

Original Article

Selection of a highly invasive cell population from a glioblastoma cell line and analysis of invasion-associated factors

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Abstract: Glioblastoma (GBM) is characterized by aggressive local invasiveness, making complete surgical resection of the cancer nearly impossible. To identify mechanisms of GBM invasion, we isolated a subpopulation of highly invasive cells and evaluated the expression of invasion-related factors, including matrix metalloproteinase-2 (MMP-2), fascin, integrin subunits, Akt, and Erk. We selected the first 10% of invading cells (U87-Inv) from the U87MG GBM cell line using laminin-2 (merosin)-coated Transwell filters. To characterize the highly invasive cells, we chose a wound-healing assay; gelatin zymography; a proliferation assay; real-time quantitative polymerase chain reaction (RT-qPCR) for integrin subunits; western blot for fascin, Akt, and Erk; and immunofluorescence for fascin and actin. The migration rate of U87-Inv cells increased approximately 20% compared with that of the relatively less invasive cells (U87-Non). U87-Inv cells demonstrated faster wound healing, but lower proliferative activity. U87-Inv cells also showed extensive lamellipodia with the expression of fascin and actin, an increase in the activity of MMP-2, but a decrease in the expression of Erk. The expression of integrins $\alpha 1$ and $\alpha 7$ in U87-Inv cells increased approximately 1.5-fold, whereas that of integrins $\alpha 6$ and $\beta 4$ was reduced by approximately 0.4- and 0.6-fold, respectively. Using a merosin substrate, we successfully isolated a subpopulation of highly invasive GBM cells. Activation of integrins $\alpha 1$ and $\alpha 7$ was correlated with the invasiveness of GBM cells. The highly invasive GBM cell line likely showed a decrease in proliferation because of Erk activation via integrins $\alpha 6$ and $\beta 4$.

Keywords: Glioblastoma, invasion, proliferation, merosin, integrin, U87MG cells

Introduction

Glioblastoma (GBM) is the most malignant form of glioma. Most patients demonstrate poor survival rates because of the aggressive and invasive character of GBM [1]. This aggressive invasiveness may be key to understanding the failure of treating GBM. A single GBM cell can detach from a primary tumor and disseminate to myelinated fiber tracts of the white matter, which contains extracellular matrix (ECM) proteins. The ECM proteins of the brain include mainly laminin, type IV collagen, tenascin, fibronectin, and hyaluronic acid [2, 3]. Laminins are a large family of multidomain $\alpha\beta\gamma$ heterotrimeric glycoproteins found in basement membranes [4]. Laminin-2 (merosin), which is composed of the $\alpha 2$, $\beta 1$, and $\gamma 1$ heterotrimeric chain, is first

found in the basement membrane of the placenta, striated muscle, and Schwann cells [5, 6]. The $\alpha 2$ subunit of merosin is expressed in skeletal muscle and other tissues, including the peripheral (PNS) and central nervous system (CNS) [7]. In the peripheral nervous system, a mutation in the *LAMA2* gene, which causes a deficiency in the merosin $\alpha 2$ chain, can cause congenital muscular dystrophy (CMD) [8]. Furthermore, merosin plays an important role in myelinogenesis and promotes axonal growth in the CNS [9, 10]. Nevertheless, an association between merosin and tumors of the CNS has not been established.

Similar to other cancers, GBM is heterogeneous at its cellular and molecular levels [11, 12]. The heterogeneity of GBM includes markedly differ-

ent karyotypes in fresh tissue specimens and cell lines [13, 14], highlighting the necessity to isolate the highly invasive GBM cells in order to study their invasive capacity. A previous study, using an invasive subpopulation of U87MG human GBM cells, evaluated their ability to invade through a collagen substrate and found that the upregulation of matrix metalloproteinase-2 (MMP-2) increases the invasive capacity of GBM cells [15]. Therefore, in our study, we selected the highly invasive GBM cells on the basis of their adhesion to a merosin substrate.

Merosin performs numerous biological functions in cell adhesion, migration, proliferation, and differentiation [16]. Merosin mediates these functions through cell-surface receptors, particularly the integrin family of receptors [17, 18]. Integrins are non-covalently linked heterodimeric transmembrane receptors for ECM molecules. Integrins are classified into several subfamilies on the basis of their ligand specificities. For example, integrins $\alpha 3$, $\alpha 6$, $\alpha 7$, and $\beta 4$ mainly bind to laminin, whereas integrins $\alpha 5$, $\beta 3$, $\beta 5$, $\beta 6$, and $\beta 8$ mainly bind to the tripeptide Arg-Gly-Asp (RGD) receptor [19]. Previous studies show that invasion in various cancers may be influenced by the integrin-mediated tumor cell-ECM interactions [20, 21]. RGD-binding integrins $\alpha 5\beta 3$ and $\alpha 5\beta 5$ are over-expressed in glioma cells and tumor-associated vasculature [22]. Cilengitide, a potent inhibitor of integrins $\alpha 5\beta 3$ and $\alpha 5\beta 5$, has been evaluated in clinical studies [23, 24]. The laminin-binding integrins $\alpha 3$ and $\alpha 6$, have been shown to regulate the stem-like glioma cells [25, 26], and integrin $\beta 4$ regulates GBM proliferation [27]. However, the role of laminin-binding integrins in GBM remains to be determined.

