

Keywords: IGFBP-2; glioma; ERK; integrin  $\beta$ 1

# Exogenous IGFBP-2 promotes proliferation, invasion, and chemoresistance to temozolomide in glioma cells via the integrin $\beta$ 1-ERK pathway

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**Background:** Insulin-like growth factor binding protein-2 (IGFBP-2) is significantly increased in the serum of patients with malignant gliomas. High plasma IGFBP-2 levels are correlated with poor prognosis in glioma patients. However, the exact role of exogenous IGFBP-2 in gliomas is unclear.

**Methods and results:** Using the MTT cell viability assay, cell cycle analysis, and the transwell migration assay, it was demonstrated that IGFBP-2 treatment stimulated proliferation and invasion in U87 and U251 cell lines and primary SU3 glioma cells. Western blot analysis and immunofluorescence staining revealed that IGFBP-2 promoted ERK phosphorylation and nuclear translocation. Moreover, blocking ERK activation using the inhibitor PD98059 markedly reduced the effects of IGFBP-2 in glioma cells. As IGFBP-2 has an integrin-binding domain, the contribution of integrin  $\beta$ 1 to these IGFBP-2-mediated processes was examined. Neutralisation or knockdown of the expression of integrin  $\beta$ 1 inhibited IGFBP-2-induced ERK activation, cell proliferation, and cell invasion. Significantly, IGFBP-2 induced temozolomide resistance in glioma cells in an integrin  $\beta$ 1/ERK-dependent manner.

**Conclusions:** Exogenous IGFBP-2 induces proliferation, invasion, and chemoresistance in glioma cells via integrin  $\beta$ 1/ERK signaling, suggesting that targeting this pathway could represent a potential therapeutic strategy for the treatment of gliomas. The identification of this pathway in glioma progression provides insight into the mechanism by which serum IGFBP-2 levels can predict the prognosis of glioma patients.

Glioblastoma is the most common primary malignant brain tumour in adults, with a median survival of about 15 months (Yan *et al*, 2012). Besides surgery, postoperative radiotherapy plus chemotherapy is the most effective treatment strategy, and significantly prolongs survival time in some patients (Stupp *et al*, 2005). However, a variety of regulators including growth factors, hormones, and cytokines promote chemoresistance in glioblastoma, resulting in treatment failure (Oliva *et al*, 2011; Sun *et al*, 2014).

Insulin-like growth factor binding protein-2 is produced by a variety of different tissues via complex regulatory processes

(Sandhu *et al*, 2002). Previous studies have demonstrated that the expression of IGFBP-2 was significantly increased in glioblastoma compared with low-grade gliomas and normal brain tissue (Sallinen *et al*, 2000; Elmlinger *et al*, 2001; Wang *et al*, 2002). Moreover, IGFBP-2 levels are significantly higher in the sera of glioblastoma patients and are negatively correlated with patient survival (Lin *et al*, 2009; Han *et al*, 2014). However, the molecular mechanism by which serum IGFBP-2 affects disease progression and patient prognosis is unclear. Although endogenous over-expression of IGFBP-2 has been associated with cell proliferation or invasion, the findings have been controversial (Wang *et al*, 2003;

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Fukushima and Kataoka, 2007; Fukushima *et al*, 2007; Mehrian-Shai *et al*, 2007; Holmes *et al*, 2012) and cannot effectively explain the prognostic role of serum IGFBP-2. These results suggest that exogenous IGFBP-2 may have an important role in regulating the function of glioma cells, and the role of exogenous IGFBP-2 needs to be clarified.

Insulin-like growth factor binding protein-2 has an Arg-Gly-Asp (RGD) cell adhesion motif that can potentially bind integrin receptors, the activation of which can, in turn, activate extracellular signal-regulated kinases (ERKs). The activated integrin-ERK pathway has been shown to induce proliferation and invasion by malignant cells in response to external stimuli (Nakada *et al*, 2013; Sun *et al*, 2013; Kale *et al*, 2014). The present study investigated the effects of exogenous IGFBP-2 on integrin-ERK pathway activation, and on proliferation and invasion by glioma cells. The results provide a mechanistic explanation for the role of serum IGFBP-2 levels in predicting the prognosis of glioblastoma patients.

## MATERIALS AND METHODS

**Cell culture.** Human glioblastoma cell lines U87, U251, and U373 were purchased from the Institute of Biochemistry and Cell Biology (Shanghai, China). The human malignant glioblastoma cell line T98G was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Primary SU3 glioma cells (Wan *et al*, 2012; Han *et al*, 2013) were kindly provided by Professor Dong Jun from the Second Affiliated Hospital of Soochow University (Taipei, Taiwan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and antibiotics (penicillin and streptomycin, each 100 U ml<sup>-1</sup>). Cultures were incubated at 37 °C in a humidified chamber with 5% CO<sub>2</sub>.

Cells were seeded in 24-well plates at 5 × 10<sup>3</sup> cells per well in a regular medium. After 24 h, the cells were washed with PBS and cultured in 0.5 ml serum-free culture medium for 48 h. The medium was then collected, and IGFBP-2 in the medium was examined by ELISA assay as described previously (Han, *et al*, 2014).

**Construction of IGFBP-2-overexpressing cells.** The human IGFBP-2 cDNA was cloned into the pEGFP-N1 plasmid (Clontech, Mountain View, CA, USA), and the resulting pEGFP-N1-IGFBP-2 plasmid or control pEGFP-N1 was transfected into U87 cells using Lipofectamine (Invitrogen). Stable cell lines were selected by G418 screening. The efficiency of IGFBP-2 overexpression was evaluated using western blotting.

**IGFBP-2 gene expression knockdown.** Specific IGFBP-2-shRNA (short hairpin RNA) sequences were synthesised as described previously (Fukushima *et al*, 2007). The IGFBP-2-shRNA sequence was 5'-ACTGTGACAAGCATGGCCTGT-3' and the control-shRNA sequence was 5'-ATCGCTAGGTCGGCGACATAT-3', which was cloned into pSUPER-puro and transfected into U251 cells. After 24 h, transfected cells were selected for 10 days with 2 mg ml<sup>-1</sup> puromycin. Cells stably expressing IGFBP-2-shRNA were used for further research. The effectiveness of IGFBP-2 silencing was assessed using western blotting.

**Integrin β1 gene expression knockdown.** U87, U251, and SU3 cells were infected with shRNA lentiviral particles (Santa Cruz Biotechnology, Santa Cruz, CA, USA) targeting β1-integrin (sc-35674-V) or control shRNA (sc-108080) according to the manufacturer's protocol. After 48 h, subcultured cells were selected in 1 μg ml<sup>-1</sup> puromycin for 1 week. The effectiveness of integrin β1 silencing was assessed using western blotting.

**PCR.** Total RNA was isolated from SU3, U87, U251, T98G, and U373 cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA was reverse transcribed into cDNA (Takara Bio Inc., Shiga, Japan), which served as the template for the PCR reaction. The primers used to amplify IGFBP-2 and GAPDH (control) were as follows: IGFBP-2 forward, 5'-AGGTTGCAGACAATGGCGAT-3' and IGFBP-2 reverse, 5'-GTAGAAGAGATGACTCTCGG-3'; GAPDH forward, 5'-GCA CCGTCAAGGCTGAGAAC-3' and GAPDH reverse, 5'-TGGTG AAGACGCCAGTGGGA-3'. Reactions were carried out in a Gradient Thermal Cycler (Biometra, Goettingen, Germany) using the following programme: 94 °C for 2 min; 30 × (94 °C for 30 s, 61 °C for 30 s, 72 °C for 90 s); and 72 °C for 10 min. Polymerase chain reaction products were resolved on a 1% agarose gel containing ethidium bromide.

