Integrin $\alpha 6 \beta 4$ is downregulated in mutant IDH1 oligodendrogliomas, promotes glioma growth, and associates with a worse outcome in glioma patients

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Abstract

The integrin $\alpha 6\beta 4$ is a laminin receptor that associates with aggressive behavior in many cancers, though its significance in gliomas has not been established. Mutations in *isocitrate dehydrogenase 1* and *2* (IDH^{mut}) are common in gliomas, especially 1p/19q co-deleted oligodendrogliomas, and correlate with improved patient survival. We sought to determine whether there is a link between integrin $\beta 4$ and malignant behavior in gliomas. An inverse relationship was identified between IDH^{mut} and integrin $\beta 4$ expression in a glioma TMA ($P < 0.0001$) and in TCGA grade II-IV gliomas ($P < 0.0001$). Methylation of CpG sites in the *ITGB4* promoter is significantly higher in IDH^{mut} gliomas when compared to wild-type tumors, and both *ITGB4* methylation and reduced *ITGB4* mRNA are not only most prominent in the 1p/19q co-deleted subset of IDH^{mut} tumors, but fully account for the lower *ITGB4* expression observed in IDH^{mut} gliomas. In fact, *ITGB4* is one of the most downregulated genes in IDH^{mut} 1p/19q co-deleted gliomas compared to IDH^{mut} 1p/19q intact gliomas ($P = 3.2 \times 10^{-39}$). In patient-derived glioma cells, we found that integrin $\beta 4$ is enriched in the stem-like population, and that knockdown of integrin $\beta 4$ slows glioma growth *in vitro* ($P < 0.001$) and *in vivo* ($P = 0.01$). Lower *ITGB4* mRNA levels were also associated with longer overall survival in multiple patient cohorts ($P = 0.0005$; $P < 0.0001$). These data suggest that integrin $\beta 4$ increases glioma malignancy, and that its relative paucity may contribute to the better prognosis in *IDH1/2* mutant, 1p/19q co-deleted oligodendrogliomas.

Keywords: Integrin; IDH; Glioma; Glioblastoma; 1p/19q; oligodendroglioma

Introduction

Gliomas account for 30% of all primary tumors of the central nervous system (CNS) and represent about 80% of malignant CNS tumors (13). Glioblastoma multiforme (GBM) accounts for over 50% of gliomas and carries an extremely poor prognosis, with a median survival of around 14 months even when treating with temozolomide and radiation therapy (57). A key reason for this dismal prognosis is the diffusely infiltrative nature of these tumors, rendering complete surgical excision impossible. Diffuse low and intermediate-grade gliomas have variable outcomes that depend on the underlying genomic alterations present in the tumor, with median survival ranging from 1 to over 12 years (8).

Mutations in *isocitrate dehydrogenases types 1 and 2* (*IDH1/2*) are present in the majority of World Health Organization (WHO) grade II and III gliomas, and are also characteristic of secondary GBMs (21, 26, 46, 61). Both enzymes normally oxidize isocitrate to α -ketoglutarate, but point mutations at R132 in *IDH1* or R172 in *IDH2* produce neoenzymatic activity, reducing α -ketoglutarate to D-2-hydroxyglutarate (D2HG) (12, 60). D2HG may contribute to gliomagenesis by promoting global DNA and histone hypermethylation and suppressing cellular differentiation (37). Yet these mutations are powerful favorable prognostic markers, as tumors with mutant *IDH1* or *IDH2* (henceforth referred to as “IDH^{mut}”) tend to be much less aggressive compared to their wildtype counterparts (24). However, IDH^{mut} gliomas that also contain 1p/19q co-deletion have an even better prognosis compared to IDH^{mut} gliomas lacking 1p/19q co-deletion: in an analysis of 654 grade II-IV gliomas from The Cancer Genome Atlas (TCGA), the median survival of IDH^{mut}, 1p/19q co-deleted gliomas was 134.3 months, compared to 87.5 months for IDH^{mut}, 1p/19q intact gliomas and 14.9 months for IDH1^{wt} gliomas (**Supplemental Figure 1**; $P < 0.0001$). The reasons for this survival difference between the major subsets of IDH^{mut} gliomas is incompletely understood.

Integrins are extracellular matrix receptors that regulate a number of diverse cellular processes including survival, motility, angiogenesis and proliferation (19, 41, 58). In addition to

their normal functions, integrins can also promote tumor initiation and progression, in part through their ability to enhance receptor tyrosine kinase activity and stimulate proliferative signaling (19). These effects are particularly notable for the integrin $\alpha 6 \beta 4$, as it promotes invasion by altering immediate downstream signaling to phosphatidylinositol 3-kinase (PI3K), AKT, MAPK and the Rho small GTPases (19, 36, 42, 43, 53). The integrin $\alpha 6 \beta 4$ is comprised of an α and a β subunit that, combined, give the integrin specificity for various laminins. The integrin $\beta 4$ subunit (referred to herein as integrin $\beta 4$) has a long cytoplasmic signaling domain that contributes to its ability to promote invasion and metastasis (55). The integrin $\beta 4$ has also been shown to regulate the expression of genes involved in cell motility, invasion and the metastatic cascade (9). Overexpression of the integrin $\beta 4$ has been observed in a variety of neoplasms where it is associated with aggressive biology, invasiveness, and poor prognosis (6, 27, 38, 55, 56). Furthermore, integrins facilitate stem cell properties in a number of human malignancies (52), including integrin $\alpha 6$, also known as CD49f, which is the binding partner for integrin $\beta 4$.

