Compression force promotes glioblastoma progression through the Piezo1-GDF15-CTLA4 axis

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Abstract. Glioma, a type of brain tumor, is influenced by mechanical forces in its microenvironment that affect cancer progression. However, our understanding of the contribution of compression and its associated mechanisms remains limited. The objective of the present study was to create an environment in which human brain glioma H4 cells experience pressure and thereby investigate the compressive mechanosensors and signaling pathways. Subsequent time-lapse imaging and wound healing assays confirmed that 12 h of compression significantly increased cell migration, thereby linking compression with enhanced cell motility. Compression upregulated the expression of Piezo1, a mechanosensitive ion channel, and growth differentiation factor 15 (GDF15), a TGF-β superfamily member. Knockdown experiments targeting *PIEZO1* or *GDF15* using small interfering RNA resulted in reduced cell motility, with Piezo1 regulating GDF15 expression. Compression also upregulated CTLA4, a critical immune checkpoint protein. The findings of the present study therefore suggest that compression enhances glioma progression by stimulating Piezo1, promoting GDF15 expression and increasing CTLA4 expression. Thus, these findings provide important insights into the influence of mechanical compression on glioma progression and highlight the involvement of the Piezo1-GDF15 signaling pathway. Understanding tumor responses to mechanical forces in the brain microenvironment may guide the development of targeted therapeutic strategies to mitigate tumor progression and improve patient outcomes.

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Introduction

Gliomas, or glial tumors, are the most common type of primary brain tumor and account for ~81% of all malignant brain tumors (1). Although relatively rare, gliomas cause marked morbidity and mortality (2). Glioblastoma (GBM) is the most aggressive and common (45%) tumor of all six glioma types and grades and presents with a median survival time of ~15 months (1). However, no significant progress has been made in glioma treatment over the last decade (3). The absence of effective treatment can be attributed to the numerous strategies used by cancer cells to escape the immune system (4). Notably, the immunosuppressive microenvironment of glioma involves checkpoints such as programmed death-ligand 1 and CTLA-4 that further contribute to the poor prognosis (5,6).

In the context of neuroanatomy, glioma presents particular difficulties as the brain is enclosed within a hard skull. This raises concerns regarding the possible effects of increasing edema on the brain by GBM, including increased intracranial pressure (ICP), compression, tension and other mechanical stresses. Thus, compression of brain tissue by the primary tumor mass is a typical feature and a key cause of the clinical symptoms observed in patients with brain cancer. As the tumor expands inside the skull, it must be pushed away from the surrounding tissue. This tumor growth-induced brain deformation can lead to severe disabilities and is associated with poor prognosis (7). In particular, cranial midline shift is a prevalent feature among patients diagnosed with GBM. Such individuals typically have considerable brain compression, which is associated with rapid development and severe neurological deficits (8). Furthermore, a previous study discovered a negative correlation between midline shift and the survival of patients who survived a biopsy but not in those who were able to undergo resection (8), indicating that resection and the ensuing reduction in compressive forces brought on by tumor growth may enhance treatment (8,9). Despite the significance of mechanical compression in brain tumors and ongoing research on its impact on other tumor types (10-12), to the best of our knowledge, no study has examined its direct effects. Furthermore, the optimal model for mimicking the physical conditions of GBM has not yet been developed (13).

Therefore, the present study aimed to investigate how glioma senses mechanical forces and responds to stress, as well as to identify the molecules involved in this process. To

simulate the GBM microenvironment, such as compressive forces, glass coverslips placed on non-metastatic H4 glioma cells were used. In the present study, it was investigated whether Piezo1, a stretch-activated calcium channel, or growth differentiation factor 15 (GDF15) might be involved in this system as mechanosensors. Additionally, it was explored whether the immune checkpoint protein, CTLA4, could be regulated by signaling induced by altered mechanical forces. Targeting compression-induced tumor progression is particularly important, as identifying the processes that drive signal transduction could reveal new therapeutic targets for treating patients with brain tumors.

Materials and methods

Cell culture and pharmacological reagents. The human neuroglioma H4 cell line was obtained from Professor Sangmyung Rhee (Chung-Ang University, Seoul, Korea), and the A172 cell line was purchased from the American Type Culture Collection (ATCC). U87MG, a GBM cell line of unknown origin cell line, was also purchased from ATCC (ATCC no. HTB-14). The cells were maintained in high-glucose Dulbecco's Modified Eagle's Medium (cat. no. LM001-05; Welgene, Inc.) containing 10% fetal bovine serum (cat. no. US-FBS-500; GW Vitek) and 1% penicillin/streptomycin antibiotics (cat. no. 15140-122; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator with 5% CO_2. The cells were incubated with or without 10 μM BAPTA-AM (cat. no. 2787; Tocris Bioscience), an intracellular calcium chelator, for 30 min prior to pressure application. Mycoplasma testing was performed on all cell lines using an eMyco™ VALiD kit (cat. no. 25239; LiliF Diagnostics; iNtRON Biotechnology).

Compression device. To apply mechanical compression to brain cancer cell monolayers, a commonly used physical contact procedure was followed (14,15). Briefly, 1-2x10^5 H4 cells were cultured for ~24 h in a 6-well plate (35-mm diameter; cat. no. 30006; SPL Life Sciences) at 37˚C in a humidified incubator. A 2% low melting agarose cushion was placed on top of the cells to prevent any direct contact between the glass weights and the cells, and to provide a uniform distribution of the applied force. The initial pressure ranges examined in this study varied from a minimum of 0.15 mmHg to a maximum of 3 mmHg, achieved by regulating the number of glass layers. However, pressures that were too low or too high (assessed based on their effects on cellular viability and motility) were excluded from further analysis. Consequently, this study focused solely on the pressures of 0.75 and 1.5 mmHg. Each pressure was applied on top of the agarose gel to compress the cells for 12 h (16). The cells covered only with an agarose cushion served as the control.