Methylation-specific PCR was performed as described previously (Han *et al*, 2014) to detect O(6)-methylguanine-DNA-methyltransferase (MGMT) promoter methylation status in U87, SU3, T98G, and U251 cells.

**Western blot.** Total protein was extracted from SU3, U87, U251, T98G, and U373 cells using a Total Cell Protein Extraction Kit (Millipore, Billerica, MA, USA) to measure the expression levels of IGFBP-2 in different cell lines. In another experiment, SU3, U87, and U251 cells were seeded at 10<sup>6</sup> cells per 100 mm dish in 10% FBS-supplemented DMEM. At 60% confluence, cells were serum starved overnight and monolayers were treated with 500 ng ml<sup>-1</sup> recombinant human IGFBP-2 (Research Diagnostics Inc., Flanders, NJ, USA) for 5, 10, or 30 min at 37 °C. Some cells were pretreated with an integrin β1-neutralising antibody (MAB1959; Chemicon International, Temecula, CA, USA) at a concentration of 2 μg ml<sup>-1</sup>. Total protein was extracted using lysis buffer to determine ERK and phospho-ERK (pERK) levels. Protein concentrations were determined using the Coomassie (Bradford) protein assay. An equivalent amount of protein from each sample was resolved by 12% SDS-PAGE and transferred to a nitrocellulose membrane. After blocking, membranes were incubated with anti-IGFBP-2 (Research Diagnostics Inc.), anti-pERK (Cell Signaling Technology, Beverly, MA, USA), or anti-ERK antibody (Cell Signaling Technology) overnight at 4 °C. All antibodies were used at a 1 : 1000 dilution. Membranes were then washed three times for 5 min with TBST/0.1% Tween-20 and incubated with a secondary antibody. Bands were detected using a Chemiluminescence ECL Kit (Santa Cruz Biotechnology).

**Immunofluorescence.** Immunofluorescence stain was performed as described previously (Han *et al*, 2013). Briefly, after overnight serum starvation, cells grown on coverslips were left untreated or treated with 500 ng ml<sup>-1</sup> IGFBP-2 for 30 min at 37 °C. The cells were washed with PBS, fixed, blocked, and probed with anti-pERK antibody (1 : 100), followed by treatment with a fluorophore-conjugated secondary antibody. Nuclei were counterstained with Hoechst 33342. Coverslips were mounted on glass slides and cells were visualised using a confocal microscope (Olympus FV1000S-SIM; Olympus, Tokyo, Japan).

**MTT cell viability assay.** Cells were seeded in 96-well plates at 1 × 10<sup>4</sup> cells per well in 10% FBS-supplemented DMEM. The following day, the cell monolayers were incubated in serum-free medium for 24 h, and then treated with 125, 250, or 500 ng ml<sup>-1</sup> IGFBP-2 for 24, 48, 72, 96, or 120 h. In another experiment, cells were treated with 100 μM temozolomide (TMZ; Tasly Pharmaceutical Co. Ltd, Tianjin, China) alone or with 500 ng ml<sup>-1</sup> IGFBP-2 for 24, 48, 72, or 96 h. In some cultures, cells were preincubated with 50 μM of the ERK inhibitor PD98059 (Calbiochem, San Diego, CA, USA) for 16 h or 2 μg ml<sup>-1</sup> integrin β1-neutralising antibody for 30 min before treatment with IGFBP-2. Cell growth was evaluated using an MTT cell viability assay system according to the

manufacturer's protocol. Following the colorimetric reaction, the optical density was determined at 490 nm using a scanning multiwell spectrophotometer (Tecan Sunrise Remote, Maennedorf, Austria).

**Bromodeoxyuridine incorporation assay.** Bromodeoxyuridine (BrdU) incorporation assay was performed to analyse cell proliferation. Briefly, cells were plated at  $2 \times 10^3$  cells per well in a 96-well plate and then treated with  $500 \text{ ng ml}^{-1}$  IGFBP-2 for 48 h after being serum starved overnight. Bromodeoxyuridine was then added and further incubated for another 24 h. The incorporation of BrdU was detected using a colorimetric immunoassay kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany).

**Cell quantification.** Cells were seeded in 24-well plates at  $5 \times 10^3$  cells per well in 10% FBS-supplemented DMEM and grown for 24 h. After overnight serum starvation, the cells were then treated with 125, 250, or  $500 \text{ ng ml}^{-1}$  of IGFBP-2 for 48 h. In another experiment, cells were treated with  $100 \mu\text{M}$  TMZ alone or together with  $500 \text{ ng ml}^{-1}$  IGFBP-2 for 48 h. In some cultures, cells were preincubated with  $50 \mu\text{M}$  PD98059 for 16 h or  $2 \mu\text{g ml}^{-1}$  integrin  $\beta 1$ -neutralising antibody for 30 min before treatment with IGFBP-2. Following treatment, cells were washed by replacing the medium with PBS, and trypsinised by adding  $200 \mu\text{l}$  of 0.25% trypsin/EDTA solution. The detached cells were counted using a haemocytometer after trypan blue stain.

**Cell cycle analysis.** Cells were plated in six-well microtitre plates and treated with 125, 250, or  $500 \text{ ng ml}^{-1}$  IGFBP-2 after overnight serum starvation. In some cultures, cells were preincubated with  $50 \mu\text{M}$  PD98059 for 16 h or  $2 \mu\text{g ml}^{-1}$  integrin  $\beta 1$ -neutralising antibody for 30 min before treatment with IGFBP-2. After 24 h, the cells were trypsinised and washed once with PBS. The cells were stained with propidium iodide (PI;  $75 \mu\text{M}$ ) in PBS with 0.1% NP-40. Analysis of DNA content was performed by collecting 10 000 events for cell cycle analysis using a FACSCalibur flow cytometer and CellQuest software (BD Biosciences, San Jose, CA, USA).

**Transwell cell migration assay.** Transwell chambers with  $8 \mu\text{m}$  pores (Corning, Corning, NY, USA) were coated with  $50 \mu\text{l}$  Matrigel (BD Biosciences). Cells ( $2 \times 10^3$ ) treated with 125, 250, or  $500 \text{ ng ml}^{-1}$  IGFBP-2 were plated in  $100 \mu\text{l}$  serum-free DMEM containing 0.1% bovine serum albumin, and added in triplicate to the transwell chambers. In another experiment, cells were treated with  $100 \mu\text{M}$  TMZ alone or together with  $500 \text{ ng ml}^{-1}$  IGFBP-2. In some cultures, cells were preincubated with  $50 \mu\text{M}$  PD98059 for 16 h or  $2 \mu\text{g ml}^{-1}$  integrin  $\beta 1$ -neutralising antibody for 30 min before treatment with IGFBP-2. Dulbecco's modified Eagle's medium with 20% FBS ( $600 \mu\text{l}$ ) was added to the bottom chamber. Cells were allowed to invade the Matrigel-coated filters toward the lower compartment for 20 h at  $37^\circ\text{C}$ . Cells that had reached the lower surface of the filter were fixed and stained, and counted using a microscope. A total of 10 fields were counted for each transwell filter.