The integrin-mediated adhesion of tumor cells influences diverse intracellular signaling pathways, including those mediated by phosphoinositide-3-kinase (PI3K)/Akt and mitogen-activated protein kinases (MAPK)/Erk [28, 29]. Previous reports show that Erk can control proliferation and invasion in GBM [30, 31], whereas Akt activation is correlated with invasiveness and stemness [32]. However, a deeper understanding of these downstream signaling effectors in GBM is needed.

To migrate, malignant cells need to remodel their cytoskeleton to form the cellular protrusions known as filopodia. Motility requires com-

plex rearrangement of multiple actin-binding proteins. One of these proteins, fascin, induces the bundling of actin for cell protrusion, adhesion, and migration [33, 34]. Fascin is upregulated in various human cancers [35-37]. The GBM cell line, with *FSCN1*-knockdown, shows attenuated invasiveness in a rat model [36]. Therefore, we investigated whether overexpression of fascin is an indicator of high invasiveness.

In this study, we isolated highly invasive cells from the U87MG cell line via adhesion to a merosin substrate and analyzed various biological features such as morphology, motility, rate of proliferation, and the expression of molecules associated with invasion, including MMP-2, fascin, integrins, Erk, and Akt.

Materials and methods

Cell line and culture

The human GBM U87MG cells were grown in minimal essential medium (MEM, Welgene, Daegu, South Korea) supplemented with 10% fetal bovine serum (FBS) and antibiotics in a humidified 5% CO₂ atmosphere at 37°C.

Cell-substrate adhesion assay

To investigate adhesion on merosin, 96-well plates were coated with various concentrations of human merosin (laminin-2; Millipore, Temecula, CA, USA) in serum-free MEM for 1 h at 37°C (**Figure 1**). Wells were washed three times with phosphate-buffered saline (PBS) and blocked with 0.5% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) solution for 30 min at 37°C. Cells were seeded at a density of 5×10^4 /well in the merosin-coated plates, which were then placed on ice for 30 min to allow for the rapid sedimentation of the cells. After incubating for 2 h at 37°C, the plates were subjected to vigorous agitation (300 rpm for 5 min on a horizontal rotor), then the medium and the non-adherent cells were aspirated and washed with PBS. The attached cells were fixed with 3.7% paraformaldehyde/PBS (v/v) for 15 min and stained with crystal violet for 15 min. After the dye solution was aspirated, cells were washed with PBS, air-dried, and eluted with 10% acetic acid on a shaker for 20 min. Absorption was measured at 540 nm using an ELISA plate reader (iMark; Bio-rad, Hercules, CA, USA).

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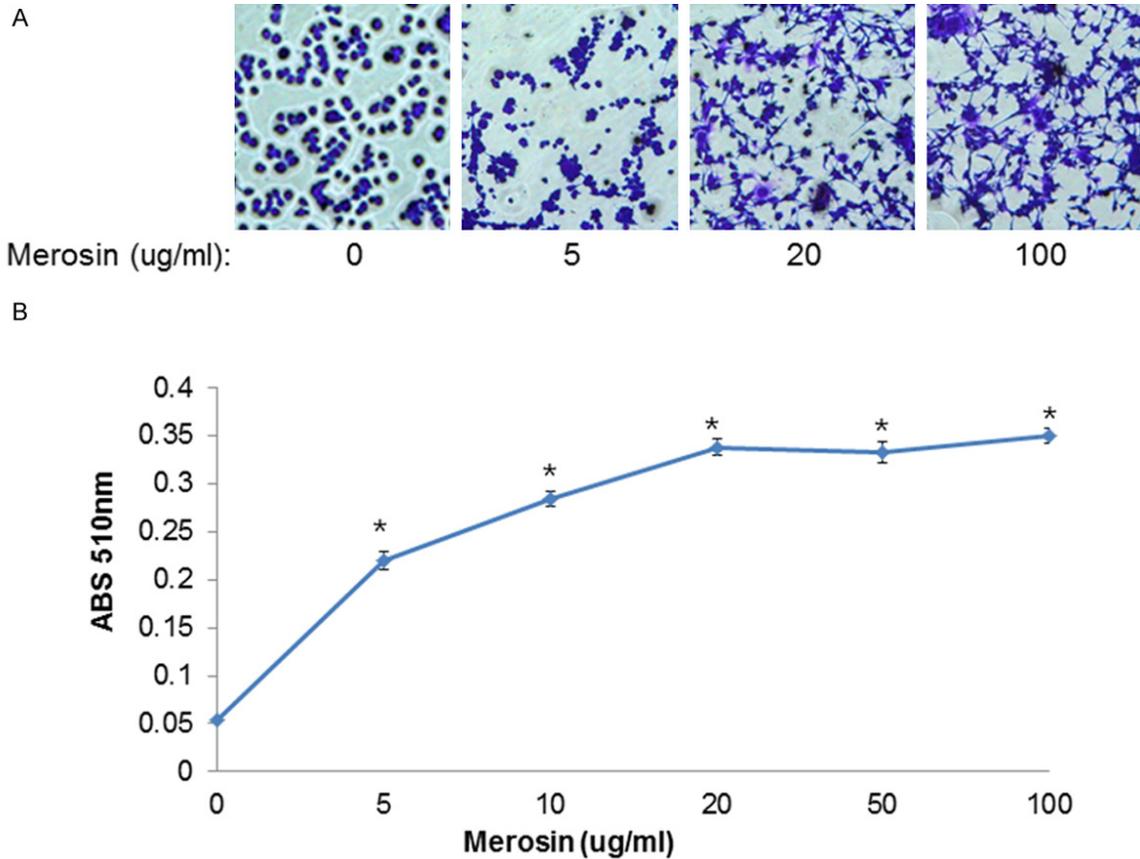