The contribution of integrin $\beta 4$ to carcinoma progression has been the subject of extensive research in other cancer types; however, expression and function of the integrin $\beta 4$ has not been studied thoroughly in gliomas. It is not known whether integrin $\beta 4$ expression is regulated by driver mutations such as in *IDH1/2*, or whether expression of the integrin $\beta 4$ is influenced by recurrent cytogenetic abnormalities such as 1p/19q co-deletion. As the integrin $\beta 4$ is known to promote proliferation in other neoplasms, we sought to determine whether integrin $\beta 4$ contributes to tumor growth and self-renewal in gliomas, and whether differential regulation of *ITGB4* could be responsible for the improved prognosis observed in the *IDH*^{mut}, 1p/19q co-deleted subset. We also sought to determine whether integrin $\beta 4$ is associated with clinicopathologic variables such as tumor grade and patient survival. Our findings may help explain why *IDH*^{mut}, 1p/19q co-deleted gliomas are even less aggressive than *IDH*^{mut} gliomas with intact 1p/19q.

Materials and Methods:

Tissue Microarray Construction

Formalin-fixed, paraffin-embedded (FFPE) glioma tissues were retrieved from the pathology archives at the University of Kentucky. Deidentified tissue microarrays (TMAs) were constructed from the gliomas as previously described (17). A total of 104 cases comprised the TMAs, including 9 nonneoplastic controls (cortical dysplasia), 9 grade II astrocytomas, 11 grade III astrocytomas, 12 anaplastic oligodendrogliomas, 16 grade II oligodendrogliomas, and 47 grade IV GBMs. Treatment and outcomes were obtained via the Kentucky Cancer Registry. Institutional Review Board approval was obtained prior to collecting the archival tissues for TMA construction and patient survival data.

Immunohistochemistry

Glioma TMA sections (4 μ m) were stained using a rat monoclonal primary antibody to the integrin β 4 subunit (CD104; clone 439-9B; BD Pharmingen, San Jose, CA) at a concentration of 5 μ g/ml according to a previously described protocol (11). Integrin β 4 expression was scored using a semiquantitative scale as follows: negative (0), minimal staining (1), weakly positive (2), moderately positive (3), strongly and diffusely positive (4). Endothelial cells were used as internal positive controls while non-neoplastic tissue cores served as negative controls. Scoring was done while blinded to WHO grade, IDH^{mut} status, and other clinicopathological variables. Results from each of three tissue cores were averaged together to produce a final score for each tumor.

R132H IDH1 immunohistochemistry of TMAs was performed as previously described (17). Of note, 5 TMA cases were immunonegative for R132H IDH1 but were clinically suspected of having a less common IDH1 or IDH2 mutation based on mutation-associated variables like WHO grade and younger patient age. Of those 5, a single anaplastic astrocytoma turned out to have R132S IDH1 via pyrosequencing, as per standard clinical workup of gliomas.

Cell culture

The LN18 (CRL-2610) glioma cell line was purchased directly from ATCC and was cultured in RPMI 1640, 10% fetal bovine serum, and 1% penicillin streptomycin, purchased from Life Technologies (Carlsbad, CA). Cells were cultured in humidified conditions at 37°C and 5% CO₂.

Cancer stem cell (CSC) derivation and xenograft maintenance

Established patient-derived GBM xenografts were previously reported (3, 20, 51) and were obtained via a material transfer agreement from Duke University (T4121) or the Mayo Clinic (GBM10). Xenografts were originally established under IRB-approved protocols in a de-identified manner using excess tissue taken from consented patients. For experimental studies, GBM cells were dissociated from established xenografts under Cleveland Clinic-approved Institutional Animal Care and Use Committee protocols as described (1). Xenografts were passaged in immune-deficient NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice (obtained from The Jackson Laboratory, Bar Harbor, ME, USA). Freshly dissociated human GBM cells were unilaterally injected into the flank of 6-week-old female mice, and animals were sacrificed by CO₂ asphyxiation and secondary cervical dislocation when the tumor volume exceeded 5% of the animal's body weight.

Tumor dissociation and GBM cell culture

Xenografted tumors were dissected and mechanically dissociated using a papain dissociation kit (Worthington Biochemical Corporation, Lakewood, NJ, USA) as previously described (1, 2, 20, 23, 31, 44). After dissociation, cells were cultured overnight in Neurobasal medium (Life Technologies, Carlsbad, CA, USA) supplemented with B27 (Life Technologies), 1% penicillin/streptomycin (Life Technologies), 1 mM sodium pyruvate, 2 mM L-glutamine, 20 ng/mL EGF (R&D Systems, Minneapolis, MN, USA), and 20 ng/mL FGF-2 (R&D Systems) in a humidified incubator with 5% CO₂.

Self-renewal of integrin β 4-high and -low populations using flow cytometry

After dissociation and overnight culture of cells isolated from xenografts, fluorescence-activated cell sorting (FACS) was used to isolate cells based on surface levels of integrin β 4 as described for other cell surface receptors (1, 20, 31). Approximately 1×10^6 cells were filtered through a 70- μ m cell strainer, centrifuged, and resuspended in 200 μ l of FACS buffer (5% BSA in unsupplemented Neurobasal medium). Two microliters of mouse anti-integrin β 4 (Abcam clone M126, ab29042) conjugated to PE/Cy7 (Abcam PE/Cy7® Conjugation Kit, ab102903) was added and incubated for 1 hour in the dark. Cells were then washed three times in unsupplemented Neurobasal medium and resuspended in 500 μ l Neurobasal complete. Using DAPI as a control for live cells and an isotype control for signal specificity, the 20% of cells with the highest and 20% of cells with the lowest expression of integrin β 4 were plated using a BD FACS Aria II at increasing cell concentrations (1, 5, 10, and 20 cells/well) in 96-well plates. After 14 days, the number of wells containing spheres larger than 10 cells across was counted, and the stem cell frequency was calculated using the Walter and Eliza Hall Institute Bioinformatics Division ELDA analyzer (<http://bioinf.wehi.edu.au/software/elda/>)(25).