Cell motility analysis. To quantify cell motility, the culture plate was placed under an inverted fluorescence microscope (ECLIPSE Ti2; Nikon Corporation) and motility was observed using phase-contrast imaging. Images were obtained by selecting a specific location on the culture plate and capturing an image every 3 min. The process was carried out for a total of 5 h in an environmental chamber maintained at 37˚C and 5% CO_2. The cells were tracked in time-lapse image sequences using the manual tracking plug-in for Fiji (ImageJ; https://imagej.net/Fiji), which comprises a passive tracking log. Cells in 1 field were analyzed by selecting an independent field with at least 20 non-overlapping cells and translating them into 0.33 μm/pixel images. The travel distance and speed of each cell were determined using the Chemotaxis and Migration Tool Version 2.0 software (ibidi GmbH).

Wound healing. Cells were seeded into a 6-well plate and allowed to reach a confluency of >90%. Then, scratches were made using 1-ml sterile tips, and the cell debris were removed by washing with PBS. After changing to medium containing 5% FBS (17), the cells were then compressed for 12 h as aforementioned, whereas the control samples were only covered with an agarose cushion. Prolonged exposure of cells to pressure for 12 h can induce significant stress, potentially leading to cell death. Therefore, in this assay, the serum concentration was reduced to 5% for the wound healing assay, but the cells were not serum-starved. Images of the wounded area were collected at 0, 12 and 24 h, using a digital camera with bright field (IX-81; Olympus Corporation). The wound area was measured using ImageJ and wound closure was calculated using the following formula: Wound closure (%)=[(width of the wound at 0 h-width of the wound at 24 h)/width of the wound at 0 h] x100.

Analysis of datasets for human samples in The Cancer Genome Atlas (TCGA). Data on differentially expressed genes in GBM [tumor (T), n=163; normal (N), n=207] were collected using the Gene Expression Profiling Interactive Tool (GEPIA; http://gepia.cancer-pku.cn/), an interactive gene expression profiling web server. The RNA-sequencing datasets used by GEPIA are based on the UCSC Xena project (http://xena.uscs.edu), which are computed by a standard pipeline. Box plots were constructed to visualize the expression of GDF15 and Piezo1 in tumors vs. matched normal tissues.

Immunohistochemistry. Paraffin-embedded brain tissue slides from the tumor and normal regions of patients with GBM were provided by the Korean Brain Bank (Korea Brain Research Institute; Table SI). The experimental procedures were approved by the Institutional Review Board of the Chung-Ang University (Seoul, South Korea; approval no. 1041078-202209-HR-199). Briefly, slides were deparaffinized and rehydrated with xylene three times for 4 min and 100, 95, 70 and 50% ethanol for 5 min each. Then, the slides were treated with sodium citrate buffer (pH 6) for 15 min at 125˚C in a pressure cooker (Bio SB, Inc.) and washed in distilled water for 5 min. For permeabilization, the slides were washed three times with 0.1% TritonX-100 in PBS for 10 min, and a circle was drawn around the tissue on the slide using a hydrophobic barrier pen. Subsequently, the sections were blocked with 5% bovine serum albumin (cat. no. A7030; Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, and then incubated with antibodies against GDF15 (1:100; monoclonal antibody; cat. no. sc-377195; Santa Cruz Biotechnology, Inc.) and Piezo1 (1:100; polyclonal antibody; cat. no. 15939-1-AP; Proteintech Group, Inc.) for 1 h at room temperature (25-26˚C). Based on the manufacturer's recommended protocol, immunohistochemistry was

performed using a Vector Laboratories VECTASTAIN Elite ABC University kit (cat. no. PK-7200; Vector Laboratories, Inc.) and DAB staining (cat. no. K3468; Dako; Agilent Technologies, Inc.). The slides were washed with water and cover-slipped with EcoMount (cat. no. BRR897L; Biocare Medical, LLC). The images were captured using an inverted microscope equipped with a camera (Leica DFC320; Leica Microsystems GmbH).

Reverse transcription-quantitative PCR (qPCR). Total RNA was extracted from H4 cells using the RNeasy Mini Kit (cat. no. 74004; Qiagen GmbH) according to the manufacturer's instructions. cDNAs were synthesized from 1-2 μg of total RNA using the Maxima First Stand cDNA synthesis kit (cat. no. K1642; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was performed using Power SYBR Green PCR Master Mix reagent (cat. no. 4367659; Applied Biosystems; Thermo Fisher Scientific, Inc.) and primers specific to the target genes [*PIEZO1*, *GDF15*, matrix metalloproteinase 9 (*MMP9*), *CTLA4* and *GAPDH*] in a StepOnePlus™ Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The sequences of the primers used in the qPCR are listed in Table SII. The 40-cycle PCR consisted of two steps: Denaturation step, 15 sec at 95˚C; and combined annealing and extension step, 30 sec at 55˚C. To calculate the fold change, the quantification cycles (Cq) were determined using the StepOne software version 2.3 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The mRNA expression was normalized to *GAPDH*. The relative gene expression ratios were analyzed using the $2^{-\Delta\Delta Cq}$ method (18). All fold changes are expressed relative to those in the control group.