Figure 1. Cell-merosin adhesion. A. Morphology of U87MG cells in merosin-coated wells coated with various concentrations of merosin. Morphology of U87MG cells is changed, showing longer neurite-like extensions in a dose-dependent manner. B. Each point represents the average optical absorbance, at 510 nm, in four independent experiments. Results are shown as mean \pm SEM (n = 3). Statistical analysis was performed by the Mann-Whitney U test; asterisks indicate a statistically significant change ($P < 0.05$) in adhesion. The maximal effect of adhesion was achieved at a merosin concentration ≥ 20 $\mu\text{g/mL}$.

Selection of highly invasive U87MG cells (U87-Inv)

U87-Inv cells were selected using a previously described method with modifications (**Figure 2**) [15]. Transwell polycarbonate membrane inserts, with 8.0- μm pores (Corning Inc., NY, USA), were coated on the underside with 100 $\mu\text{g/mL}$ of merosin. Cell suspensions were seeded onto the filters and allowed to migrate for approximately 3 h in a CO_2 incubator in the absence of serum. Cells that had migrated to the bottom of the inserts were detached and labeled with a cell dissociation solution (Trevigen, Gaithersburg, MD, USA) and the fluorescent dye calcein acetoxymethylester (Calcein AM, BD, Bedford, MA, USA) for 1 h in a CO_2 incubator. Calcein AM is internalized by the cells, and hydrolyzed by intracellular esterases, into the fluorescent anion calcein. The fluorescence

of free calcein was used to quantify the number of cells, which had invaded out of the total seeded cells. After removing the inserts, the detached cells were transferred to a black plate, which was read at the excitation of 494 nm/emission 517 nm. Data are presented as a percentage of invading cells. The invading cells were visualized and photographed under a fluorescent microscope. To select for U87-Inv cells, the first 10% of the invading cells from U87MG were collected using the merosin-coated Transwell under serum-free conditions. The incubation time, required for approximately 10% of the cells to invade, was circa 3 h; the invading cells were harvested by brief, gentle trypsinization and cultured in new dishes. The selected cells were subsequently expanded and designated as U87-Inv. U87-Non (non-invading cells) on the upper surface of the filters were transferred to another dish and cultured separately.

Zymography

The production of MMP-2 was detected by gelatin zymography. Serum-free conditioned media were electrophoresed for 48 h on 8% SDS-PAGE, containing 0.1% gelatin, under non-reducing conditions. Then, the gels were washed with 2.5% Triton X-100 for 30 min at room temperature (21°C), and incubated with activation buffer (50 mM Tris, 0.2 M NaCl, 5 mM CaCl₂, pH 7.6) overnight at 37°C. The gels were then stained with Coomassie Brilliant Blue R-250 and destained with a 30% methanol/10% acetic acid solution. Gelatinolytic activity was visualized as a transparent band against a blue background.

Wound-healing assay

The ability to migrate was assessed by a wound-healing assay. After confluent monolayers were prepared, a yellow p200 pipet tip was used to make a straight scratch across each monolayer. The wounded areas were imaged immediately after the induction of the wound (0 h) and captured at 8 and 24 h post wound induction. At the indicated time points, the images of 10 different areas were acquired, then the gap distance was measured and compared with that of the initial scratch.

Cell proliferation assay

The Ez-Cytox cell viability assay kit (Daeil Service, Seoul, South Korea) was used to measure proliferation. The cells were seeded in a 96-well plate at a density of 5×10^3 cells/well and cultured for the indicated times (1, 2, or 3 days) in MEM containing 1% FBS. The reagent from the kit was then added to each well. After incubating for 2 h, the plate was measured at the absorbance wavelength of 450 nm using an ELISA plate reader.

Immunofluorescence (IF) double staining

First, the cells were cultured in chamber slides (Lab-Tek II chamber slide™ system, Nunc, Naperville, USA), then fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.5% Triton X-100 for 5 min. Non-specific binding was blocked with 1% BSA for 1 h and the cells were incubated with an anti-fascin monoclonal antibody (Fascin 1 [55K-2]: sc-21743, Santa Cruz Biotechnology Inc., TX, USA) overnight at 4°C.