Immunoblotting

Cells were harvested and lysed in RIPA buffer (150 mM NaCl, 0.5 mM EGTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 50 mM Tris-HCl pH 7.4, 15 μ g/ml protease inhibitor cocktail, 1 mM PMSF, 50 mM NaF and 10 mM sodium pyrophosphate). Total cell lysates (80 μ g) were subjected to 8% SDS-PAGE, transferred and immunoblotted with mouse anti-CD104 (integrin β 4, BD Bioscience) antibody; β -actin antibodies (Sigma-Aldrich) were used as the loading controls. Data are representative of at least three experiments.

Quantitative real time PCR (Q-PCR)

Total RNA was extracted using TRizol reagent (Life Technologies/ThermoFisher, Grand Island, NY). Total RNA (1 μ g) was used to reverse-transcribe into cDNA using the High Capacity

cDNA Reverse Transcription Kit (Applied Biosystems/ThermoFisher, Grand Island, NY). Target sequence for *ITGB4* shRNA: #4: 5'CGGCTCCTCAGCTACTCCATCCTTC-TCGAGAAGGATGGAGTAGCTGAGGAGTTTTTG and #5: 5'CCGGCCAGCGACTACAC-TATTGGATCTCGAGATCCAATAGTGTAGTCGCT-GGTTTTTG. Expression of *ITGB4* was assessed by Comparative Ct ($\Delta\Delta C_t$) using commercially available probes and Master Mix reagent and performed on a StepOnePlus™ 96-well instrument as described by the manufacturer (Applied Biosystems). The expression level of each gene was normalized by β -actin RNA and reported as a relative level.

ITGB4 knockdown by shRNA and Xenograft experiments

Patient derived primary GBM stem cells were obtained and isolated as described above. Cells were labeled with CD133 antibody conjugated with APC and sorted to enrich for CD133+ stem cells using FACS. The CD133+ cells were then cultured in Neurobasal Media supplemented with B27 without vitamin A (Invitrogen), 10 ng/mL bFGF (Life Technologies, PHG0026), and 10 ng/mL EGF (Life Technologies, PHG0313). For stable knockdown of *ITGB4* in CD133+ primary GBM stem cells, lentivirus was produced and cells were infected as described previously (54). The cells were then placed under the selection of puromycin. The knockdown efficiency of *ITGB4* was assessed by immunoblotting and Q-PCR. Intracranial tumor implantation was performed by injecting 10 μ L (10^5 cells) into the right cerebral cortex of 5-week-old female NU/J nude mice as previously described (3). Survival was tracked after tumor implantation. Any mouse showing distress, neurological signs (lethargy, hemiparesis, ataxia, seizures) or weight loss of greater than 10% was euthanized in accordance with a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee.

Bioinformatics analysis of external cohorts

To validate TMA data with external cohorts, the relationship of integrin $\beta 4$ to tumor type and patient survival was done using the Oncomine microarray database (Oncomine,

Compendia Bioscience, Ann Arbor, MI <http://www.oncomine.org>). Specific correlations between *ITGB4* mRNA and IDH^{mut} status were done via direct query of The Cancer Genome Atlas (TCGA Research Network, <http://cancergenome.nih.gov/>). Additional survival data in a separate cohort of GBMs was obtained via the National Cancer Institute REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT, <https://caintegrator.nci.nih.gov/rembrandt/>). TCGA and REMBRANDT datasets were visualized and downloaded using the GlioVis web application (<http://gliovis.bioinfo.cnio.es/>) (7). Datasets include TCGA grade II-IV gliomas ($N = 654$) analyzed using RNA-Seq, and REMBRANDT gliomas analyzed using Affymetrix HG U133 v2.0 Plus microarrays.

Statistical Analysis

Differences between groups were analyzed via two-tailed Student's t-test, one-way ANOVA with post hoc Tukey's test, or Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons where noted. Significance was reached when $P < 0.05$. Survival differences were assessed via log-rank tests for univariate analyses and Cox proportional hazards for multivariate. All univariate statistical analyses were performed using GraphPad Prism, Version 7.01 (Graph Pad Software, La Jolla, CA), and multivariate analyses were done with www.statpages.org.

Results

To determine the range of integrin $\beta 4$ expression in gliomas, immunohistochemistry (IHC) for the integrin $\beta 4$ subunit was performed on a set of glioma TMA sections that included 104 cases (see Materials and Methods). We found that integrin $\beta 4$ expression was uniformly weak in nonneoplastic control tissues as measured by IHC, but that there was a greater range of staining intensities in grade II-IV gliomas (**Figures 1A-B**). The overall mean staining intensity was significantly higher in grade IV GBMs when compared to grade II-III tumors and nonneoplastic controls ($P < 0.0001$; **Figure 1B**). TCGA analysis confirmed this association, showing elevated *ITGB4* mRNA in GBMs compared to grade II and III tumors, though the difference in means was relatively modest (**Figure 1C**).

IDH^{mut} has been shown to be a strong favorable prognostic factor in diffusely infiltrative gliomas (24), and high integrin $\beta 4$ expression can contribute to cancer invasiveness and aggression (55). Therefore, we sought to determine if a correlation between these two molecules existed. Interestingly, a strong inverse relationship between IDH^{mut} and integrin $\beta 4$ expression was observed in our TMA cohort ($P < 0.0001$; **Figure 2A-B**). Likewise, an analysis of TCGA gliomas showed that IDH^{mut} tumors had lower average *ITGB4* mRNA levels than did IDH^{wt} tumors ($P = < 0.0001$; **Figure 2C**).