**Glioma tissue samples.** A total of 154 clinical samples were collected from the Chinese Glioma Genome Atlas (<http://www.cgca.org.cn/>), including 83 primary glioblastomas (P), eight anaplastic astrocytomas (AA), 58 astrocytomas (A) and five normal brain tissue samples (N). Three normal brain samples were obtained from patients with severe brain trauma who required surgery, and the remaining two samples were from patients who underwent surgery for primary epilepsy. All patients received surgical resection between January 2005 and December 2009. Samples were flash frozen in liquid nitrogen immediately after resection. Histologic diagnosis was established according to the 2007 World Health Organisation classification guidelines, and verified by two neuropathologists. Before the study, the percentage of tumour cells was evaluated for each sample using a

haematoxylin and eosin-stained frozen section. Only samples with  $>80\%$  tumour cells were selected for analysis. The study was approved by the institutional review board at the hospital, and written informed consent was obtained from every patient.

**Microarray analysis.** Total RNA was extracted using a mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. RNA concentration and quality were assessed using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

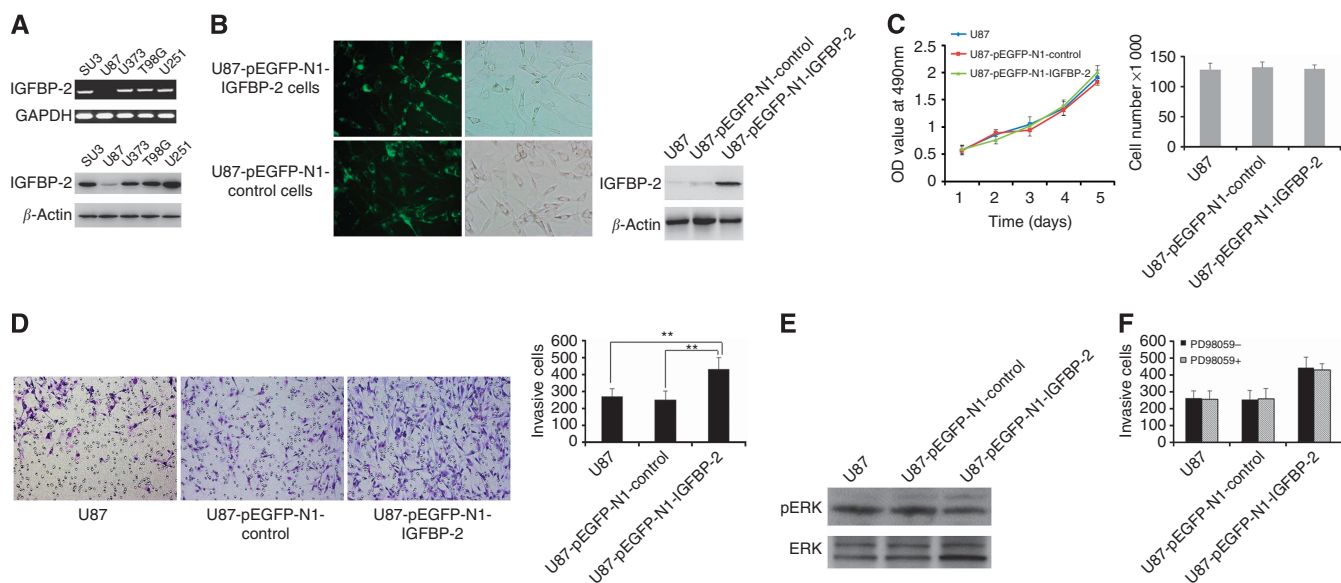
A total of 154 samples (83 P, 8 AA, 58 A, and 5 N) were analysed by microarray using the Agilent Whole Human Genome Array (Agilent Technologies, Palo Alto, CA, USA) according to the manufacturer's instructions. The integrity of total RNA was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies). cDNA and biotinylated cRNA were synthesised and hybridised to the array. The Agilent G2565BA Microarray Scanner System (Agilent Technologies) and Agilent Feature Extraction Software (version 9.1, Agilent Technologies) were used for data acquisition. Probe intensities were normalised using GeneSpring GX 11.0 (Agilent Technologies).

**Statistical analysis.** Integrin  $\beta 1$  and ERK expression levels obtained from microarray analysis of tumorigenic and normal tissue were subjected to cluster analysis (Cluster 2.20) using the hierarchical clustering method with average linkage. The result was visualised using TreeView software (Stanford University, Palo Alto, CA, USA). Pearson's correlation between the expression levels of integrin  $\beta 1$  and ERK was computed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The microarray data set can be accessed at the website <http://www.cgga.org.cn/medical.php?mod=search>. Publicly available data set was also used to verify the expression of integrin  $\beta 1$  and ERK in gliomas, which can be accessed at <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE4290>. The Student's *t*-test and ANOVA were used to assess statistical significance. Each experiment was performed in triplicate, and all data are presented as the mean  $\pm$  s.e. of three independent experiments. A two-tailed *P*-value of  $<0.05$  was considered significant.

## RESULTS

**Endogenous IGFBP-2 overexpression or knockdown in glioma cells affects invasion but not proliferation.** The expression level of IGFBP-2 in four different glioblastoma cell lines and primary SU3 glioma cells was evaluated using RT-PCR and western blot analysis (Figure 1A). As U87 cells express endogenous IGFBP-2 at low levels, they were chosen to generate stable cell lines. Insulin-like growth factor binding protein-2 expression was stably upregulated in U87 cells, by transfection with the pEGFP-N1-IGFBP-2 plasmid (Figure 1B). Despite the differences in the levels of IGFBP-2 expression, cells did not show marked differences in their proliferation (Figure 1C). However, significantly higher rate of invasion was observed in IGFBP-2-overexpressing cells and the invasive potential was increased nearly one-fold ( $P < 0.01$ ; Figure 1D) compared with the control cells. Further, we examined the effect of IGFBP-2 overexpression in U87 cells on ERK signaling pathway by western blot analysis. We found that ERK phosphorylation was not significantly affected by the levels of IGFBP-2 expression (Figure 1E). Moreover, PD98059, an ERK inhibitor, had no effect on IGFBP-2 overexpression-induced invasion (Figure 1F). These results suggest that ERK signaling pathway is not linked to endogenous IGFBP-2 overexpression.

Furthermore, IGFBP-2 expression was stably knocked down in relatively high IGFBP-2-expressing U251 cells by transfection with an IGFBP-2-specific shRNA. The loss of IGFBP-2 expression was confirmed by western blot (Supplementary Figure 1A). MTT assay and cell counting showed that the downregulation of IGFBP-2 in



**Figure 1.** Insulin-like growth factor binding protein-2 overexpression in glioma cells enhances invasion but not proliferation. **(A)** Insulin-like growth factor binding protein-2 expression in four glioblastoma cell lines and primary SU3 glioma cells, as determined by RT-PCR and western blot analysis. **(B)** pEGFP-N1-IGFBP-2 plasmid or control pEGFP-N1 was effectively transfected into U87 cells. **(C)** Insulin-like growth factor binding protein-2 overexpression did not significantly affect cell proliferation as shown by MTT assay and cell counting. **(D)** Invasion assay showed that compared with cells transfected with control vector and untransfected cells, the invasive ability of U87-pEGFP-N1-IGFBP-2 cells was markedly increased. **(E)** The phosphorylation of ERK was not markedly altered by the levels of IGFBP-2 expression. **(F)** Inhibition of ERK did not affect the invasion induced by IGFBP-2 overexpression.

U251 cells had no significant effect on cell proliferation (Supplementary Figure 1B–D). However, the knockdown of IGFBP-2 resulted in a significant reduction of the invasion through the Matrigel in U251 cells (Supplementary Figure 1E).