After washing, the cells were incubated with a goat anti-mouse IgG-FITC (goat anti-mouse IgG-FITC: sc-2010, Santa Cruz Biotechnology Inc.) secondary antibody for 1 h at room temperature (21°C). For double staining with F-actin, the cells were additionally incubated with Alexa Fluor568 phalloidin (Invitrogen, MA, USA), then mounted and visualized using a Zeiss microscope (Carl Zeiss, Jena, Germany). The fluorescent images were captured with an AxioCam camera (Carl Zeiss).

SYBR green real-time quantitative PCR

Total RNA was isolated using the TRIzol (TRIzol Reagent, Invitrogen, CA, USA) method according to the manufacturer's protocol. Four micrograms of total RNA was reverse-transcribed using the PrimeScript™ 1st strand cDNA synthesis kit (TaKaRa Biotechnology, Shiga, Japan). Transcripts were quantified by SYBR green PCR amplification (BIOLINE, London, UK). The primers were designed, and RT-qPCR performed, as described previously [38]. Primers were targeted against integrin $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 6$, $\alpha 7$, $\beta 1$, $\beta 4$, or β -actin (**Figure 5A**). β -actin was used as an internal control gene for calculation of a RT-PCR normalization factor. Data were analyzed by the comparative CT method as follows [39]:

$2^{-\Delta\Delta C_t}$, $\Delta\Delta C_t = (C_t \text{ gene of interest} - C_t \text{ internal control; sample A}) - (C_t \text{ gene of interest} - C_t \text{ internal control; sample B})$.

Western blotting

Total cell lysates were extracted in lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, and 0.1% SDS) containing a protease inhibitor cocktail (Roche, IN, USA). Lysates were separated by SDS-PAGE and transferred to a nitrocellulose membrane (BioScience, Dassel, Germany). The membrane was blocked with 3% skim milk and incubated overnight at 4°C with the following primary antibodies: anti-phospho-Erk1/2 (Thr202/Tyr204) (Cell Signaling Technology, MA, USA), anti-phospho-Akt (Cell Signaling Technology), anti-fascin monoclonal (Santa Cruz Biotechnology Inc.), or anti- β -actin (Santa Cruz Biotechnology Inc.). Subsequently, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit IgGs; Santa Cruz Biotechnology Inc.) and developed with ECL detection reagents (Pierce, IL, USA).

