAAC
			R: CGATGGTGTAGACCAGATCCTC
mCSPG4	Mouse Chondroitin sulfate proteoglycan 4	qPCR	F: GCTGTGCGTCGTTTGAGTTT
			R: CGTAAGGGCTTTGGTCCCAT
ACTB	Actin, beta	qPCR	F: CACCATTGGCAATGAGCGGTTC
			R: AGGTCTTTGCGGATGTCCACGT
CHST11	Carbohydrate sulfotransferase 11	siRNA	ON-TARGETplus SMARTpool siRNA ID: 50515
siRNA control	Non-targeting siRNA	siRNA	Assay ID: s736
C4Sp	C4S binding peptide	peptide	Biotin-AMDIAYRTHREP
Scrambled	C4S scrambled peptide	peptide	Biotin-RDYHPARMITEA

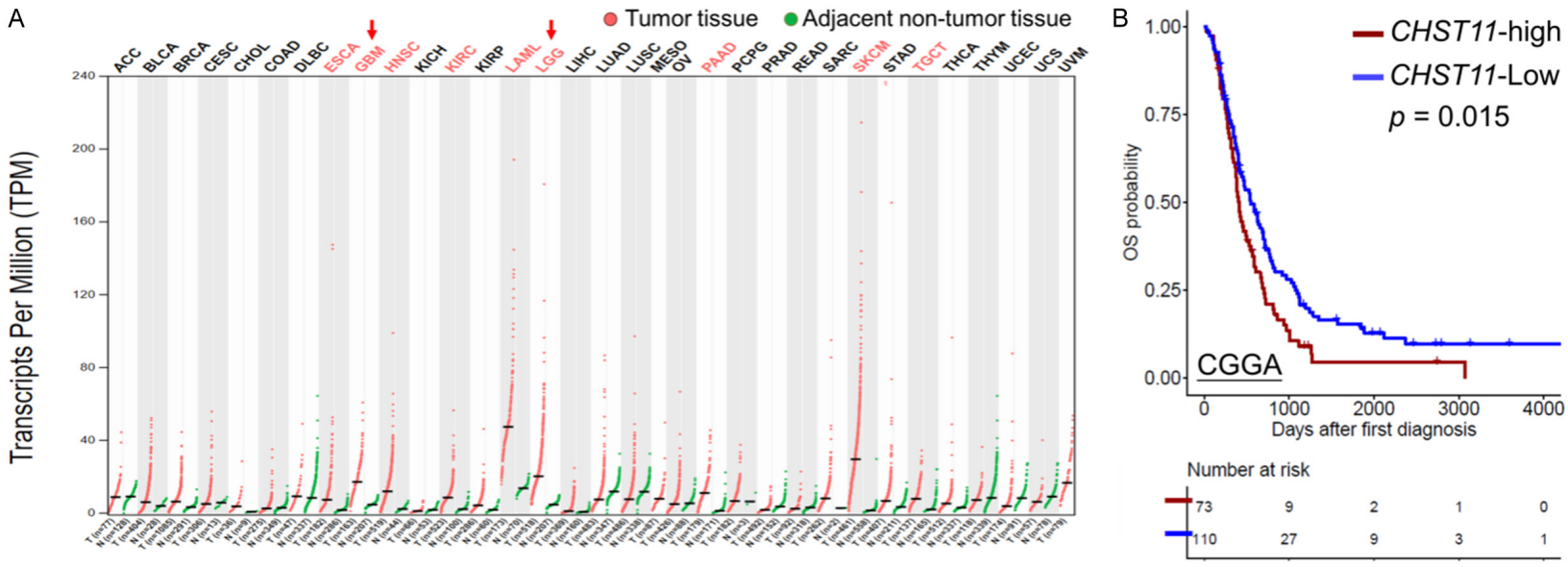

Figure S1. Expression of CHST11 in TCGA and CGGA datasets. A. Transcripts per million count of mRNA in various type of tumor tissue (red dots) and adjacent non-tumor tissue (green dots). Red arrows pointed glioblastoma (GBM) and low grade glioma (LGG). This plot was generated by GEPIA website (http://gepia2.cancer-pku.cn/#index). B. Overall survival (OS) analysis of the Chinese Glioma Genome Atlas (CGGA). 183 GBM patients were included. Kaplan-Meier analysis was used.