**Exogenous IGFBP-2 promotes proliferation and invasion by glioblastoma cells.** Subsequently, the effect of exogenous IGFBP-2 on glioblastoma cells was examined using recombinant human IGFBP-2 purchased from Research Diagnostics Inc. U87 cells were used to minimise the effects of endogenous IGFBP-2. U251 cells were used to test the effects of exogenous IGFBP-2 on glioblastoma cells, which have high levels of endogenous IGFBP-2 expression. To exclude the possible bias introduced by cell lines that have undergone multiple passages, primary SU3 glioma cells derived from fresh clinical samples were also used in this study.

The effects of exogenous IGFBP-2 on the proliferation of U87, SU3, and U251 cells were investigated at different time points. It was observed by phase-contrast microscopy that IGFBP-2 treatment significantly stimulated cell growth (Figure 2A). The results from the MTT assay and cell counting revealed that the IGFBP-2-induced increase in cell proliferation was dose-dependent (Figure 2B). Insulin-like growth factor binding protein-2 of 125 and 250 ng ml<sup>-1</sup> increased proliferation by 33.8–62.7%, and IGFBP-2 of 500 ng ml<sup>-1</sup> increased proliferation by about 1.14-fold.

To assess whether the observed increase in proliferation in response to exogenous IGFBP-2 was owing to an increase in the number of cells entering the cell cycle, the cell cycle profile was evaluated by PI staining 24 h after treatment. The results showed that IGFBP-2 promoted the S- and G2/M-phase entry in a dose-dependent manner in U87, SU3, and U251 cells (Figure 2C). IGFBP-2 of 125 and 250 ng ml<sup>-1</sup> increased the G2/M phase cells from 0–2.7 to 8.1–17.4%, and IGFBP-2 of 500 ng ml<sup>-1</sup> increased that number to ~30%. In addition, BrdU assay also confirmed that exogenous IGFBP-2 promoted cell proliferation and cell cycle entry (Supplementary Figure 1G).

To evaluate the effects of exogenous IGFBP-2 on cell migration, a Matrigel invasion assay was performed using U87, SU3, and

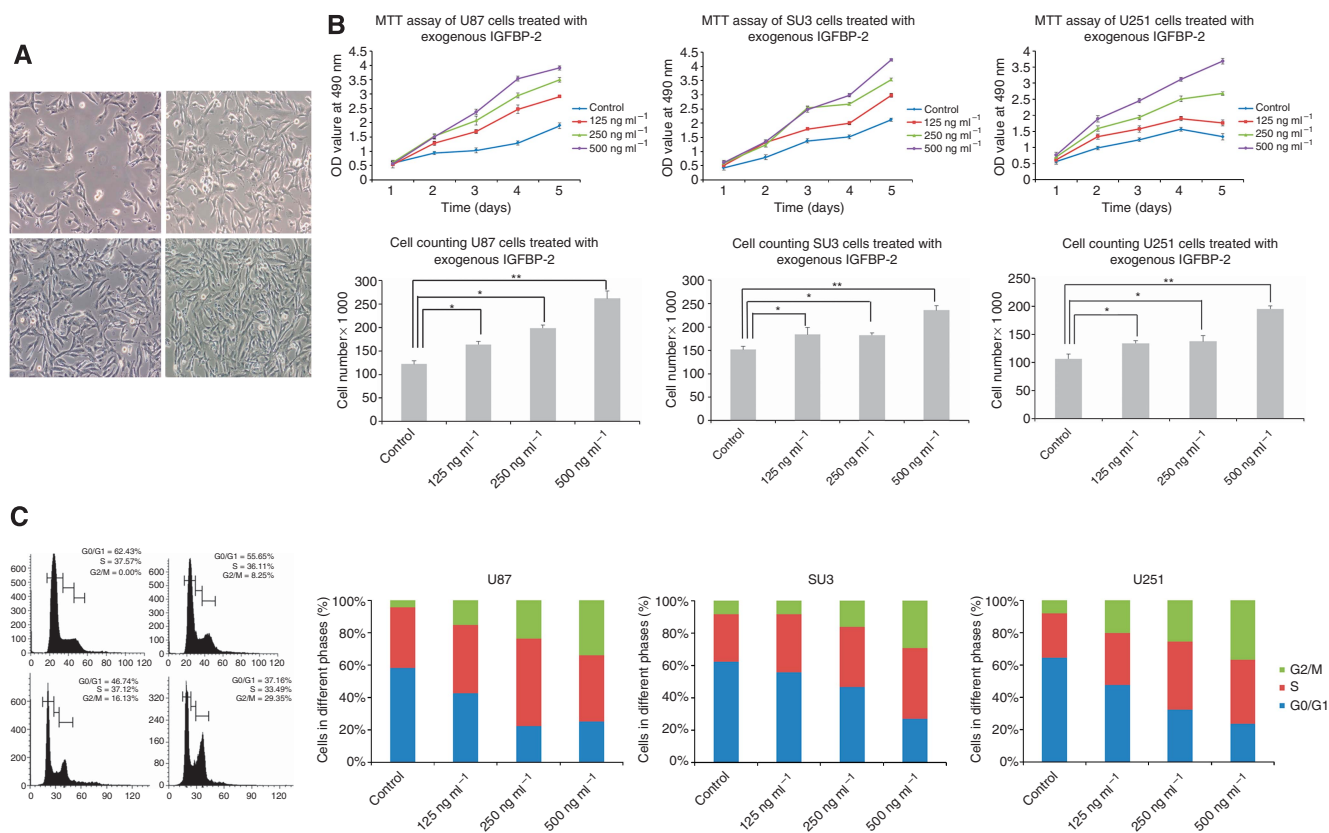
U251 cells. The invasive potential of glioblastoma cells was significantly increased by IGFBP-2 in a dose-dependent manner (Figure 3A). Insulin-like growth factor binding protein-2 of 125 and 250 ng ml<sup>-1</sup> increased invasive cells by 37.8–87.4%, and IGFBP-2 of 500 ng ml<sup>-1</sup> increased invasive cells by ~1-fold (Figure 3B).

**Exogenous IGFBP-2 enhances ERK activation in glioblastoma cells.** To identify the signaling pathway through which IGFBP-2 promotes proliferation and invasion, the activation of ERK was examined in U87, SU3, and U251 cells, since the ERK signaling pathway has been associated with IGFBP2s (Chakrabarty and Kondratick, 2006; Kiepe *et al*, 2008). Cellular protein extracted from cultures treated with IGFBP-2 was analysed by western blot. Although the levels of total ERK protein did not increase, pERK levels were significantly elevated (2–4.5-fold) with respect to control cells 30 min after IGFBP-2 administration (Figure 3C).

The localisation of pERK in glioblastoma cells after IGFBP-2 administration was examined by immunofluorescence. Administration of exogenous IGFBP-2 for 30 min potentiated ERK phosphorylation and nuclear translocation (Figure 3D). These results indicate that exogenous IGFBP-2 enhances the activation of ERK signaling in glioblastoma cells.

**Inhibition of ERK blocks the effects of IGFBP-2 in glioblastoma cells.** To confirm that activation of the ERK signaling pathway is required for exogenous IGFBP-2-induced proliferation and invasion, U87, SU3, and U251 cells were preincubated with the ERK inhibitor PD98059. The inhibition of ERK abrogated exogenous IGFBP-2-induced proliferation, cell cycle progression, and invasion by glioblastoma cells (Supplementary Figures 2 and 3A, B). These findings suggest that exogenous IGFBP-2 promotes proliferation and invasion of glioblastoma cells through ERK signaling.