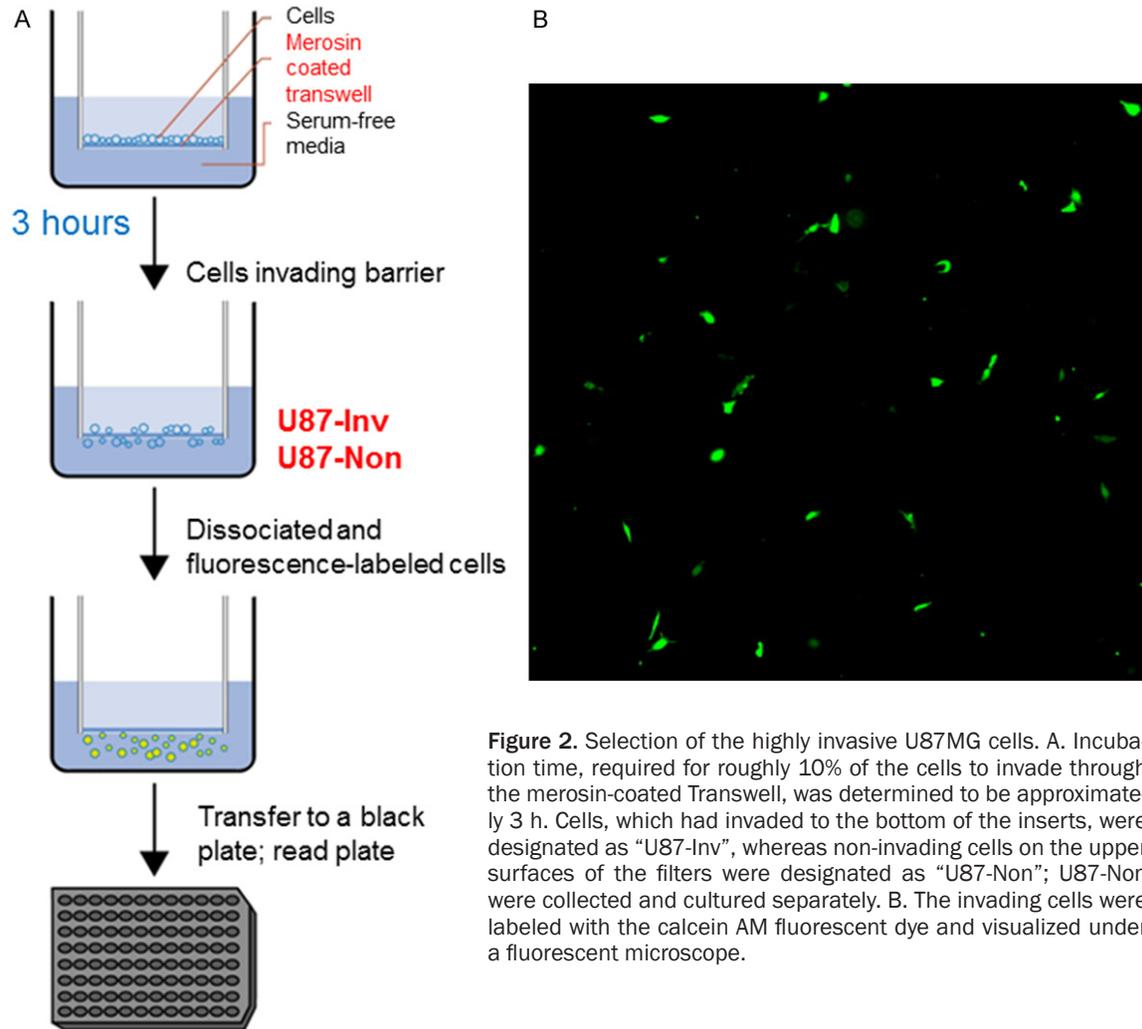


Figure 2. Selection of the highly invasive U87MG cells. A. Incubation time, required for roughly 10% of the cells to invade through the merosin-coated Transwell, was determined to be approximately 3 h. Cells, which had invaded to the bottom of the inserts, were designated as “U87-Inv”, whereas non-invading cells on the upper surfaces of the filters were designated as “U87-Non”; U87-Non were collected and cultured separately. B. The invading cells were labeled with the calcein AM fluorescent dye and visualized under a fluorescent microscope.

Statistical analysis

Data were expressed as means \pm standard error of the mean (SEM). Different treatments were compared by the Mann-Whitney U test, as indicated in the figure legends. Statistical analyses were performed using the SPSS 21 software (IBM Analytics, USA). For all analyses, $P < 0.05$ was considered to be statistically significant. All experiments were performed at least three times.

Results

Effect of merosin on U87MG adhesion

We performed an adhesion assay, which removes weakly attached cells by mechanical agitation, to assess whether U87MG cells can adhere to merosin (Figure 1). The cells were plated on surfaces coated with various concen-

trations of human merosin. In a dose-dependent manner, merosin induced an increase in the adhesive capacity of U87MG cells and led to a change in the morphology of the cells, which developed longer neurite-like extensions. Maximal adhesion was achieved at a 20 $\mu\text{g}/\text{mL}$, or greater, concentration of merosin. Merosin was the most effective substrate for U87MG cell adhesion. Micrographs were acquired 2 h after incubating the cells on merosin-coated plates.

U87-Inv cells showed increased motility

A migration assay was performed using a non-coated membrane. Cells were plated under serum-free conditions, and medium, containing 10% FBS as the chemoattractant, was added to the lower chamber. After 4 h of incubation, cells that had migrated to the bottom side of the inserts were quantified using calcein AM

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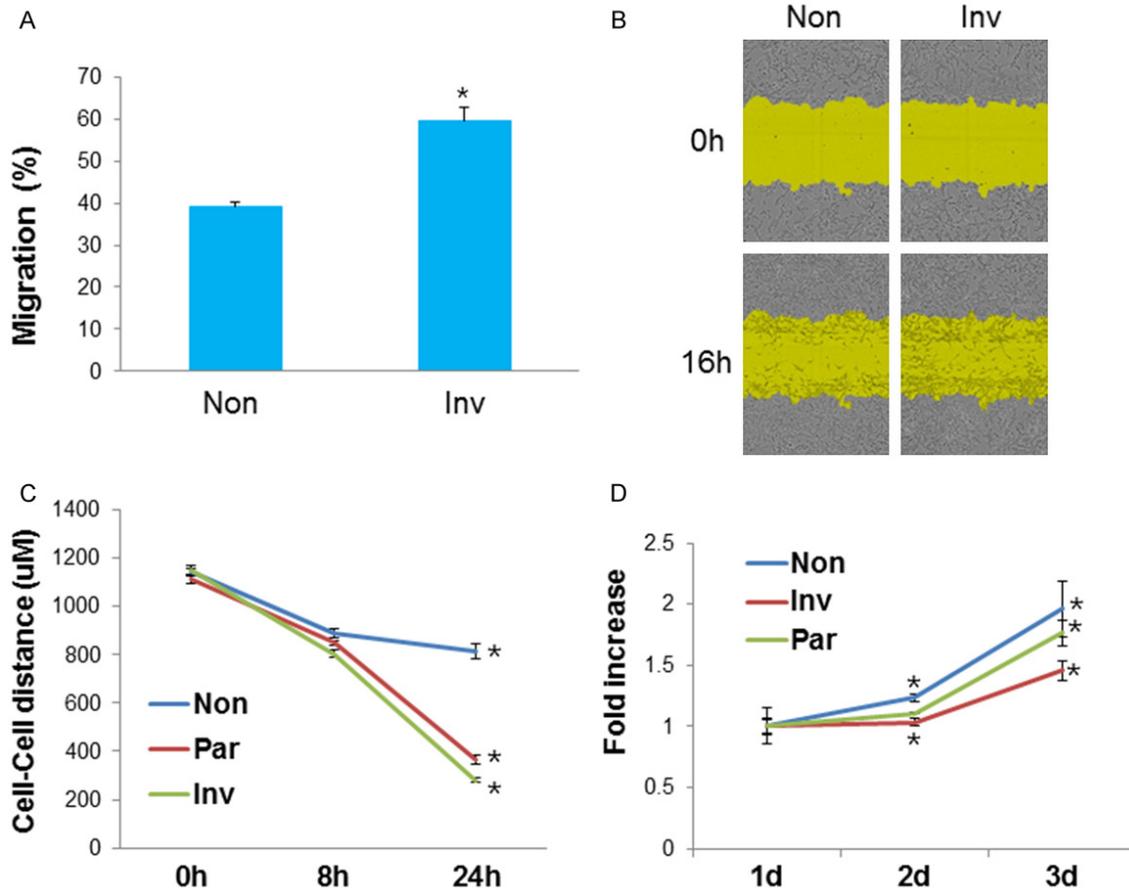