IDH^{mut} causes global hypermethylation and gene suppression (24). Therefore, we examined CpG sites in the integrin $\beta 4$ gene (*ITGB4*) and compared its methylation status in IDH^{wt} versus IDH^{mut} tumors. We identified 6 CpG sites in *ITGB4* that are significantly hypermethylated in IDH^{mut} tumors when compared to IDH^{wt} tumors (**Figure 3A, Supplemental Table 1**). Methylation at each of the 6 sites individually correlates with reduced *ITGB4* mRNA (**Figure 3B, Supplemental Table 1**), and 4 of the sites are in the *ITGB4* promoter.

Gliomas that harbor IDH^{mut} along with 1p/19q co-deletion are histologically oligodendrogliomas and have a particularly good prognosis, even when compared to IDH^{mut} astrocytomas lacking this co-deletion (8, 14). As *ITGB4* promotes tumor invasiveness and

aggression, we determined whether differential regulation of *ITGB4* could contribute to the substantially better prognosis observed in this IDH^{mut} subset. By comparing methylation of CpG islands in the *ITGB4* gene between IDH^{mut}, 1p/19q intact and IDH^{mut}, 1p/19q co-deleted tumors, we found that, while methylation was increased in IDH^{mut}, 1p/19q intact astrocytomas relative to IDH^{wt} gliomas, it is even higher (up to 3.7 fold) in the IDH^{mut}, 1p/19q co-deleted subset of tumors (**Figure 4A**). Remarkably, only the IDH^{mut}, 1p/19q co-deleted oligodendrogliomas showed reduced *ITGB4* mRNA relative to IDH^{wt} gliomas, not the IDH^{mut}, 1p/19q intact astrocytomas ($P < 0.0001$; **Figure 4B**). Among 20,530 genes analyzed in the TCGA via RNA-Seq, *ITGB4* is the 56th most downregulated gene in IDH^{mut} 1p/19q co-deleted gliomas relative to IDH^{wt} 1p/19q intact gliomas $P=3.2 \times 10^{-39}$, **Supplemental Table 2**). We also compared integrin $\beta 4$ IHC between the IDH^{mut} and IDH^{mut}, 1p/19q co-deleted subsets in the TMA. However, we did not detect a significant difference in expression in this cohort ($P = 0.60$; **Figure 4C**); the TMA cohort was likely underpowered to detect such a difference.

Glioma stem cells (GSCs) represent a subset of cells that contribute to glioma growth and self-renewal, and are believed to promote therapy resistance and evasion of anticancer host immunity (32). Previous studies have demonstrated that integrins can mediate cancer stem-cell properties (52), and this led us to investigate the relationship between integrin $\beta 4$ expression and self-renewal in patient-derived, CD133+ IDH^{wt} primary GBM cells. Tumors were sorted for integrin $\beta 4$ surface expression using FACS, and limiting dilution analysis was used to estimate the stem cell frequency of cells isolated from freshly dissociated GBM xenografts. We found that across multiple specimens, integrin $\beta 4$ -high cells had higher stem cell frequencies than was observed in integrin $\beta 4$ -low cells (**Figure 5A-D**). To further investigate the role of integrin $\beta 4$ in glioma growth, we performed loss-of-function studies in cell lines and in an intracranial patient-derived xenograft model of glioblastoma. Efficient knockdown of *ITGB4* (**Figure 6A-B**) in LN18 cells dramatically decreased *in vitro* glioma growth ($P < 0.001$; **Figure 6C**), and significantly extended the survival of mice engrafted with patient-derived, CD133+ IDH^{wt} primary GBM cells

(median survival 24.5 days vs. 33 days ; $P = 0.01$; **Figure 6D-E**).

To date, no data on integrin $\beta 4$ and outcome in gliomas has been published. In our TMA cohort, elevated integrin $\beta 4$ expression was significantly associated with shorter overall survival among all grade II-IV gliomas (median survival not reached for integrin $\beta 4$ IHC score <1 ; 63.5 months for 1-1.9; 18.1 months for 2+, $P = 0.0005$; **Figure 7A**). We also examined the relationship between *ITGB4* mRNA expression and patient survival in the TCGA glioma cohort ($N = 656$), where we found that increasing levels of *ITGB4* mRNA correlated with worse overall survival (**Figures 7B**). Moreover, in the TCGA glioma cohort, *ITGB4* expression quartile was found to be an independent prognostic factor, even when including variables such as IDH^{mut} and 1p/19q co-deletion status (**Table 1**).

Discussion

Our results demonstrate that integrin $\beta 4$ is highly expressed in GBMs compared to lower grade gliomas and non-neoplastic brain tissue. We found that elevated integrin $\beta 4$ expression is an adverse prognostic marker, which is consistent with work done in other cancers showing that integrin $\beta 4$ overexpression imparts more aggressive behavior (55). We provide evidence that integrin $\beta 4$ is enriched in the stem-like subset of glioma cells and that it contributes to glioma growth and self-renewal. We find that integrin $\beta 4$ expression is inversely correlated with IDH^{mut} status, and that methylation and expression of *ITGB4* differ according to 1p/19q co-deletion status. Given the role of integrin $\beta 4$ in tumor malignancy, these findings to help explain why IDH^{mut}, 1p/19q co-deleted oligodendrogliomas generally are even less aggressive than IDH^{mut} astrocytomas with intact 1p/19q (50, 62).