Western blotting. Whole cells were harvested by washing twice with ice-cold PBS (cat. no. LB001-01; Welgene, Inc.) and then lysed in RIPA lysis buffer (cat. no. 89900; Thermo Fisher Scientific, Inc.) supplemented with a phosphatase and protease inhibitor cocktail (cat. no. P3100-001; cat. no. 3200-001; GenDEPOT, LLC). Total protein content was quantified using a Bradford protein assay kit (cat. no. 5000006; Bio-Rad Laboratories, Inc.) following the manufacturer's instructions. Next, ~40 μg of total protein from each sample was separated by 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were then transferred to a nitrocellulose membrane. The membranes were blocked in 5% (w/v) skimmed milk for 1 h at room temperature and then incubated with anti-Piezo1 (1:1,000; cat. no. 15939-1-AP; polyclonal antibody; Proteintech Group, Inc.), anti-GDF15 (1:1,000; cat. no. sc-377195; monoclonal antibody; Santa Cruz Biotechnology, Inc.) and anti-β-actin (1:1,000; cat. no. sc-47778; monoclonal antibody; Santa Cruz Biotechnology, Inc.) overnight at 4˚C with gentle shaking. Then, horseradish peroxidase-conjugated mouse anti-rabbit (1:5,000; cat. no. sc-2357; Santa Cruz Biotechnology, Inc.) and goat anti-mouse IgG (1:5,000; cat. no. GTX 213111-01; GeneTex, Inc.) secondary antibodies were applied for 1 h at room temperature. The signals were detected with a chemiluminescent reagent using an enhanced chemiluminescence imaging system (ImageQuant™ LAS 4000; Cytiva).

Small interfering (si)RNA transfection. Transient siRNA transfection was performed using DharmaFECT1 (cat. no. T-2001-02; GE Healthcare Dharmacon, Inc.) according to the manufacturer's instructions. In preparation for siRNA transfection, the H4 cells were plated at 70% confluency with $1.5x10^5$ cells per well in a 6-well plate. The following day SMARTpool siRNAs against *GDF15* (cat. no. J-019875-05-0002), *PIEZO1* (cat. no. J-020870-09-0002) and control siRNA (cat. no. D-001210-01-05), all from GE Healthcare Dharmacon, Inc., were transfected at a final concentration of 25 nM at 37˚C for 24 h. Experiments were then performed with or without pressure.

Statistical analysis. All data were analyzed using GraphPad Prism version 10.2.3 software (Dotmatics) and are present as the mean ± standard error of the mean. Differences between two groups were analyzed using unpaired Student's t-test, whereas those among three or more treatment groups were assessed using one-way ANOVA and Tukey's multiple comparison method. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Mechanical compression promotes the migration of human brain glioma cells. Brain glioma cells experience compression stress within the confined space of the skull. In the present study, pressure was applied to mimic the brain tumor microenvironment, as illustrated in Fig. 1A. To determine the optimal pressure for compressing glioma cells without causing cell death, different pressures were applied to the human non-metastatic H4 glioma cell line for 12 h, before releasing the pressure. The control was subjected to no pressure. Pressure equivalent to 0.75, 1.5 and 0 mmHg was applied on top of the cells for 12 h using an agarose cushion. It was found that under 1.5 mmHg pressure, the cell population decreased (Fig. S1). Therefore, the applied pressure was set at 0.75 mmHg. To determine whether compression affects cell motility, the cells were cultured for 12 h under 0.75 or 0 mmHg pressure. Cell motility was then monitored for 5 h using time-lapse imaging (Datas S1 and 2). When the movement path and location of the cells were plotted, the cells to which pressure was applied were found to move over long distances (Fig. 1B). The average migration speed of cells in the control was 0.18±0.005 μm/min and that of cells exposed to pressure was 0.23±0.007 μm/min (Fig. 1C). These results indicated a significant increase in cell motility under pressure. Additionally, wound closure was assessed using a wound healing assay to verify whether pressure affects cell migration. The cell monolayer was wounded, placed under pressure for 12 h and the wound closure was examined at 0, 12 and 24 h (Fig. 1D). At 12 h, the cells under pressure showed a higher closure rate (46.98±0.97%) than the control group (26.07±1.72%), which persisted at 24 h (Fig. 1E). These results suggest that mechanical compression enhances the migration of human brain cancer cells.

Compressive solid stress enhances the expression of the GDF15 and Piezo1 mechanosensors in glioma cells. As shown in Fig. 1, compressive stress increased the migration of glioma cells. We hypothesized that mechanosensors present in the cell