Figure 3. Comparison of motility and proliferation rate between U87-Non and U87-Inv cells. A. A migration assay was first performed with the non-coated membrane. After 4 h of incubation, cells that had migrated to the bottom side of the inserts were quantified using the calcein AM fluorescent dye. U87-Inv cells show a 1.5-fold increased motility compared with that of the U87-Non cells. Results are shown as mean \pm SEM ($n = 3$). Statistical analysis was performed by the Mann-Whitney U test; asterisks indicate statistically significant change ($P < 0.05$). B. Wound-healing was monitored at 8, 16, and 24 h after scratching the surface of the plate with a pipette tip. C. The area of the gap between the migrating edges of the cells was used to quantify the assay by measuring 10 different distances at random. The cells to recover more rapidly (in order) were the U87-Inv cells, followed by the U87MG parental cell line (U87-Par), and the U87-Non cells. Results are shown as mean \pm SEM ($n = 10$). Statistical analysis was performed by the Mann-Whitney U test; asterisks indicate statistical significance ($P < 0.05$). D. Cell proliferation was determined using Ez-Cytox. U87-Inv cells show lower proliferation compared with that of U87-Non cells. Results are shown as mean \pm SEM ($n = 3$). Statistical analysis was performed by the Mann-Whitney U test; asterisks indicate statistically significant changes ($P < 0.05$).

fluorescent dye. The migration rate of U87-Inv cells was approximately 20% higher than that of U87-Non cells (**Figure 3A**). A wound-healing assay was performed to confirm this difference in migration capacity. Cells were seeded as a high-density monolayer, and wound healing was observed at 8, 16, and 24 h after inducing a scratch with a p200 pipette tip (**Figure 3B**). After 8 h, U87-Inv cells rapidly migrated into the wounded area, and the wounds were nearly healed after 24 h. Conversely, the parental (Par) and U87-Non cells showed slower migration rate than that of the U87-Inv cells; conse-

quently, closure of the wound was also slower (**Figure 3C**). Interestingly, the proliferation rate of U87-Inv cells was significantly reduced compared with that of U87-Non cells (**Figure 3D**).

U87-Inv cells showed an increased level of MMP-2

The expression of MMP-2, an important factor in GBM invasion, was investigated by zymography [40, 41]. Our result showed that the expression of MMP-2 increased in U87-Inv cells compared with that in U87-Non cells (**Figure 4A**).

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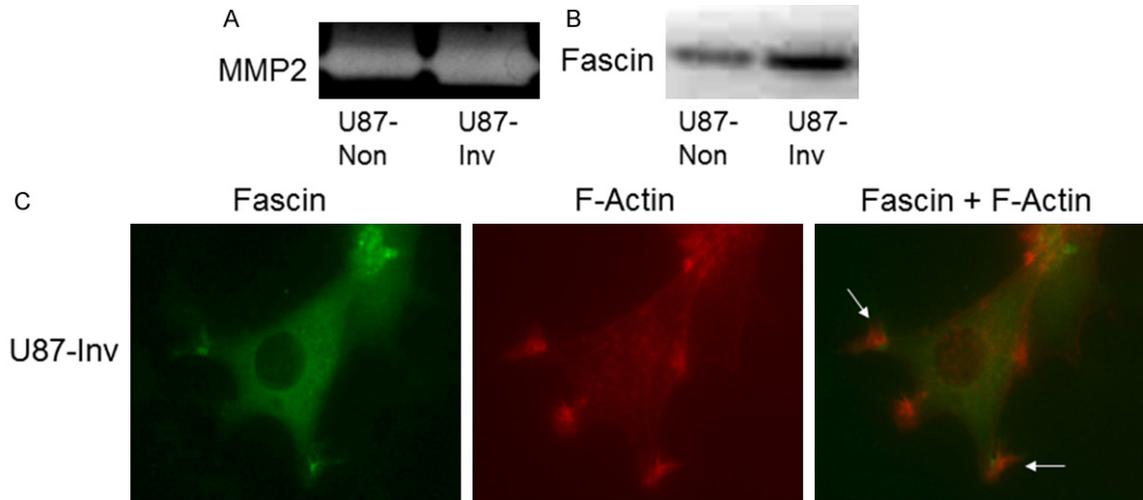