Global hypermethylation contributes to tumorigenesis by virtue of its ability to suppress cellular differentiation, yet the effects of generalized hypermethylation can eventually lead to changes that inhibit tumor malignancy (24). For example, IDH^{mut} is associated with hypermethylation of the *MGMT* promoter and suppression of *MGMT* (40), thereby reducing the ability of the tumor to repair DNA damage caused by alkylating chemotherapeutic agents such as temozolomide (22). We find that methylation of CpG islands in the *ITGB4* promoter is significantly higher in IDH^{mut} tumors with 1p/19q co-deletion, and that this hypermethylation is associated with a concomitant reduction in *ITGB4* gene expression. In agreement with these findings, epigenetic regulation of integrin $\beta 4$ expression through promoter hypermethylation and histone modification has been previously described (35).

Mutations in *IDH1* are known to promote global DNA and histone methylation, although the exact genes that are methylated can differ between tumor types (18, 28). We find that while *ITGB4* promoter methylation is generally higher in IDH^{mut} gliomas, this phenomenon is mostly due to the 1p/19q co-deleted subset of IDH^{mut} tumors. At this point, it is unclear what mechanism is responsible for this observation. However, in one study of IDH^{mut} gliomas, *MGMT*

promoter methylation was even higher in 1p/19q co-deleted oligodendrogliomas compared to 1p/19q intact astrocytomas, suggesting that secondary genetic alterations may affect the type and degree of IDH^{mut}-induced methylation (34). Genes such as *FGFR2* and *TNFRFS1B* have been shown to be differentially methylated in 1p/19q co-deleted oligodendrogliomas compared to IDH^{mut} astrocytomas (28). Furthermore, secondary mutations, such as in *ATRX* and *TP53* in 1p/19q intact gliomas, and *CIC* and *FUBP1* in 1p/19q co-deleted gliomas, may also influence which genes are hypermethylated in IDH^{mut} tumors.

Our results provide further evidence for the importance of integrins in the GSC population, as we found that integrin $\beta 4$ is enriched in CD133+ glioma stem cells and that it contributes to glioma self-renewal. A variety of stem cell populations use integrins to associate with extracellular matrix proteins, a relationship that is important for stem cell homing and niche interaction (10). The integrin $\beta 4$ interacts with laminins in the extracellular matrix (55), and laminin-integrin signaling plays a role in the development of neural stem cells (33). In the neoplastic setting, high expression of integrin $\alpha 6$ is a characteristic of GBM stem cells, and its interaction with laminin $\alpha 2$ in the extracellular matrix mediates stem cell properties like self-renewal and tumor formation (29, 30). In glioblastoma, the integrins $\alpha 3$, $\alpha 6$ and $\beta 1$ cooperate with CD151 to regulate stemness and self-renewal (59). Integrin $\beta 4$ is also a marker for cancer stem cells in other tumor types, and a recent paper demonstrated that integrin $\beta 4$ identifies a stem-cell enriched population of partially mesenchymal cells in patients with triple negative breast carcinoma (6).

In addition to their ability to mediate stem-cell properties, integrins have been shown to contribute to other aspects of glioma biology. Integrin $\beta 8$ is expressed in gliomas, positively correlates with overexpression of *Egfr* and *Pdgfr- α* , and may promote *Fak* and *Erk* activity in regions rich in extracellular matrix (49). The $\alpha \nu \beta 3$ and $\alpha \nu \beta 5$ integrins have been implicated in tumor-induced angiogenesis (16), which is a common feature of GBMs (15). The $\alpha \nu \beta 3$ and $\alpha \nu \beta 5$ integrins increase with increasing histologic grade in gliomas, and tumor cells at the periphery of

high-grade gliomas have higher $\alpha\beta3$ expression (4). Attempts to target $\alpha\beta3$ and $\alpha\beta5$ in GBM led to multiple clinical trials with cilengitide, a small molecule inhibitor of the $\alpha\beta3$, $\alpha\beta5$ and $\alpha5\beta1$ integrins (39).

To date, few studies have examined integrin $\beta4$ expression in gliomas. However, expression of the integrin $\beta4$ has been identified in astrocytomas, oligodendrogliomas, and GBMs, as well as in reactive astrocytes (5, 45, 47). One study found that the integrin $\beta4$ was expressed in a mouse model of GBM, that integrin $\beta4$ was present early in tumor development, and that expression was more intense within the neoplastic cells of proliferative centers (48).

In summary, we demonstrate for the first time that expression of the $\beta4$ integrin is specifically decreased in IDH^{mut}, 1p/19q co-deleted oligodendrogliomas, and that high integrin $\beta4$ correlates with poor prognosis in glioma patients. Further work is needed to clarify the mechanisms by which IDH^{mut} and 1p/19q co-deletion may alter the expression of cellular adhesion molecules, and to examine the role of integrin $\alpha6\beta4$ in gliomagenesis and invasion. Gaining a deeper understanding of why IDH^{mut} gliomas, especially those with 1p/19q co-deletion, are less aggressive than their IDH^{wt} counterparts will not only help develop ways of targeting IDH^{mut} gliomas, but also of making IDH^{wt} tumors behave more like IDH^{mut} tumors.

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FIGURES AND TABLES

Figure 1: Integrin $\beta 4$ expression by WHO grade.

(A) Representative integrin $\beta 4$ IHC in WHO grade II-IV gliomas, as well as control non-neoplastic brain tissue. The inset in the grade IV integrin panel highlights membranous staining of integrin $\beta 4$ in GBM cells (arrowheads). Magnification = 400x for all images. (B) Semi-quantitative integrin $\beta 4$ IHC in control and grade II-IV gliomas. Overall $P < 0.0001$ via one-way ANOVA, $P < 0.0001$ between grade IV GBMs and each other group via post-hoc Tukey's test. (C) *ITGB4* mRNA in grade II-IV TCGA gliomas. Overall $P < 0.0001$ via one-way ANOVA.