**Integrin  $\beta$ 1 knockdown or neutralisation inhibits IGFBP-2-induced proliferation and invasion.** As IGFBP-2 has an RGD adhesion motif that can potentially bind and activate integrin



**Figure 2.** The effects of exogenous IGFBP-2 on glioblastoma cell proliferation and cell cycle kinetics. (A) Representative photomicrographs of untreated or IGFBP-2-treated U87 cells. Cell proliferation was stimulated by treatment with exogenous IGFBP-2 in a dose-dependent manner. (Upper) left: control; right: 125 ng ml<sup>-1</sup> IGFBP-2. (Lower) left: 250 ng ml<sup>-1</sup> IGFBP-2; right: 500 ng ml<sup>-1</sup> IGFBP-2. (B) Viability of U87, SU3, and U251 cells treated with various concentrations of IGFBP-2, assessed by the MTT assay (upper). Cell quantification following treatment of glioblastoma cells with various concentrations of IGFBP-2 for 48 h (lower). (C) Cell cycle phase was determined from the incorporation of propidium iodide (PI). Cells were seeded in triplicate and analysed 24 h after plating by flow cytometry. The fraction of cells in each phase of the cell cycle is indicated in the graphs. Treatment with IGFBP-2 induced the S- and G2/M-phase entry in a dose-dependent manner. (Upper) left: control; right: 125 ng ml<sup>-1</sup> IGFBP-2. (Lower) left: 250 ng ml<sup>-1</sup> IGFBP-2; right: 500 ng ml<sup>-1</sup> IGFBP-2. Results are presented as mean  $\pm$  s.e. of triplicate samples from three independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$ .

receptors, it was hypothesised that IGFBP-2 and integrin receptors can interact in glioblastoma cells. mRNA expression data obtained by microarray for integrin receptors and ERK in 154 clinical samples was subjected to cluster and correlation analyses. Integrin  $\beta 1$  was highly expressed in glioblastoma cells, compared with low-grade gliomas and normal brain tissue ( $P < 0.001$ ; Supplementary Figure 3C). Moreover, the expression of integrin  $\beta 1$  was significantly correlated with that of ERK ( $R = 0.490$ ;  $P < 0.001$ , Supplementary Figure 3E). These results were validated on publicly available data sets (Sun, *et al*, 2006); (Supplementary Figure 3D).

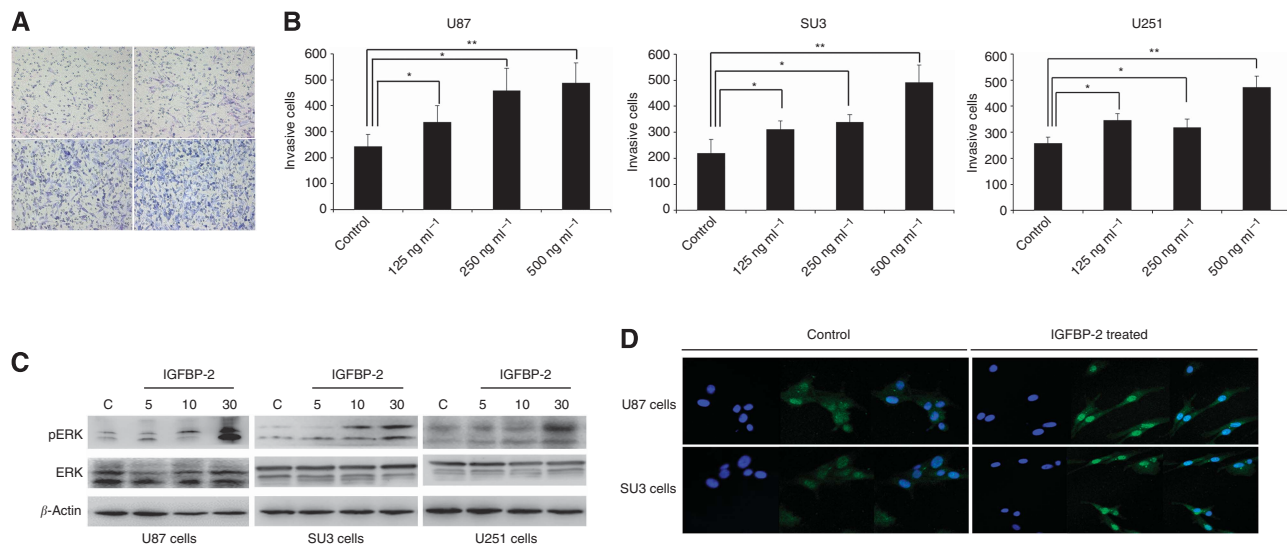
First, integrin  $\beta 1$  expression was stably knocked down in U87 cells by transfection with an integrin  $\beta 1$ -specific shRNA. The loss of integrin  $\beta 1$  expression was confirmed by western blot analysis (Figure 4A). Integrin  $\beta 1$  knockdown resulted in a significant decrease in cell proliferation and invasion. Moreover, integrin  $\beta 1$  knockdown also abrogated exogenous IGFBP-2-induced ERK activation, tumour cell proliferation, and invasion (Figures 4B–G).

Then, to further investigate the role of integrin  $\beta 1$  in IGFBP-2-induced proliferation and invasion, U87, SU3, and U251 cells were pre-treated with anti-integrin  $\beta 1$ -neutralising antibody or non-immune IgG before IGFBP-2 administration. Blocking integrin  $\beta 1$  function by neutralisation inhibited IGFBP-2-induced ERK activation, tumour cell proliferation, cell cycle progression, and invasion (Figures 5 and 6A). Thus, IGFBP-2 mediates its effects on proliferation and invasion through the integrin  $\beta 1$ -ERK pathway in glioblastoma cells.