Figure 4. Expression of matrix metalloproteinase-2 (MMP-2) and fascin. A. Expression of MMP-2 was analyzed by zymography. U87-Inv cells show an increased expression level of MMP-2 compared with that of U87-Non cells. B. U87-Inv cells show an increase in fascin expression as assessed by western blotting. C. Expression and localization of fascin was detected by immunofluorescence. Arrows show co-localization of fascin and F-actin, demonstrating bundles of filopodia.

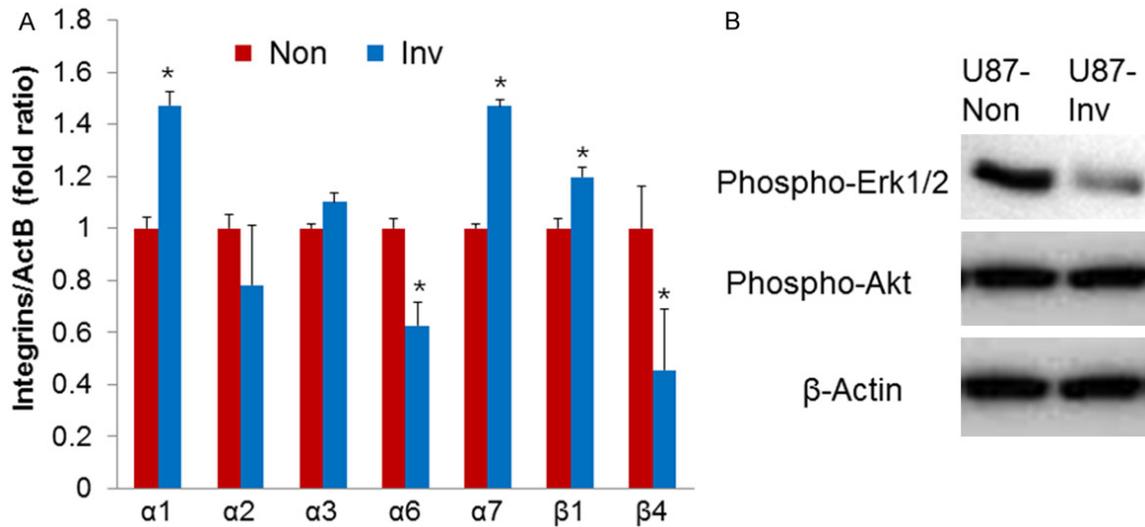


Figure 5. Expression of integrin subunits and downstream signaling molecules in U87-Non and U87-Inv cells. A. Expression of mRNAs encoding integrins $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 6$, $\alpha 7$, $\beta 1$, and $\beta 4$, which are known receptors for merosin, was investigated by real-time quantitative PCR (RT-qPCR). U87-Inv cells show an increased expression level of integrins $\alpha 1$ and $\alpha 7$ compared with that in U87-Non cells, whereas the expression of integrins $\alpha 6$ and $\beta 4$ is reduced in U87-Inv cells. Results are shown as mean \pm SEM ($n = 3$). Statistical analysis was performed by the Mann-Whitney U test; asterisks indicate statistically significant changes ($P < 0.05$). B. Cellular proteins were resolved on 6-10% SDS-polyacrylamide gels. Membranes were probed with anti-phospho-Erk1/2 or phospho-Akt primary antibodies. β -actin was used as loading control. U87-Inv cells show a decrease in the levels of phosphorylated Erk1/2.

U87-Inv cells displayed cytoskeletal features of high motility

We assessed the localization of fascin expression because fascin is a major regulator of cell

motility and cytoskeletal alterations. Western blotting indicated that U87-Inv cells had an increased expression of fascin (Figure 4B). Additionally, U87-Inv cells showed extensive lamellipodia. Fluorescence double-staining revealed

that fascin was expressed in the part of filamentous actin (F-actin)-rich protrusive structures in U87-Inv cells (**Figure 4C**).

Expression of integrin receptors in U87-Non and U87-Inv cells

We analyzed whether the expression levels of merosin-binding integrins differed between U87-Non and U87-Inv cells (**Figure 5A**). The expression of mRNAs encoding integrins $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 6$, $\alpha 7$, $\beta 1$, and $\beta 4$ was examined by RT-qPCR [6]. The expression of integrins $\alpha 1$ and $\alpha 7$ was increased by approximately 1.5-fold in U87-Inv cells. The expression of integrins $\alpha 6$ and $\beta 4$ was reduced by approximately 0.4- and 0.6-fold, respectively, in U87-Inv cells compared with that in U87-Non cells. There was no significant difference in the expression of integrins $\alpha 2$, $\alpha 3$, and $\beta 1$ between U87-Inv and U87-Non cells.