Figure 2: Integrin β4 expression by *IDH* mutation status.

(A) Representative photomicrographs of H&Es and IHC for R132H IDH1 and integrin β4 in IDH^{wt} GBM (left panels) and IDH^{mut} GBM (right panels). Magnification = 400x for all images. (B) Semi-quantitative integrin β4 IHC in IDH^{wt} versus IDH^{mut} gliomas. $P < 0.0001$ via two-tailed t-test. (C) *ITGB4* mRNA in IDH^{wt} and IDH^{mut} TCGA gliomas. $P < 0.0001$ via two-tailed t-test.

Figure 3: Hypermethylation of *ITGB4* in IDH^{mut} gliomas.

(A) Fold difference in methylation of each CpG site in the *ITGB4* gene among IDH^{mut} TCGA gliomas relative to IDH^{wt} TCGA gliomas. (B) Methylation score of significantly hypermethylated CpG sites in the *ITGB4* gene from (A), plotted according to *ITGB4* mRNA levels among all TCGA gliomas.

Figure 4: Differential *ITGB4* expression and methylation between major subtypes of IDH^{mut} gliomas.

(A) Methylation of CpG sites in *ITGB4* gene in IDH^{mut}, 1p/19q co-deleted oligodendrogliomas compared with 1p/19q intact IDH^{mut} astrocytomas. (B) *ITGB4* mRNA levels IDH^{mut}, 1p/19q co-deleted oligodendrogliomas compared with 1p/19q intact IDH^{mut} astrocytomas ($P < 0.0001$) and IDH^{wt}, 1p/19q intact GBM ($P < 0.0001$). (C) Integrin β 4 IHC semi-quantitative score between IDH^{mut}, 1p/19q co-deleted gliomas and those with intact 1p/19q ($P = 0.60$ via two-tailed t test) in our institutional TMA cohort.

Figure 5: Integrin $\beta 4$ in glioma stem cells.

(A-D) Limiting dilution analysis was utilized to estimate the stem cell frequency of cells isolated from freshly dissociated GBM xenografts. The 20% of cells with the highest and lowest expression of integrin $\beta 4$ were plated in a limiting dilution manner, and the number of wells containing spheres were counted after 14 days to generate stem cell frequencies.

Figure 6: *ITGB4* knockdown suppresses glioma growth.

(A-C) Effect of *ITGB4* knockdown on *in vitro* growth of LN-18 cells. (D-E) Effect of *ITGB4* knockdown on *in vivo* growth of patient-derived, CD133+ primary GBM cells xenografted into the brains of immunocompromised mice.

Figure 7: Integrin $\beta 4$ and survival in gliomas.

(A) Overall survival according to integrin $\beta 4$ IHC in all grade II-IV gliomas from our institutional TMA cohort ($P = 0.0005$ via log-rank test). (B) Overall survival according to *ITGB4* mRNA in grade II-IV TCGA gliomas ($N = 656$, $P < 0.0001$ via log-rank test).

Variable	Risk ratio	95% CI	P Value
GBM	2.9	2.1-4.1	<0.001
IDH ^{mut}	0.19	0.13-0.27	<0.001
1p/19q code1	0.83	0.48-1.4	0.52
<i>ITGB4</i> quartile	1.2	1.1-1.4	0.0015

Table 1: Multivariable analysis of TCGA gliomas according to specific prognostic markers. Cox proportional hazards modeling of TCGA gliomas, including GBM, IDH^{mut} status, 1p/19q codeletion, and *ITGB4* mRNA quartile (see **Figure 7B**) as variables.

SUPPLEMENTAL DATA

Supplemental Figure 1: Molecular subtype and overall survival in TCGA gliomas.

In a TCGA cohort ($N = 654$) the median survival for patients with IDH^{mut}, 1p/19q co-deleted gliomas was significantly higher (134.3 months) when compared to that of patients with IDH1mut, 1p/19q intact gliomas (87.5 months) and IDH^{wt} gliomas (14.9 months; $P < 0.0001$).