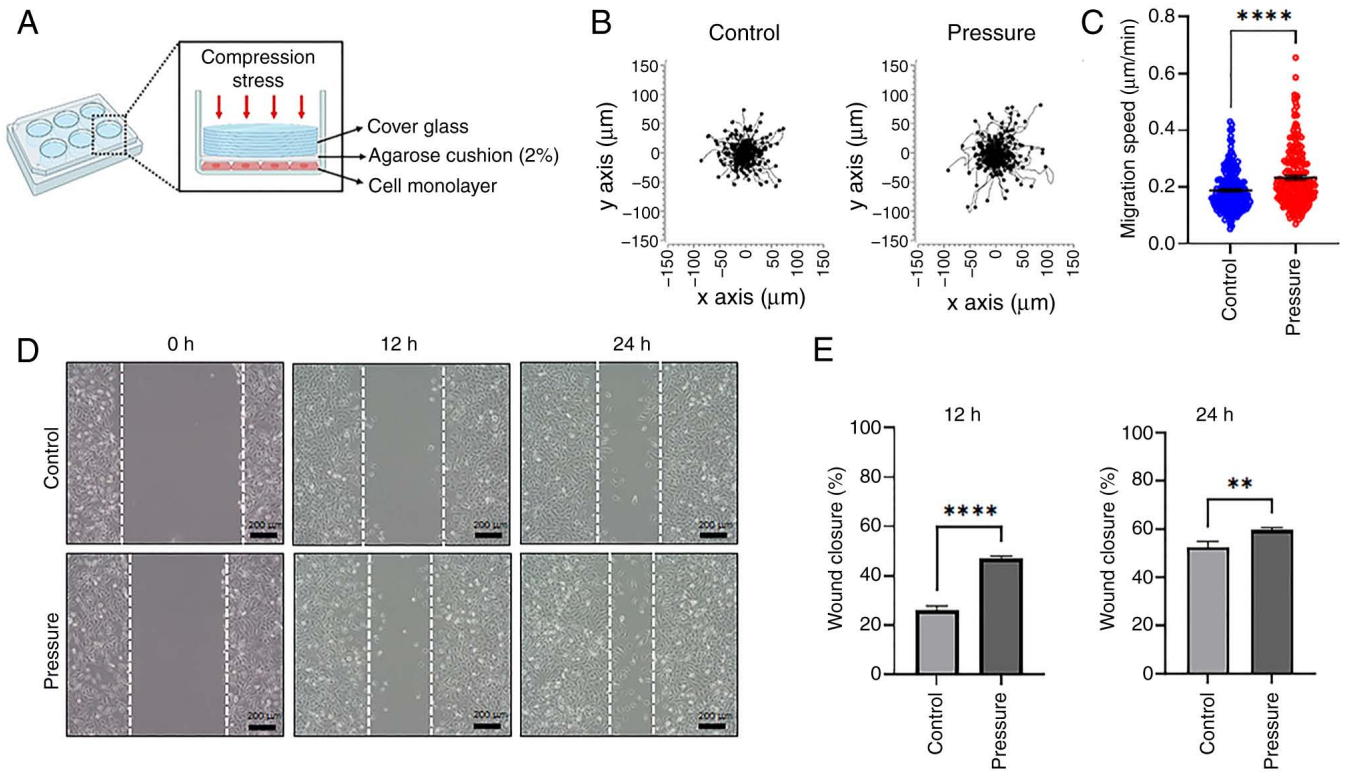

Figure 1. Mechanical compressive stress increases the motility of human brain glioma cells. (A) A schematic of the mechanical pressure device. H4 glioma cells were cultured in a 6-well plate to form a monolayer, and compressive stress was applied with a predefined weight using glass coverslips. (B) A pressure of 0.75 mmHg was applied to the H4 cells for 12 h, followed by a 5-h observation under a time-lapse imaging microscope, where each cell lies at the origin (0,0) at t=0 h. The plots depict the motility of individual cells in 1 representative experiment. (C) Quantification of the migration speed of the individual cells in the control and pressure groups (n=3 independent experiments). (D) The effect of compression/compressive force on H4 cell migration was confirmed using a wound healing assay. Representative images of the wound healing assay for the control and pressure groups after 0, 12 and 24 h of pressure application. (E) Graph showing the percentage wound closure as quantified using ImageJ software (n=3). **P<0.01, ****P<0.0001, determined using unpaired t-test.

membrane that sense mechanical force could initiate signal transduction and ultimately affect motility. Therefore, these mechanosensors were identified according to our previous study (19). Notably, TCGA profiling through GEPIA confirmed that the expression of *GDF15* and *PIEZO1* was significantly higher in GBM tissues than in normal tissues (Fig. 2A). Additionally, the expression of GDF15 and Piezo1 was examined in human GBM tissues using immunohistochemistry. The expression of these proteins was markedly enhanced in GBM tissues compared with the adjacent non-tumor tissues (Fig. 2B and C). The changes in gene expression in H4 cells with or without compressive stress for 12 h were also examined. The gene expression levels of *GDF15* and *PIEZO1* significantly increased 2-fold and 1.5-fold, respectively, under compressive solid stress. Pressure-induced *GDF15* and *PIEZO1* expression was also observed in the A172 and U87MG glioma cell lines (Fig. S2). Furthermore, the expression of *MMP9*, which plays an important role in cancer progression, including extracellular matrix (ECM) remodeling and metastasis, also increased by ~2-fold (Fig. 2D) in H4 cells. Piezo1 and GDF15 protein expression increased under pressure at 12 h and was further enhanced after exposure for 24 h in a time-dependent manner (Fig. 2E). Notably, enhanced expression of *GDF15* and *PIEZO1* was gradually reduced in H4 cells and returned to basal level 24 h after the pressure was removed (Fig. S3). In summary, consistent with observations in the samples from patients with GBM (Fig. 2B and C), mechanical compression stress increased the expression of the mechanical sensors GDF15 and Piezo1 in H4 cells.

Piezo1-mediated mechanotransduction promotes the motility of glioma cells through GDF15 regulation. To determine whether the changes in glioma cells caused by mechanical compression were initiated by GDF15 and Piezo1, their expression was suppressed using respective siRNAs (Figs. 3E and S4). When pressure was applied to cells transfected with control siRNA, cell motility significantly increased, as observed in the previous experiment. However, when pressure was applied to cells in which *GDF15* expression was suppressed using siRNA, there was no increase in motility (Fig. 3A and B). Furthermore, there was no increase in motility when pressure was applied after the knockdown of *PIEZO1* using siRNA (Fig. 3C and D). Gene expression was confirmed with or without pressure applied to *PIEZO1*-knockdown cells. *PIEZO1* expression increased under pressure, but in siPiezo1, the expression of *PIEZO1* did not increase whether pressure was applied or not (Fig. 3E). Similarly, *GDF15* expression increased with pressure, but was not enhanced regardless of the presence or absence of pressure when Piezo1 was ablated (Fig. 3F). Furthermore, the intracellular calcium chelator, BAPTA-AM, was applied to H4 cells during pressure exposure, as Piezo1 is a stretch-activated calcium channel (20). Consequently, when intracellular calcium levels were suppressed, *GDF15* expression was not significantly changed, even in the presence of