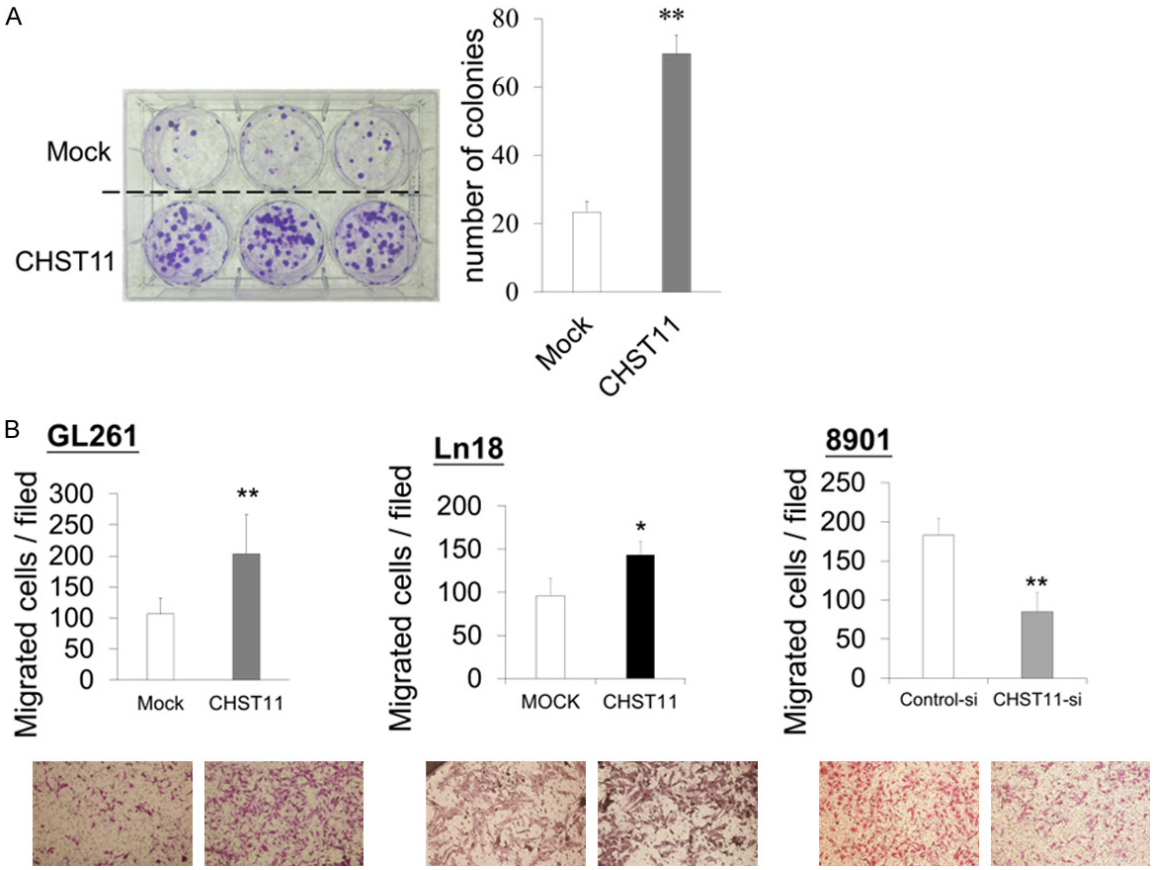

Figure S2. Effects of CHST11 on colony formation and transwell migration. A. Anchoring dependent colony formation assay of empty plasmid transfected cells (Mock) and CHST11 overexpressed GL261 cells. $^{**}P<0.01$. B. Transwell cell migration assay comparing mock and CHST11 overexpressed cells (GL261 cells and Ln18 cells), and CHST11 siRNA silenced GBM8901 cells (8901). $^{*}P<0.05$; $^{**}P<0.01$. Representative images are shown at bottom.