ID#	position relative to island	coordinate	relation to ITGB4 coding region	IDH1wt	IDH1mut	fold change	P value	slope	95% CI	R square
cg12146151	N_Shore	73716945	TSS1500	0.35	0.58	1.66	7.60572E-41	-0.04328 ± 0.005710	-0.05447 to -0.03208	0.15
cg16047490	Island	73717197	TSS1500	0.02	0.02	1.12	0.021987713			
cg04094723	Island	73717212	TSS1500	0.02	0.03	1.46	0.002947797			
cg23913400	Island	73717552	5'UTR	0.03	0.09	2.78	3.47511E-15	-0.02593 ± 0.002361	-0.03056 to -0.02130	0.26
cg09513209	Island	73717562	5'UTR	0.02	0.10	6.64	7.4081E-14	-0.04422 ± 0.003424	-0.05093 to -0.03751	0.33
cg22162848	Island	73717736	5'UTR	0.31	0.65	2.07	1.8728E-51	-0.06526 ± 0.007446	-0.07986 to -0.05067	0.19
cg27346988	S_Shore	73719693	5'UTR	0.77	0.80	1.04	7.64758E-09			
cg18326022	S_Shelf	73719885	5'UTR	0.97	0.97	1.00	0.038764352			
cg20667796	S_Shelf	73720234	5'UTR	0.70	0.78	1.10	1.16883E-17			
cg18709893	S_Shelf	73720591	5'UTR	0.83	0.86	1.04	4.85836E-09			
cg15700850	S_Shelf	73720840	Body	0.77	0.84	1.10	3.22756E-25			
cg16379248	N_Shelf	73723681	Body	0.92	0.93	1.01	0.000561755			
cg15958938	Island	73726239	Body	0.90	0.92	1.03	5.12804E-06			
cg16603176	Island	73726241	Body	0.88	0.91	1.04	9.10955E-07			
cg23738681	Island	73726337	Body	0.88	0.91	1.03	7.78461E-17			
cg10949753	Island	73726372	Body	0.77	0.95	1.22	9.79778E-37			
cg13347255	Island	73726430	Body	0.81	0.88	1.08	7.56851E-19			
cg19159842	S_Shore	73727435	Body	0.73	0.82	1.13	4.48068E-26			
cg27029445	S_Shore	73727713	Body	0.93	0.94	1.01	6.73622E-05			
cg24037380	S_Shore	73727879	Body	0.84	0.92	1.09	3.60522E-11			
cg26314765	S_Shore	73727957	Body	0.94	0.98	1.04	2.82777E-07			
cg20942310	S_Shore	73728061	Body	0.85	0.89	1.04	1.18098E-07			
cg06128893	S_Shelf	73729655	Body	0.97	0.98	1.01	0.000958105			
cg00145838	S_Shelf	73729676	Body	0.99	0.99	1.00	0.130023661			
cg18566702	N_Shore	73736931	Body	0.73	0.79	1.08	3.4272E-14			
cg06550704	Island	73738580	Body	0.87	0.82	0.94	0.000728413			
cg11025757	Island	73738713	Body	0.97	0.98	1.02	0.002526145			
cg12842409	Island	73738809	Body	0.91	0.94	1.03	2.54487E-07			
cg24079672	S_Shore	73739840	Body	0.96	0.99	1.03	2.13744E-05			
cg25120705	S_Shore	73739874	Body	0.90	0.97	1.08	1.85209E-21			
cg21850852	N_Shelf	73746186	Body	0.06	0.06	1.04	0.440958354			
cg03601229	N_Shore	73748344	Body	0.85	0.89	1.05	1.8649E-12			
cg11223622	Island	73749654	Body	0.20	0.58	2.88	2.9742E-110	-0.04934 ± 0.006558	-0.06219 to -0.03649	0.14
cg16916914	Island	73749694	Body	0.04	0.51	11.29	1.06347E-75	-0.07510 ± 0.009054	-0.09285 to -0.05735	0.17
cg07436562	S_Shore	73750299	Body	0.40	0.36	0.90	8.81742E-05			
cg20626964	S_Shelf	73753034	Body	0.80	0.82	1.02	4.15866E-06			
cg08668997	S_Shelf	73753076	Body	0.98	0.99	1.01	0.000232337			
cg10231126	S_Shelf	73753800	3'UTR	0.85	0.89	1.04	6.75818E-08			

Supplemental Table 1: Methylation of CpG sites within *ITGB4* gene in TCGA gliomas. See Figure 3 in main text.

gene	1p19q codel	1p19q intact	codel vs intact	rank	overall percentile
psiTPTE22	5.052291716	9.233551357	-4.181259641	1	0.0
LEFTY2	2.017285207	6.00421531	-3.986930103	2	0.0
OR4N2	1.40726213	4.852731395	-3.445469265	3	0.0
COL11A1	5.359632544	8.631916279	-3.272283735	4	0.0
RPE65	5.01631716	8.235792636	-3.219475476	5	0.0
HOXA13	0.180984024	3.394731589	-3.213747565	6	0.0
POU4F1	1.688097633	4.726536047	-3.038438413	7	0.0
ZNF560	2.719194083	5.68216686	-2.962972778	8	0.0
MMEL1	0.919946154	3.826736047	-2.906789893	9	0.0
AQP7P1	1.686335503	4.430685078	-2.744349575	10	0.0
VCAM1	7.266555621	9.994897868	-2.728342247	11	0.1
HS3ST1	5.897610059	8.591846899	-2.69423684	12	0.1
HOXA10	0.714897041	3.365995349	-2.651098307	13	0.1
HOXA11	0.201991716	2.768375581	-2.566383865	14	0.1
SCN7A	2.553143787	5.107155426	-2.554011639	15	0.1
PGA3	1.871360947	4.410335853	-2.538974906	16	0.1
IRX1	4.802995266	7.335825194	-2.532829928	17	0.1
FOXD3	1.045983432	3.536804651	-2.490821219	18	0.1
HAND2	0.507229586	2.982290891	-2.475061306	19	0.1
PI16	6.370411834	8.843281395	-2.472869561	20	0.1
C4orf31	3.932259763	6.40235814	-2.470098376	21	0.1
REM1	3.289690533	5.744200388	-2.454509855	22	0.1
SULF1	7.186999408	9.518338566	-2.331339158	23	0.1
F2RL1	4.384781065	6.687350194	-2.302569129	24	0.1
SPOCD1	4.982308876	7.24385562	-2.261546744	25	0.1
BMP8B	4.944255621	7.20565	-2.261394379	26	0.1
SLC34A2	1.061142604	3.304915891	-2.243773288	27	0.1
DEFB119	0.031581657	2.267439922	-2.235858266	28	0.1
CHST9	8.221537278	10.43607345	-2.214536172	29	0.1
CX3CR1	8.57230355	10.77805969	-2.20575614	30	0.1
TNFSF12-TNFSF13	2.607685799	4.793801938	-2.186116139	31	0.2
RIPK4	0.884013018	3.064812791	-2.180799773	32	0.2
FOXD1	5.301037278	7.47670407	-2.175666792	33	0.2
CTSH	8.859095266	11.01925562	-2.160160354	34	0.2
HCG22	3.251015385	5.409302713	-2.158287329	35	0.2
C1QL4	4.722183432	6.876132171	-2.153948739	36	0.2
PGA4	0.333223669	2.47680155	-2.143577882	37	0.2
VIM	12.12637751	14.25978314	-2.133405625	38	0.2
HOXA11AS	0.152640828	2.283547674	-2.130906846	39	0.2
SCIN	6.663278107	8.78400969	-2.120731583	40	0.2
MIA	1.229165089	3.345486628	-2.116321539	41	0.2
AR	4.004425444	6.111171512	-2.106746068	42	0.2