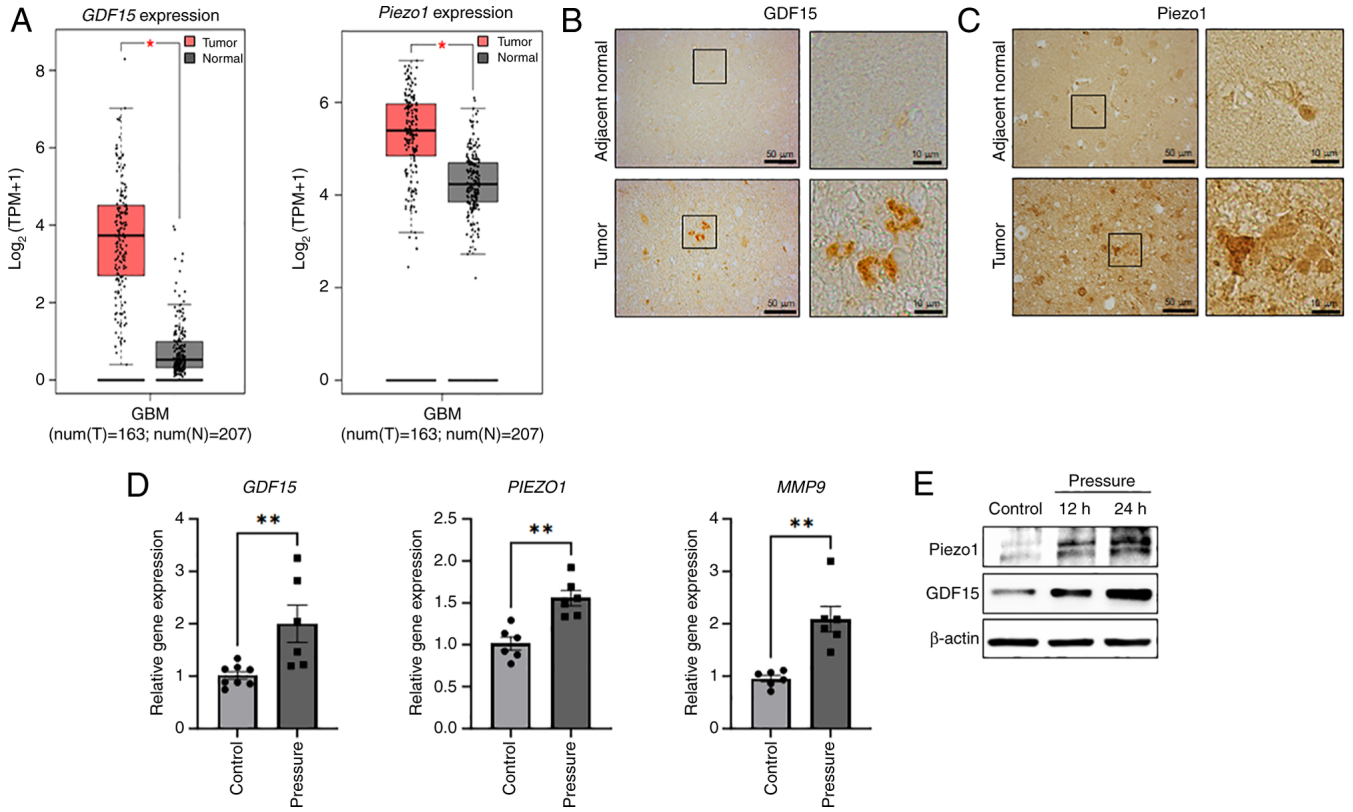

Figure 2. Compressive solid stress increases the expression of the GDF15 and Piezo1 mechanosensors. (A) Comparisons of *GDF15* and *PIEZO1* gene expression between human GBM and normal brain tissues using data from The Cancer Genome Atlas and GTEx, analyzed with GEPIA. The red box plots represent GBM tissues (n=163) and the gray box plots represent non-GBM tissue (n=207). The figure was obtained from GEPIA. The expression levels of (B) GDF15 and (C) Piezo1 in human GBM tissue were compared with normal brain tissue by immunohistochemistry. The tissues were obtained from the Korea Brain Bank Network. Scale bars represent 50 μm and 10 μm. (D) H4 cells were subjected to compression for 12 h, and the gene expression levels of *GDF15*, *PIEZO1* and *MMP9* were evaluated using reverse transcription-quantitative PCR (n=3 independent experiments). (E) Piezo1 and GDF15 protein expression in H4 cells exposed to pressure for 12 and 24 h measured via immunoblotting analysis. *P<0.05, **P<0.01. GBM, glioblastoma multiforme; GDF15, growth differentiation factor 15; MMP9, matrix metalloproteinase 9; N, normal tissue; T, tumor tissue; TPM, transcripts per million.

pressure (Fig. 3G and H). These results suggest that Piezo1 acts as a mechanosensor for compressive forces and regulates *GDF15* expression as an upstream regulator. Overall, it can be hypothesized that mechanical pressure in the GBM microenvironment promotes the progression of glioma cells through the Piezo1-GDF15 axis.

Mechanical compression induces CTLA4 expression in glioma cells via Piezo1 and GDF15. CTLA4, an immune checkpoint protein, contributes to immune evasion via antitumor activity. High CTLA4 expression has also been reported to be correlated with the poor prognosis of patients with GBM (21). To determine whether *CTLA4* expression was increased in the brain tumor microenvironment system, the gene expression of *CTLA4* was examined. When a pressure of 0.75 mmHg was applied to the cells for 12 h, the expression of *CTLA4* increased 1.5-fold (Fig. 4A). To confirm its relationship with the mechanical sensors, the gene expression of *CTLA4* was measured when *GDF15* expression was knocked down by siRNA. Application of pressure to cells transfected with siGDF15 failed to increase *CTLA4* expression (Fig. 4B). Additionally, *PIEZO1*-knockdown suppressed *CTLA4* expression under solid compressive stress (Fig. 4C). These results confirm that ICP in brain glioma cells increases the expression of *CTLA4*, and mechanosensors such as Piezo1 and GDF15 affect the regulation of *CTLA4* expression.