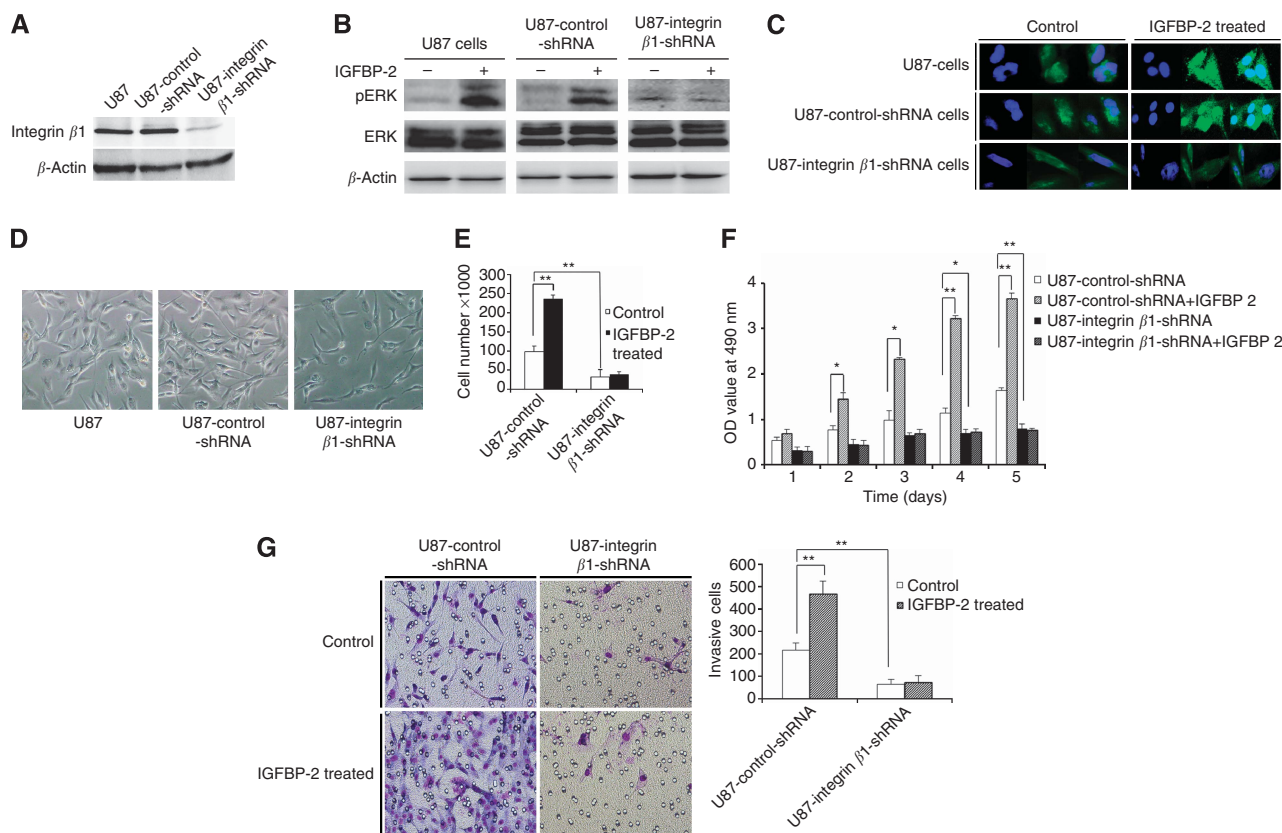
**IGFBP-2 induces chemoresistance to TMZ.** In the previous study, plasma IGFBP-2 levels after standard postoperative radiotherapy plus chemotherapy were found to be correlated with prognosis of glioblastoma patients (Han *et al*, 2014), indicating that exogenous IGFBP-2 may affect TMZ chemosensitivity. As shown in Supplementary Figure 3F, MGMT promoter was methylated in U87, SU3, and U251 cells. In the present study, TMZ administration was found to inhibit significantly the proliferation and invasion of these glioblastoma cells (proliferation was inhibited by 64.2% and invasion was decreased by 44.7%); however, the presence of IGFBP-2 abrogated this effect. Moreover, inhibition of ERK, as well as integrin  $\beta 1$  neutralisation, countered the effect of IGFBP-2 (Figures 6B–E). Nevertheless, endogenous IGFBP-2 overexpression or knockdown had no significant effect on TMZ chemosensitivity in glioblastoma cells (Supplementary Figures 4 and 5). Taken together, these results suggest that rather than endogenous IGFBP-2, exogenous IGFBP-2 induces chemoresistance to TMZ in glioblastoma cells via the integrin  $\beta 1$ -ERK signaling pathway.

## DISCUSSION

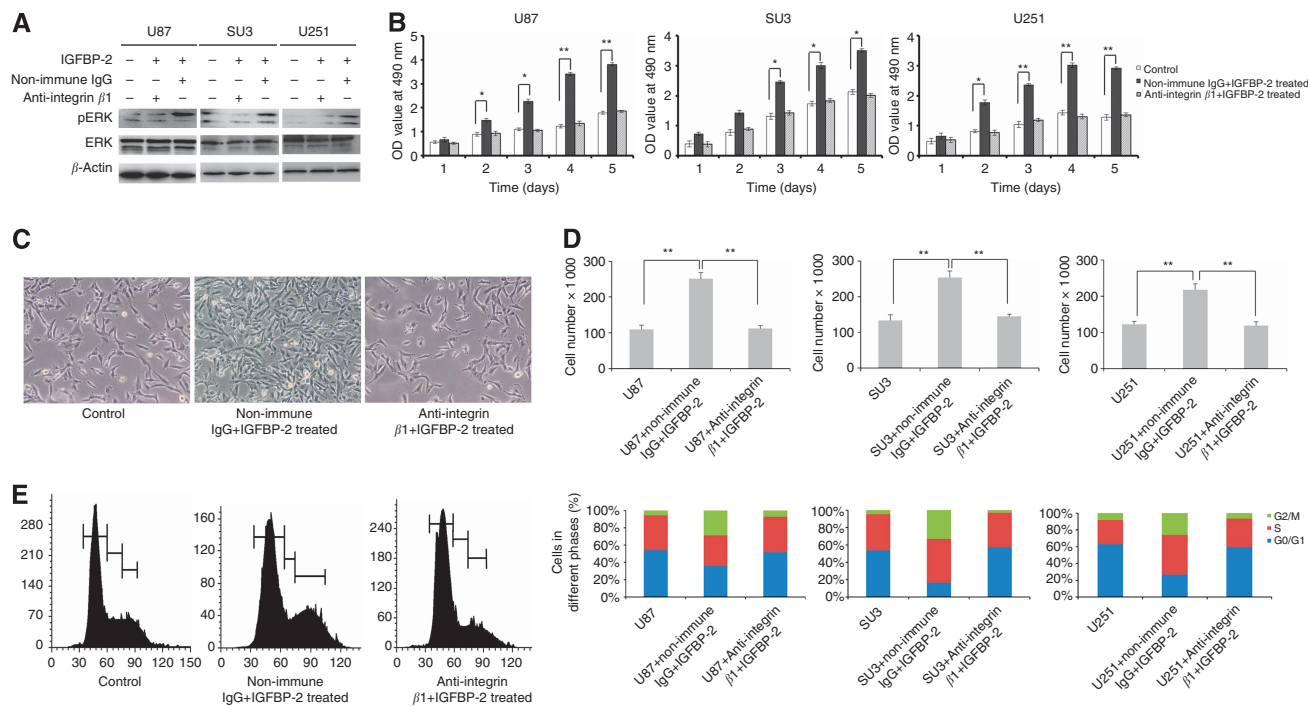
Standard therapy for glioblastoma is postoperative TMZ administration, initially in conjunction with radiotherapy, and



**Figure 3.** Effects of exogenous IGFBP-2 on invasive potential of glioblastoma cells and ERK activation. **(A and B)** Exogenous IGFBP-2 significantly enhanced invasion by glioblastoma cells in a dose-dependent manner, as determined using the Matrigel assay. **(A)** U87 cells were presented as examples. (upper) left: control; right: 125 ng ml<sup>-1</sup> IGFBP-2. (Lower) left: 250 ng ml<sup>-1</sup> IGFBP-2; right: 500 ng ml<sup>-1</sup> IGFBP-2. **(B)** Effects of exogenous IGFBP-2 on the invasive potential of various glioblastoma cells. **(C and D)** Exogenous IGFBP-2 enhances ERK activation in glioblastoma cells. **(C)** A 30-min treatment with 500 ng ml<sup>-1</sup> IGFBP-2 induced an increase in phospho-ERK expression levels, whereas total ERK protein levels were not significantly altered, as assessed by western blot analysis. **(D)** Extracellular signal-regulated kinase (ERK) phosphorylation and nuclear translocation resulting from a 30-min treatment with 500 ng ml<sup>-1</sup> IGFBP-2, as visualised by immunofluorescence staining. The mean values from triplicate samples of three independent experiments are shown. \**P*<0.05 and \*\**P*<0.01.