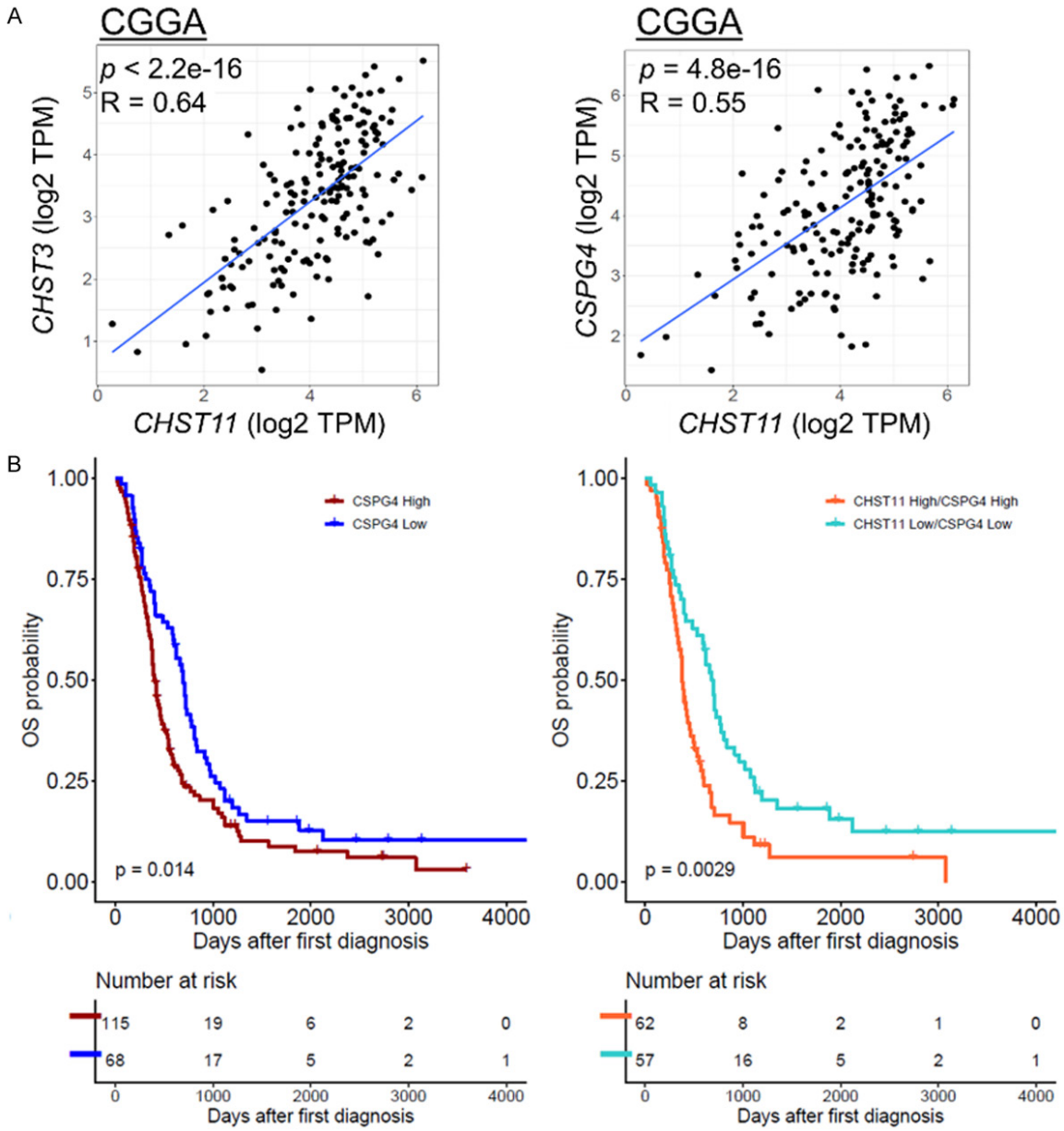

Figure S3. Analyzing the expression of CSPG4 and CHST11 by the CGGA dataset. A. CHST11 is positively associated with CHST3 and CSPG4, which is similar to that in the TCGA dataset. B. GBM patients with both CSPG4 and CHST11 high expression revealed short survival time.

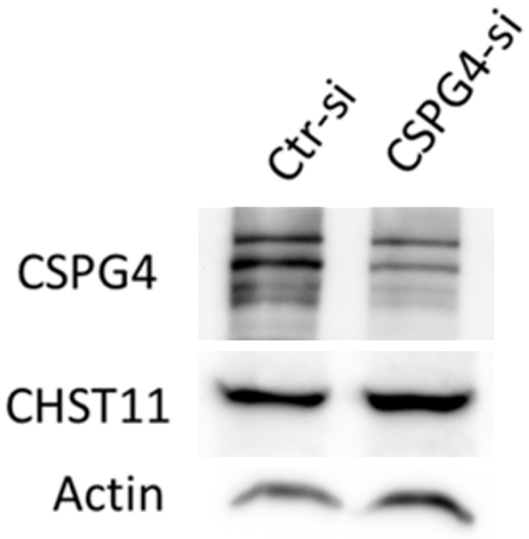

Figure S4. Western blots of control and CHST11 silenced GBM8901 cells. Protein levels of CSPG4 and CHST11 were measured, and actin was used as loading control.