Discussion

In the tumor microenvironment, mechanical forces and matrix stiffness are two distinct biomechanical anomalies (22,23). However, while extensive research has explored the effect of matrix stiffness on tumor progression, the impact of mechanical stress on cancer cell behavior, particularly brain cancer cell migration and progression, remains largely unexplored. Thus, to mimic the brain microenvironment in the present study, compression forces were applied to non-metastatic glioma H4 cells. To investigate the effect of compressive stress on glioma, an agarose cushion was used to apply pressure to the glioma cells. In the present study, agarose cushions were used for both the control and pressure groups. A previous report demonstrated that chondrocytes embedded in agarose gel maintained a survival rate >95% after 24 h of incubation, which was maintained up to 72 h (24), suggesting that applying pressure via agarose gel does not significantly affect cell viability. The results of the present study demonstrated that compressive solid stress increased the motility of glioma cells and the protein expression of Piezo1 and GDF15 in H4 cells. When *PIEZO1* expression was reduced using siRNA, *GDF15* expression was also suppressed, and an increase in cellular motility under a compression force was no longer observed. This suggested that Piezo1 regulates GDF15 expression,

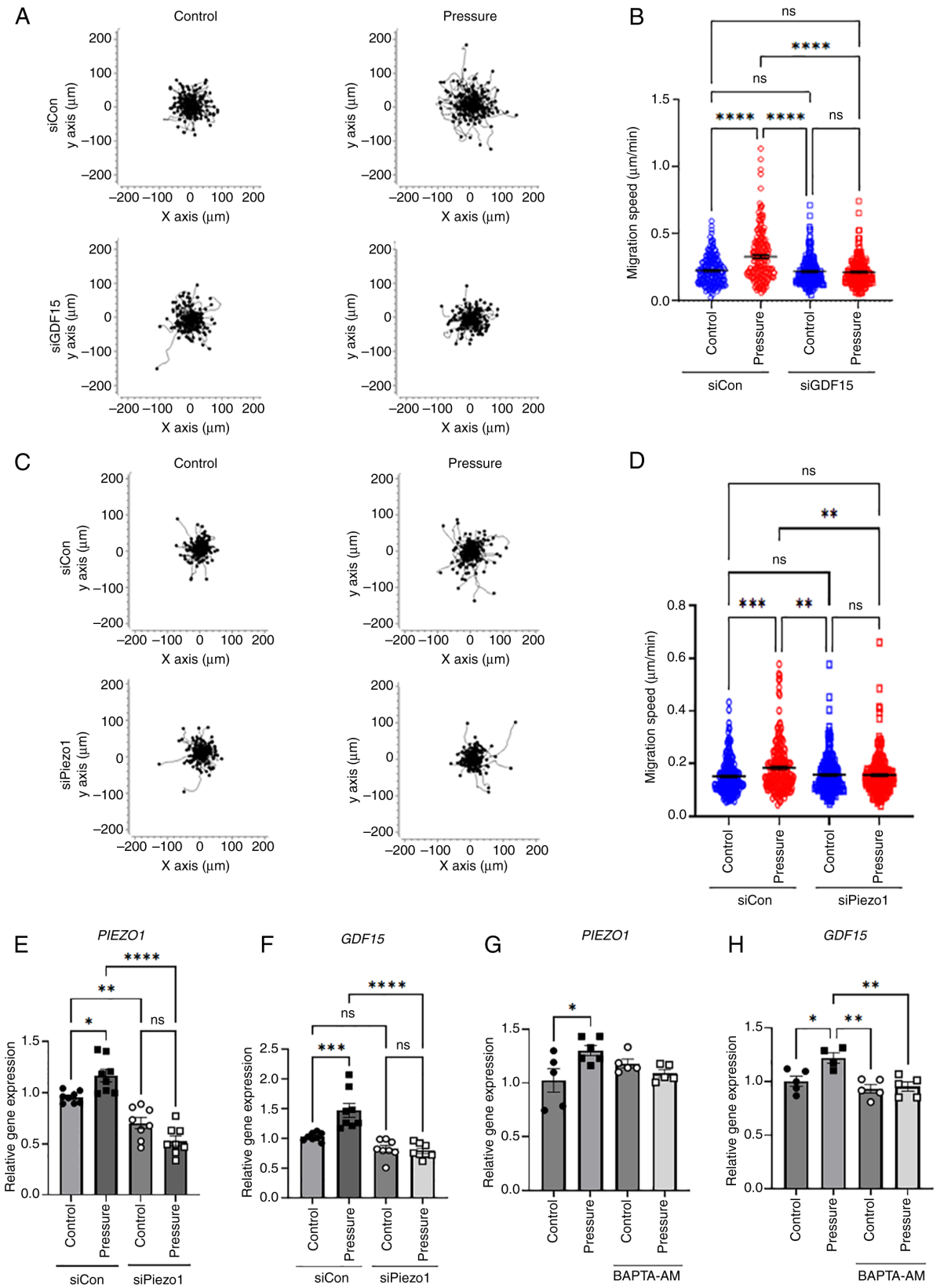

Figure 3. Knockdown of *GDF15* and *PIEZO1* using siRNA significantly reduces cell motility under pressure. (A) Motility plots of siCon or siGDF15 transfected cells subjected to pressure for 12 h and then observed for 5 h under a time-lapse imaging microscope. Plots depict the motility of individual cells in 1 representative experiment. (B) Quantification of the migration speed of individual cells (n=200). (C) Motility plots of siCon or siPiezo1 transfected cells subjected to pressure for 12 h and then observed for 5 h under a time-lapse imaging microscope. Plots depict the motility of individual cells in 1 representative experiment. (D) Quantification of the migration speed of individual cells (n=200). Cells with or without *PIEZO1* knockdown were subjected to pressure for 12 h, and the gene expression levels of (E) *PIEZO1* and (F) *GDF15* were evaluated using RT-qPCR (n=3 independent experiments for each gene). Cells were subject to pressure with or without the addition of 10 μM BAPTA-AM for 12 h, and the gene expression levels of (G) *PIEZO1* and (H) *GDF15* were assessed using RT-qPCR (n=3 independent experiments for each gene). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, determined using one-way ANOVA followed by Tukey's test. Con, control; GDF15, growth differentiation factor 15; ns, not significant; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA.