**Figure 4.** The effects of exogenous IGFBP-2 are integrin  $\beta$ 1-dependent. **(A)** The loss of integrin  $\beta$ 1 expression was confirmed by western blotting in U87-integrin  $\beta$ 1-shRNA cells. **(B and C)** Integrin  $\beta$ 1 knockdown inhibited ERK activation induced by 500 ng ml<sup>-1</sup> exogenous IGFBP-2 as shown by western blot analysis and immunofluorescence staining. **(D–G)** Cell counting **(E)**, MTT assay **(F)**, and Matrigel assay **(G)** showed a significant reduction in the proliferation and invasion of U87-integrin  $\beta$ 1-shRNA cells compared with cells transfected with non-silencing control shRNA and untransfected U87 cells. Moreover, the proliferation and invasion induced by 500 ng ml<sup>-1</sup> exogenous IGFBP-2 were also inhibited by integrin  $\beta$ 1 knockdown. The mean values from triplicate samples of three independent experiments are shown. \**P*<0.05 and \*\**P*<0.01.



**Figure 5.** Insulin-like growth factor binding protein-2-mediated effects on ERK activation, cell proliferation, and cell cycle kinetics are integrin  $\beta 1$ -dependent. **(A)** Integrin  $\beta 1$  neutralisation inhibited IGFBP-2-induced ERK activation, as determined by western blot analysis. **(B–D)** The IGFBP-2-induced increase in cell proliferation was abrogated by integrin  $\beta 1$  neutralisation, as assessed by the MTT assay and cell counting. **(E)** Treatment of cells with IGFBP-2 had no effect on cell cycle after neutralisation of integrin  $\beta 1$ . Results are presented as mean  $\pm$  s.e. of triplicate samples from three independent experiments. \* $P < 0.05$  and \*\* $P < 0.01$ .

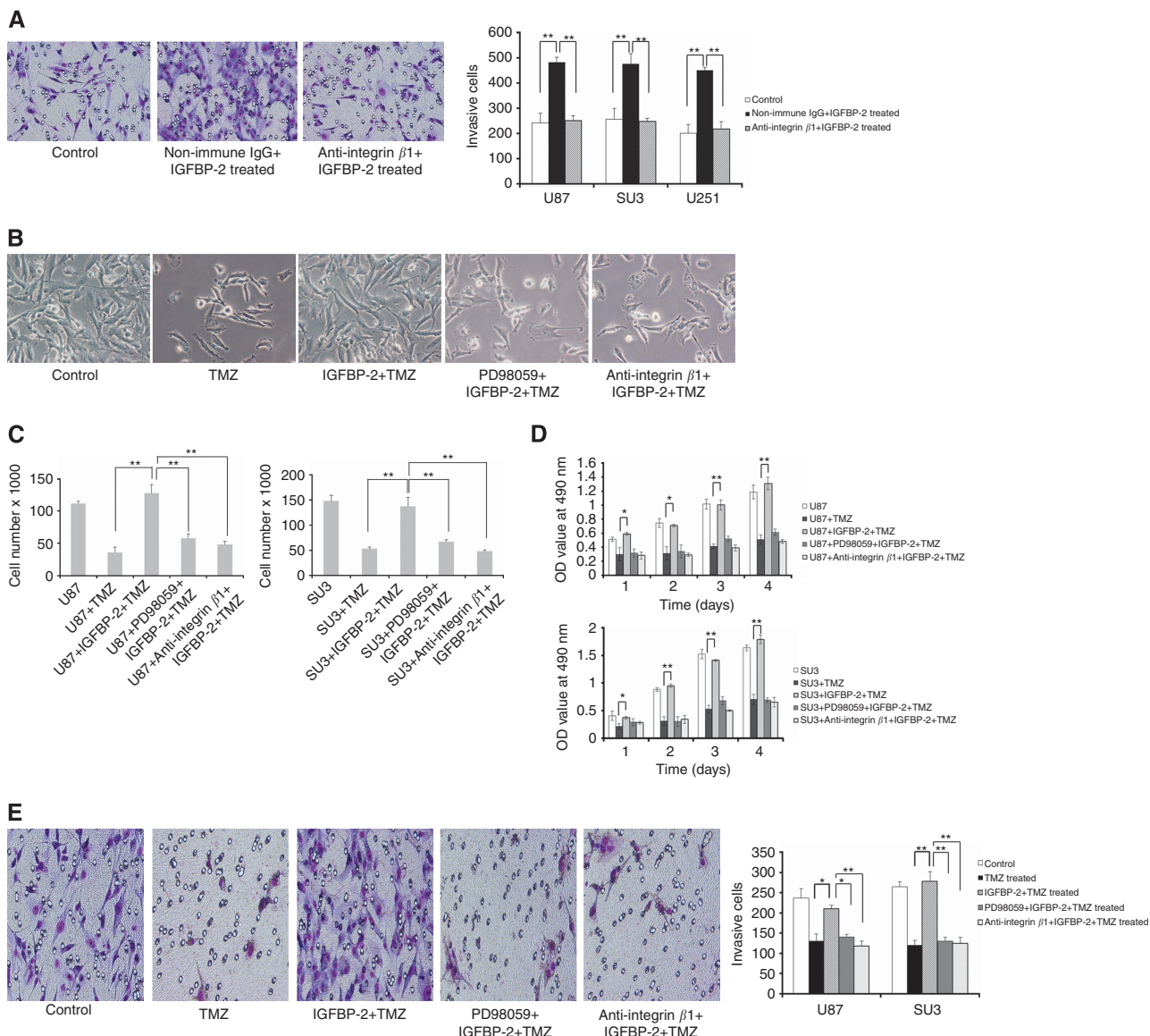
subsequently as maintenance therapy. This strategy has given patients a remarkable survival advantage (Stupp *et al*, 2005). However, there are still large numbers of patients who cannot benefit from this approach owing to the development of chemoresistance to TMZ; thus, identifying factors that determine TMZ chemosensitivity to be clinically relevant. Previous studies have identified several such factors that mediate chemosensitivity through different mechanisms, including O<sup>6</sup>-methylguanine-DNA methyltransferase promoter methylation, isocitrate dehydrogenase 1 mutation, and 1p/19q status among others (Weller *et al*, 2012; Wick *et al*, 2013). Analysing these requires tumour tissue samples that are not always readily available; therefore, molecular markers that can be detected in blood samples would be preferable to assess the response of tumours to chemotherapy and patient prognosis.

It was previously demonstrated that preoperative plasma IGFBP-2 levels were higher in patients with high-grade gliomas, as compared with patients with low-grade gliomas and healthy subjects, and these were significantly correlated with disease recurrence and disease-free survival in glioblastoma patients who received postoperative radiotherapy and chemotherapy (Lin *et al*, 2009). Recently, we have found that after postoperative standard radiotherapy plus chemotherapy, plasma IGFBP-2 levels were significantly higher in elderly than in young patients, and could predict the prognosis of elderly patients (Han *et al*, 2014). Moreover, plasma IGFBP-2 levels were negatively correlated with patients' Karnofsky performance status. Interestingly, the main source of serum IGFBP-2 may not have been the tumour in this case, as the tumour had mostly been removed from the body, and plasma IGFBP-2 levels were remarkably high in some radiologically tumour-free patients after combined therapy. One group has consistently reported a lack of correlation between plasma IGFBP-2 levels and tumour IGFBP-2 expression, as well as tumour size in glioblastoma patients (Lin *et al*, 2009). Insulin-like growth factor binding protein-2 is expressed in many tissues and organs, including the liver and kidney, and is subject to regulation by

numerous factors, such as treatment regimens and nutritional status, which significantly influence plasma IGFBP-2 levels (van den Beld *et al*, 2003). All these results indicated that plasma IGFBP-2 may comprise of IGFBP-2 unrelated to glioma cells. As shown by several researches, increased serum IGFBP-2 levels are associated with tumour progression in a number of different cancers including colon, ovarian, lung, and prostate (el *et al*, 1994; Lee *et al*, 1999; Shariat *et al*, 2002; Baron-Hay *et al*, 2004). Therefore, it is possible that exogenous stimulation from serum IGFBP-2 significantly affects tumour chemosensitivity and progression; in the present study, the effects of exogenous IGFBP-2 on the proliferation, invasion, and chemosensitivity of glioma cells were examined to test this hypothesis.