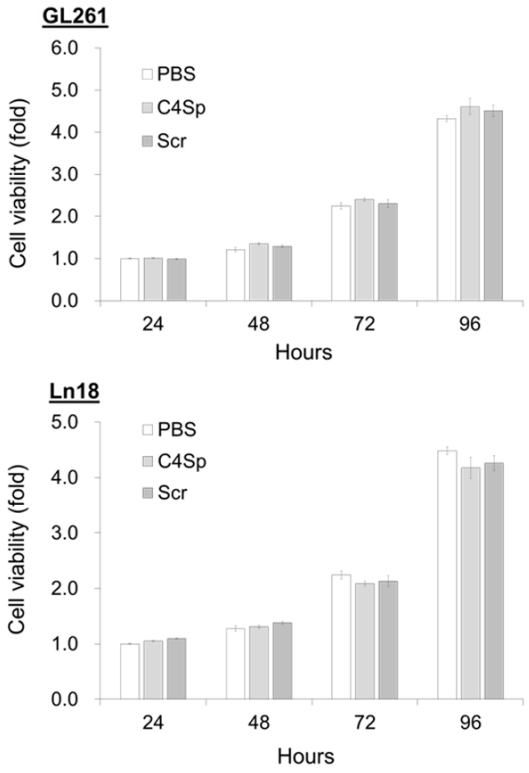

Figure S5. Effects of C4S binding peptide (C4Sp) and scrambled peptide (Scr) on GBM cell viability. CCK8 assay was used every 24 hours until 96 hours. GL261 cells and Ln18 cells were measured using CCK8 assays at indicated time points. Data were represented as means ± SD from three independent experiments.

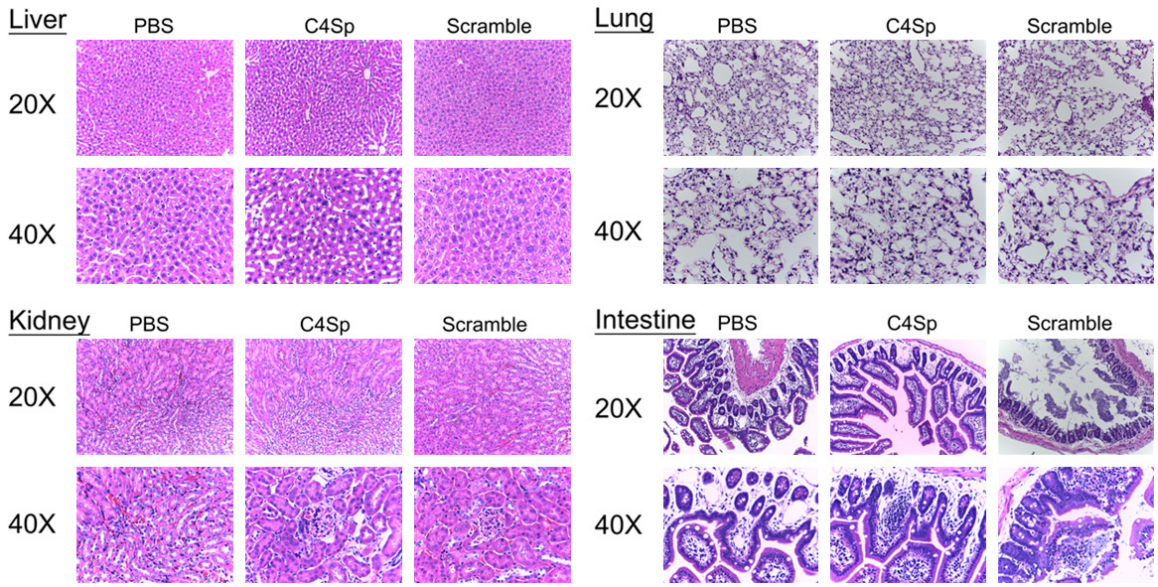

Figure S6. Histology examines tissue morphology of liver, kidney, lung, and small intestine after three weeks of peptide treatment.