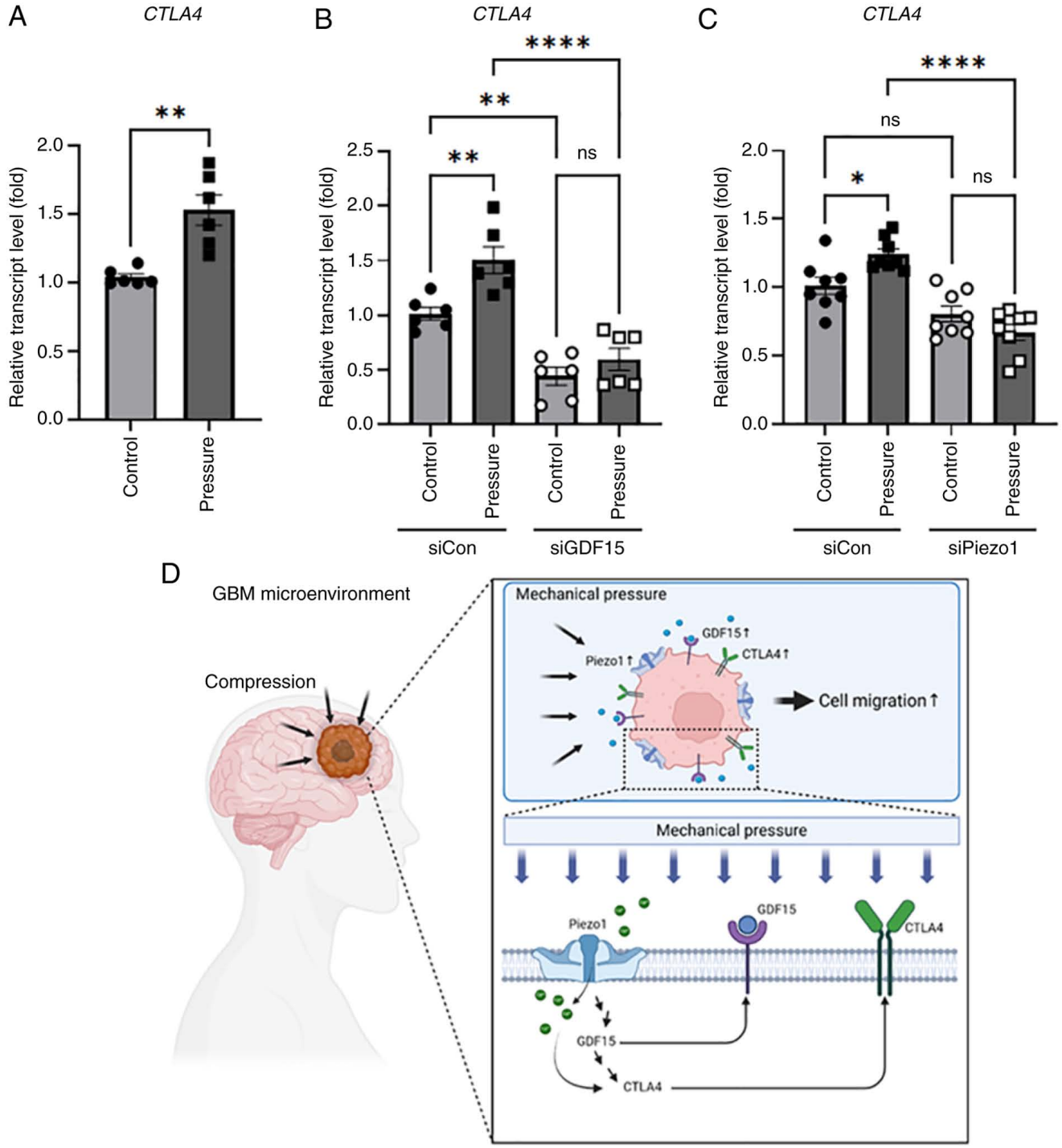

Figure 4. Compressive stimuli regulate the expression of CTLA4 in neuroglioma cells. (A) H4 cells were subjected to 12 h of compressive stimuli and the expression of *CTLA4* was analyzed using RT-qPCR. n=6 independent experiments; **P<0.01, determined by unpaired t-test. Cells transfected with (B) siCon or siGDF15 and (C) siCon or siPiezo1 were subjected to compressive stimulation for 12 h, and expression of *CTLA4* was analyzed using RT-qPCR. n=3 independent experiments for each transfection procedure; *P<0.05, **P<0.01, ****P<0.0001 determined by one-way ANOVA followed by Tukey's test. (D) A proposed mechanism that promotes glioma progression through a mechanosensor that detects mechanical pressure in the brain tumor microenvironment. As GBM grows in a limited space, pressure builds between cells and surrounding tissues. The expression of Piezo1, a mechanosensor present in the cell membrane, increases, followed by the expression of GDF15. Subsequently, the immune checkpoint protein, CTLA4, is upregulated, enhancing the poor prognosis of glioma. Con, control; GBM, glioblastoma; GDF15, growth differentiation factor 15; ns, not significant; RT-qPCR, reverse transcription-quantitative PCR; siRNA, small interfering RNA.

followed by cellular motility, in response to mechanical stimuli. Furthermore, the use of BAPTA-AM, a calcium chelator, inhibited the expression of *GDF15*, indicating that Piezo1 channels, activated by pressure, increase intracellular calcium levels, which in turn regulates GDF15 expression. Notably, compression highly enhanced *CTLA4* expression in H4 cells, which may be regulated by Piezo1-GDF15 signaling.