Erk and Akt expression in U87-Non and U87-Inv cells

We investigated the expression of integrin-related molecules Erk and Akt, which are involved in proliferation and invasion (**Figure 5B**). The expression of phospho-Erk was significantly upregulated in U87-Non cells compared with that in U87-Inv cells. There was no difference in the expression of phospho-Akt between U87-Non and U87-Inv cells.

Discussion

GBM extensively invades into the surrounding normal brain tissue, rendering complete surgical resection nearly impossible and resulting in a poor prognosis. We examined the highly invasive GBM cells for their heterogeneity at the cellular level [11, 12]. A highly invasive subpopulation of GBM cells was selected according to previously described methods [15]. Unlike previous studies, we used the merosin substrate to examine adhesion because the ECM of the CNS is composed mainly of merosin rather than collagen [42, 43]. To investigate whether the selected cells were more invasive, we performed a migration assay to examine the motility of the cells, zymography to examine the expression of MMP-2, and immunofluorescence to assess the expression of fascin. Our results indicated that we had successfully selected a subpopulation of highly invasive cells.

We compared the expression of integrin receptors between U87-Non and U87-Inv cells (**Figure 5A**). The U87-Inv cells showed an increase in the expression of integrins $\alpha 1$ and $\alpha 7$. Integrin $\alpha 1$, a collagen receptor that can activate the Ras/Shc/mitogen-activated protein kinase pathway [19, 44], is also reported to drive the progression of non-small cell lung and colorectal cancers [45, 46]. Integrin $\alpha 7$ is a laminin receptor that serves as a link between the extracellular matrix and the actin cytoskeleton [47]. Hence, integrin $\alpha 7$ may suppress tumor growth and retard metastasis in several malignant tumors [48]. The U87-Inv cells, overexpressing integrins $\alpha 1$ and $\alpha 7$, showed an increased ability to migrate but a decreased rate of proliferation. However, the mechanism of integrin $\alpha 1$ and $\alpha 7$ in GBM remains unclear and requires further investigation.

In contrast with the expression of integrin $\alpha 1$ and $\alpha 7$, our results indicate that U87-Inv cells showed a decrease in the expression of integrin $\alpha 6$ and $\beta 4$. Integrin $\beta 4$ binds distinctly only to $\alpha 6$ subunits [19]. The expression of the laminin receptor of integrin $\alpha 6\beta 4$ is correlated with tumor survival and angiogenesis in various malignancies [49-52]. A previous study reported that integrin $\alpha 6$ enhances the proliferation of U87MG cells [53]. GBM stem cells overexpress integrin $\alpha 6$ and their interaction with laminin on endothelial cells directly regulates tumorigenicity [26, 54]. Other studies suggest that integrin $\alpha 6\beta 4$ signaling promotes tumorigenesis and angiogenesis by enhancing the nuclear translocation of Erk [52], and that integrin $\beta 4$ is involved in the regulation of GBM proliferation [27]. Taken together, Erk activation, via integrins $\alpha 6$ and $\beta 4$, may be correlated with the proliferation, rather than with invasion, of GBM cells. The results of these previous studies are in agreement with our results, indicating that U87-Inv cells showed a decrease in cell proliferation with the downregulation of integrin $\alpha 6\beta 4$ and Erk. Interestingly, we found that highly invasive GBM cells showed decreased proliferation. Several previous studies confirm that the levels of invasiveness are inversely related to the rate of proliferation [32, 55]. These studies also indicate that the invasiveness of GBM was correlated with stem-like characteristics and suggest that tumor cells with stem-like characteristics have a lower rate of proliferation [32]. The invasiveness and proliferation of

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GBM may be controlled by different and exclusive mechanisms. Previous studies report that Akt is activated by the RTK/PTEN/PI3K pathway, which helps glioma cells grow uncontrollably and become invasive [32, 56]; however, we could not find the same correlation (**Figure 5B**).

In summary, using a merosin substrate, we selected a highly invasive subpopulation of cells from the U87MG cell line and showed that U87-Inv cells possess highly invasive characteristics. Our findings indicate that merosin, major component in the microenvironment of GBM, stimulates GBM invasiveness. The U87-Inv cells showed an increased expression of integrins $\alpha 1$ and $\alpha 7$ but a decreased expression of Erk and the integrins $\alpha 6$ and $\beta 4$. Our results suggest that activation of the integrins $\alpha 1$ and $\alpha 7$ enhanced the invasiveness, whereas the activity of the MAPK/Erk pathway, stimulated via integrins $\alpha 6$ and $\beta 4$, enhanced the proliferation of GBM. The various integrin subunits and related molecules should be further investigated in human GBM tissues.

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Disclosure of conflict of interest

None.

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