We examined the levels of IGFBP-2 in the medium of several glioma cell lines and genetically engineered cells. The IGFBP-2 level in the medium was generally lower than 40 ng ml<sup>-1</sup> (Supplementary Figure 1F). However, it was reported that levels of plasma IGFBP-2 of healthy people and glioblastoma patients were 458.68  $\pm$  91.41 and 622.3  $\pm$  201.6 ng ml<sup>-1</sup>, respectively (Lin *et al*, 2009), whereas after standard therapy, the plasma IGFBP-2 level of glioblastoma patients was 637.0  $\pm$  52.3 ng ml<sup>-1</sup> (Han *et al*, 2014). The blood–brain barrier (BBB) of glioblastoma patients might be damaged owing to local inflammatory reaction, seizures and the use of mannitol, and so on, especially after surgery and radiochemotherapy (Marchi *et al*, 2007, 2009). The opening size of BBB could be as large as allowing the delivery of 70 kDa molecule (Chen and Konofagou, 2014), while IGFBP-2 is 36 kDa. It is possible that *in vivo* the level of IGFBP-2 in the local environment of glioblastoma cells could be much higher than 40 ng ml<sup>-1</sup> (even near the plasma IGFBP-2 levels) owing to the permeation of serum IGFBP-2 through the leakage of BBB. Thus, the exogenous IGFBP-2 concentration used in this study was from 125 to 500 ng ml<sup>-1</sup>.

Insulin-like growth factor binding protein-2 contains an RGD adhesion motif that is a known integrin-binding domain



**Figure 6.** Insulin-like growth factor binding protein-2 induces chemoresistance to TMZ via the integrin  $\beta$ 1-ERK pathway. **(A)** Neutralisation of integrin  $\beta$ 1 reversed the IGFBP-2-induced increase in invasive potential in glioblastoma cells, as assessed by the Matrigel assay. **(B–D)** Temozolomide (TMZ) administration significantly inhibited glioblastoma cell proliferation; this effect was abrogated by treatment with 500 ng ml<sup>-1</sup> IGFBP-2. This IGFBP-2-induced increase in chemoresistance to TMZ was not observed when integrin  $\beta$ 1 was neutralised or ERK was blocked. **(E)** Insulin-like growth factor binding protein-2 reversed the inhibition of invasion induced by TMZ through the activation of integrin  $\beta$ 1-ERK signaling. Results are presented as mean  $\pm$  s.e. of triplicate samples from three independent experiments. \* $P$ <0.05 and \*\* $P$ <0.01.

(Kawai *et al*, 2011), suggesting that exogenous IGFBP-2 may function through binding integrin on the surface of glioma cells. Although there is no direct evidence that IGFBP-2 binds to integrin  $\beta$ 1 receptors, this hypothesis was supported by our results. In the present study, exogenous IGFBP-2 was found to stimulate proliferation, invasion, and chemoresistance to TMZ in an integrin  $\beta$ 1-dependent manner in glioblastoma cells. The invasion promoting role of IGFBP-2 on glioma cells was supported by Wang *et al* (2006) and Mendes *et al* (2010). Other studies have also described the integrin-binding function of IGFBP-2, especially of integrin  $\beta$ 1, in various cancer cells. Insulin-like growth factor binding protein-2 specifically bound integrin  $\alpha$ 5 $\beta$ 1 at the cell surface in a Ewing’s sarcoma cell line, resulting in increased cell migration (Schutt *et al*, 2004). In addition to cell migration, proliferation and chemoresistance can also be stimulated by exogenous IGFBP-2, acting via integrin receptors, in some tumour cells such as mammary tumour cells (Perks *et al*, 2007; Foulstone *et al*, 2013) and prostate cancer

cells (Uzoh *et al*, 2011). As observed in the microarray analysis, integrin  $\beta$ 1 is highly expressed in glioblastoma clinical samples, suggesting the possibility of a physical interaction with IGFBP-2, and predicting poor outcome for TMZ chemotherapy and rapid progression of glioblastoma in patients with high serum IGFBP-2 levels. This was verified by the study of Lin *et al* (2009) and our study Han *et al* (2014).

Integrin activation can, in turn, activate ERK signaling, which transduces cell-specific growth signals and is a major participant in cancer-related cellular processes (Lin *et al*, 1997; Renshaw *et al*, 1997). Expression of integrin  $\beta$ 1 was significantly correlated with that of ERK in glioma clinical samples, reflecting an interaction between the two signaling pathways. In this study, exogenous IGFBP-2 was shown to activate ERK in an integrin  $\beta$ 1-dependent manner in glioblastoma cells, and this was essential for IGFBP-2-induced proliferation, invasion, and chemoresistance to TMZ. Therefore, the effects of exogenous IGFBP-2 were mainly exerted



through the integrin  $\beta$ 1-ERK signaling pathway. After IGFBP-2 administration, the cellular morphology might change (Figure 3D) as a result of the interaction between IGFBP-2 and integrin  $\beta$ 1 (Wu *et al*, 2011). Similar phenomena were also present in tumours other than glioblastoma. Exogenous IGFBP-2 stimulated proliferation and activated the ERK pathway in NIH-OVCAR3 human epithelial ovarian carcinoma cells (Chakrabarty and Kondratik, 2006), and promoted proliferation in rat growth plate chondrocytes via MAPK/ERK1/2. This activity of IGFBP-2 was associated with cell membrane binding (Kiepe *et al*, 2008). In contrast, endogenous IGFBP-2 overexpression may activate different signaling pathways and have other effects as shown by this study. Consistently, Mendes *et al* (2010) reported that rather than ERK, JNK was specifically involved in IGFBP-2-mediated migration in IGFBP-2-overexpressing glioma cells. Different from exogenous IGFBP-2, which affects both invasion and proliferation, endogenous IGFBP-2 in glioblastoma cells may not regulate cell growth (Wang *et al*, 2003). And, endogenous IGFBP-2 overexpression cannot increase the IGFBP-2 in the medium to a high enough level (Supplementary Figure 1F). Thus, exogenous IGFBP-2 administration is more suitable for exploring the mechanisms by which serum IGFBP-2 mediates chemosensitivity and tumour recurrence since, as stated above, serum IGFBP-2 may not originate from the tumour. Moreover, our study emphasises the significance of therapeutic intervention for glioblastoma patients to decrease plasma IGFBP-2 levels. The effectiveness of chemical reagents or specific antibody targeting plasma IGFBP-2 should be explored in future *in vivo* studies using suitable animal models.

## CONCLUSIONS

To summarise, in the present study, exogenous IGFBP-2 was shown to stimulate proliferation, invasion, and chemoresistance to TMZ via the integrin  $\beta$ 1-ERK pathway in glioblastoma cells. These findings have clinical implications. First, they reveal a mechanism by which serum IGFBP-2 can affect the prognosis of glioblastoma patients who received postoperative standard radiotherapy plus TMZ chemotherapy. Second, endogenous IGFBP-2 overexpression and exogenous IGFBP-2 stimulation may have different pathophysiological effect via different signaling pathways. Furthermore, targeting the integrin  $\beta$ 1-ERK pathway may represent a new approach for the treatment of glioblastoma in patients with high serum IGFBP-2 levels.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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