Regarding the enhanced motility of glioma H4 cells observed in the present study, Kalli *et al* (25) reported that, through actin remodeling by ras homolog family member B

GTPase and Rac family small GTPase 1, compressive forces (2 or 4 mmHg) promote cellular motility in non-metastatic H4 glioma cells, but not in metastatic A172 glioma cells. As 1.5 mmHg of pressure decreased the density of H4 cells after 12 h of incubation in the present study, its effect on glioma was not test any further. The lower pressure of 0.75 mmHg used in the present study was considered sufficient to increase cellular motility and glioma progression. Notably, different levels of compressive stress have been applied to gliomas in various studies, with reported values of 2.8-6.1 (10), 4-28 (26), 28-120 (27) and 3.7-16.0 (28) mmHg. Depending on the measurement method, the equivalent compressive solid stress is 0.02 kPa (0.15 mmHg) in *ex vivo* measurements but reaches 0.1 kPa (0.75 mmHg) in *in situ* measurements. Furthermore, the precise magnitude of pressure or solid stress exerted within the brain by a tumor remains unknown and is challenging to study, as it is highly dependent on the location and size of the tumor as well as the stage of progression, which is a limitation of the present study. However, what is important is that pressure or solid stress is a key factor in the progression of brain cancer. This suggests that the effect of compressive force on the tumor is more significant than the absolute value of the pressure itself. In the present study, it was demonstrated that the initial compressive force could induce cell migration through the Piezo1-GDF15-CTLA4 pathway during glioma progression.

In the present study, Piezo1 expression was increased by pressure in glioma cells, suggesting that Piezo1 acts as a mechanosensor. Piezo1 is a member of a non-selective cationic mechanosensitive channel family expressed in mammalian cells (29). *In vivo*, Piezo channels respond to a number of various forces including laminar flow and cellular compression (30-32) and are highly expressed in the bladder where they respond to mechanical stretching (33). The ablation of the *PIEZO1* gene is lethal during early embryonic development (32), and vascular abnormalities occur due to the targeted deletion of the *PIEZO1* gene in the endothelium shortly after the onset of cardiac activity (20). In our previous study, Piezo1 was found to sense interstitial fluid-like flow and initiate signal transduction of the Src-Yes associated transcriptional regulator axis to promote cancer progression and metastasis in the tumor microenvironment of prostate cancer (19). Furthermore, the expression of Piezo1 in GBM has been reported by a number of other groups. Notably, Piezo1 has been reported to be sensitively activated by the stiffness of the brain matrix in GBM, and further ECM remodeling is followed by integrin-FAK signaling, which accelerates GBM progression (34). Overall, these results suggest that mechanical pressure, which is a unique physical stimulus in GBM, activates the Piezo1 channel in the membrane of glioma cells and initiates a signaling cascade in GBM through calcium influx, followed by GBM progression.

In the present study, it was also demonstrated that *GDF15* expression was regulated by Piezo1 activation. GDF15 is a tumor suppressor in the early stages of tumor formation (35,36), but subsequently promotes the growth of high-grade tumors (37,38). In particular, GDF15 controls immune evasion and cell proliferation in glioma (39). GDF15 has also been linked to poor prognosis and is thought to be an oncogenic factor in glioma (40). Notably, the results of the present study showed that *GDF15* affected the expression of *CTLA4*, and that compressive force-induced *CTLA4* expression was significantly reduced by knocking down *GDF15* expression using siRNA. Although CTLA4 has been almost exclusively studied in terms of the T cell lineage, certain studies have shown that its expression is not limited to T cells and is found in other cells, including solid tumors (41,42). In the present study, mechanical stress directly increased the expression of *CTLA4* in H4 cells, which was reduced by *PIEZO1* or *GDF15* knockdown. Studies exploring the transcriptional regulation of CTLA4 are limited; however, a report has indicated that transcription factors such as NFAT, STAT1, Fox and Myc may promote CTLA4 transcription (43). The involvement of those transcription factors in the system were not investigated in the present study. Further research is necessary to fully understand the molecular mechanisms underlying CTLA4 regulation, as epigenetic regulation, direct regulation through microRNAs and transcription factors are additional mechanisms that govern this protein. Furthermore, intracellular calcium levels may regulate *CTLA4* expression. Linsley *et al* (44) reported that increasing intracellular calcium levels rapidly increased the cell surface expression of CTLA4 in T cells, indicating that CTLA4 expression may be increased by intracellular calcium levels through Piezo1 activation.

Overall, the findings of the present study indicate that mechanical pressure in the glioma microenvironment enhances glioma aggression through the Piezo1-GDF15-CTLA4 axis (Fig. 4D). Piezo1 likely senses the compressive pressure from outside the glioma and initiates signal transduction, such as GDF15 activation, which in turn enhances glioma motility. Although the role of CTLA4 expression in glioma remains to be elucidated, increased CTLA4 expression in H4 cells might drive immune evasion or poor prognosis in glioma, similar to that in other diseases. To further investigate the detailed role of CTLA4 in gliomas, we plan to further verify these findings in animal models to explore potential therapeutic drugs. Thus, the present study demonstrates the molecular mechanisms by which physical stimuli originating in the GBM microenvironment increase the aggressiveness of cancer cells and will help to develop strategies to target these molecules for the treatment of GBM in the future.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

OHK, IJT and HJL conceived and designed the experiments. OHK, IJT, HK and ESC performed the experiments. OHK and IJT analyzed the data. OHK and HJL wrote the manuscript. HJL revised the manuscript. OHK and HJL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The experiments using human brain tissue were performed according to the guidelines of the Institutional Review Board (approval no. 1041078-202209-HR-199) of Chung-Ang University (Seoul, Korea).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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