TNFRSF8	2.198569822	4.305189147	-2.106619325	43	0.2
HOXD8	2.668682249	4.767345736	-2.098663488	44	0.2
TMEM119	7.450131361	9.530624612	-2.080493251	45	0.2
C1orf194	3.714478698	5.792873256	-2.078394558	46	0.2
ZNF662	5.578250296	7.65445407	-2.076203774	47	0.2
MSTN	6.418646154	8.487937597	-2.069291443	48	0.2
S100A3	2.315573964	4.378482558	-2.062908594	49	0.2
ACY3	4.631452071	6.681450388	-2.049998317	50	0.2
FCGBP	8.546024852	10.58877151	-2.04274666	51	0.2
CYP4F12	1.961363905	4.000445155	-2.03908125	52	0.3
TFPI	5.819252071	7.851904651	-2.03265258	53	0.3
ANKRD22	4.402957396	6.433193992	-2.030236596	54	0.3
HOXA7	0.475179882	2.488847093	-2.013667211	55	0.3
ITGB4	8.78182071	10.78912287	-2.007302158	56	0.3
FPR1	6.438223669	8.417505233	-1.979281564	57	0.3
ID3	10.50520473	12.48383236	-1.978627631	58	0.3
ZNF518B	6.24534497	8.221807752	-1.976462782	59	0.3
IRX2	5.353023077	7.328514729	-1.975491652	60	0.3
C3	11.27972367	13.23971802	-1.959994355	61	0.3
ALOX5AP	6.499092308	8.457922093	-1.958829785	62	0.3
FOX E1	0.534759172	2.471917636	-1.937158464	63	0.3
CYP21A2	5.053733136	6.986347093	-1.932613957	64	0.3
HAS2	5.038509467	6.967007752	-1.928498284	65	0.3
GBX2	4.280851479	6.206899806	-1.926048327	66	0.3
OLFML2B	6.514059172	8.439076744	-1.925017573	67	0.3
HAMP	3.169220118	5.092927907	-1.923707789	68	0.3
CLEC9A	3.838704734	5.747956202	-1.909251468	69	0.3
MMP7	0.984807101	2.88794845	-1.903141349	70	0.3
ADAMTS15	4.085476331	5.981587403	-1.896111072	71	0.3
LHX9	2.206942604	4.101913178	-1.894970575	72	0.4
YSK4	1.119053846	2.99480562	-1.875751774	73	0.4
COL28A1	4.858940828	6.732570736	-1.873629908	74	0.4
RNASE2	2.980555621	4.853441667	-1.872886045	75	0.4
BLNK	5.109388166	6.981067054	-1.871678889	76	0.4
STOX1	7.59614142	9.463448837	-1.867307417	77	0.4
LOC100233209	2.777894083	4.63970814	-1.861814057	78	0.4
GBP2	7.611135503	9.46765407	-1.856518567	79	0.4
SLC2A5	8.077614793	9.919391085	-1.841776292	80	0.4
FNBP1L	8.228352663	10.0656595	-1.837306833	81	0.4
CYBB	8.356531361	10.18905504	-1.832523678	82	0.4
HOXC4	2.739497041	4.563863566	-1.824366524	83	0.4
WDR78	4.37518284	6.198200581	-1.823017741	84	0.4
CCDC135	2.22823432	4.046305039	-1.818070719	85	0.4

FCGR1A	6.604588757	8.414325194	-1.809736436	86	0.4
GEM	6.901491124	8.69917093	-1.797679806	87	0.4
IRX5	1.322022485	3.11927907	-1.797256585	88	0.4
GNG12	8.021589941	9.818064147	-1.796474206	89	0.4
CSF1R	10.32822959	12.12169826	-1.79346867	90	0.4
VSIG4	8.577107692	10.35946105	-1.782353354	91	0.4
CELSR1	3.862850888	5.635051163	-1.772200275	92	0.4
PLD4	7.359911834	9.129910659	-1.769998825	93	0.5
IFI44L	8.467472781	10.23136202	-1.763889234	94	0.5
PGA5	0.877906509	2.640264922	-1.762358414	95	0.5
EMP1	9.08548284	10.84461008	-1.759127237	96	0.5
C5orf38	4.682923669	6.439860078	-1.756936409	97	0.5
EN2	3.065754438	4.811746124	-1.745991686	98	0.5
LOC400043	5.53614497	7.278914535	-1.742769564	99	0.5
SPNS3	3.24407574	4.984414729	-1.740338989	100	0.5

Supplemental Table 2: List of the 100 most downregulated genes in IDH^{mut} 1p/19q co-deleted oligodendrogliomas relative to IDH^{mut} 1p/19q intact astrocytomas.

Among 20,530 genes analyzed in TCGA gliomas via RNA-Seq, *ITGB4* is the 56th most downregulated gene in IDH^{mut} 1p/19q co-deleted oligodendrogliomas relative to IDH^{mut} 1p/19q intact astrocytomas ($P = 3.2 \times 10^{-39}$).