

Integrin $\alpha 5\beta 1$ Plays a Critical Role in Resistance to Temozolomide by Interfering with the p53 Pathway in High-Grade Glioma

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Abstract

Integrins play a role in the resistance of advanced cancers to radiotherapy and chemotherapy. In this study, we show that high expression of the $\alpha 5$ integrin subunit compromises temozolomide-induced tumor suppressor p53 activity in human glioblastoma cells. We found that depletion of the $\alpha 5$ integrin subunit increased p53 activity and temozolomide sensitivity. However, when cells were treated with the p53 activator nutlin-3a, the protective effect of $\alpha 5$ integrin on p53 activation and cell survival was lost. In a functional p53 background, nutlin-3a down-regulated the $\alpha 5$ integrin subunit, thereby increasing the cytotoxic effect of temozolomide. Clinically, $\alpha 5\beta 1$ integrin expression was associated with a more aggressive phenotype in brain tumors, and high $\alpha 5$ integrin gene expression was associated with decreased survival of patients with high-grade glioma. Taken together, our findings indicate that negative cross-talk between $\alpha 5\beta 1$ integrin and p53 supports glioma resistance to temozolomide, providing preclinical proof-of-concept that $\alpha 5\beta 1$ integrin represents a therapeutic target for high-grade brain tumors. Direct activation of p53 may remain a therapeutic option in the subset of patients with high-grade gliomas that express both functional p53 and a high level of $\alpha 5\beta 1$ integrin. *Cancer Res*; 72(14); 3463–70. ©2012 AACR.

Introduction

Glioblastoma multiforme (GBM) are the most aggressive brain tumors and remain a challenge for oncologists. New therapies are urgently needed. Gene expression profiling of high-grade glioma revealed that genes of extracellular matrix components and their regulators are often affected in the patients. Fibronectin is overexpressed in glioblastoma versus normal brain (1) and belongs to the cluster of genes associated with a more malignant phenotype (2, 3). It has recently been shown that fibronectin knockdown delays tumor growth in a mouse glioma model (4). The $\alpha 5\beta 1$ integrin is a fibronectin receptor that was recently shown to have an important role in tumor progression (5), metastasis (6), and/or resistance to therapies (7) in lung, ovarian, and breast cancer, respectively.

Few works addressed directly the issue of $\alpha 5\beta 1$ integrin in glioma. Through the use of non-peptidic $\alpha 5\beta 1$ integrin antagonists and GBM cell lines, we previously showed that $\alpha 5\beta 1$ integrin may be a therapeutic target for these tumors (8, 9) and that concomitant addition of $\alpha 5\beta 1$ antagonists sensitizes p53 wild-type (p53-wt) glioma cells to chemotherapeutic drugs (10). The presence of p53 mutations in high-grade glioma varied across GBM subtypes with 0%, 21%, 32%, and 54% in classical, neural, mesenchymal, and proneural subtypes, respectively (11). There is increasing evidence that gliomas harboring a p53-wt resist to therapies through inhibitory pathways upstream of p53. Nutlin-3 belongs to the family of small-molecule inhibitors of the MDM2–p53 interaction (12). Nutlin-3 has been shown, alone or in combination with chemotherapeutic agents, to increase the degree of apoptosis in hematologic malignancies (13). Recent studies extended its therapeutic window for use in solid tumors (14, 15).

The aim of this study was to investigate the role of $\alpha 5\beta 1$ integrin in glioma resistance to temozolomide chemotherapy using *in vitro* and *in vivo* models. We found that a high expression of $\alpha 5$ subunit inhibited the temozolomide-induced p53 pathway and that reactivation of p53 by nutlin-3a restores the sensitivity to temozolomide by decreasing the expression of the $\alpha 5\beta 1$ integrin. Finally, we found that high $\alpha 5$ integrin gene expression is associated with a more aggressive phenotype in brain tumors and a decrease in survival of patients. Our results provide a clinical rationale for including $\alpha 5\beta 1$ integrin-targeted therapy in a subpopulation of patients with glioma.

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Materials and Methods

Reagents

Temozolomide was a kind gift from Schering-Plough. Nutlin-3a, the active enantiomer of nutlin, was from Cayman. Temozolomide was prepared before use at 10 mmol/L in 50/50 ethanol/H₂O. Other drugs were prepared as stock solutions in ethanol at 10 mmol/L and were kept at -20°C until use.

Cell culture and transfection

The U87MG cells (p53-wt) was from American Type Culture Collection; the U373 cells (p53-mutated) from ECACC (Sigma) and not authenticated in the laboratory. The LN18 (p53-mutated) and LNZ308 [p53 knockout (KO)] cells were kindly provided by M. Hegi (University Hospital, Lausanne, Switzerland). Cells were cultured as described elsewhere (10). The identity of cell lines was regularly checked by morphologic criteria, and importantly p53 status was routinely checked by the yeast functional assay (16), Western blot quantification of p53 stability and phosphorylation, and by quantitative PCR (qPCR) quantification of p53 target genes after treatment with ellipticine. Cells were stably transfected to overexpress (by transfecting a pcDNA3.1 plasmid containing the human $\alpha 5$ integrin gene; provided by Dr. Ruoshlati, University of California, Santa Barbara, CA) or to repress [by transfecting a pSM2 plasmid coding for a short hairpin RNA (shRNA) targeting the $\alpha 5$ mRNA; Open Biosystems] the $\alpha 5$ integrin subunit by using jetPRIME (Polyplus transfection) according to the manufacturer's instructions. The vector for the p53-wt transfection was a kind gift from Dr. C. Blattner (Karlsruhe Institute of Technology, Karlsruhe, Germany). Cells were transfected with specific siRNA for human p53, the $\alpha 5$ integrin subunit, or nontargeting siRNA (Thermo Scientific Dharmacon) with jetPRIME (Polyplus transfection) according to the manufacturer's instructions.

Western blot

Western blotting was carried out as previously described (10). Antibodies used were against $\alpha 5$ integrin Ab1928 (Millipore) or H104 (Santa-Cruz), $\beta 1$ integrin Ab1952, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Millipore), p53 (BD Biosciences), or p53^{pser15} (Cell Signaling).

Flow cytometry

After detachment with EDTA, cells were incubated for 30 minutes at 4°C under agitation in the presence of primary antibodies: anti- $\alpha 5$ integrin antibody IIA1 (BD Biosciences) and anti- $\beta 1$ integrin antibodies (TS2/16 from Santa Cruz; 9EG7 and mab13 from BD Biosciences). After washing, cells were incubated for 30 minutes with secondary antibody (Alexa488-conjugated goat anti-mouse or rat; Jackson ImmunoResearch). After washing, cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson), and the mean fluorescence intensity characterizing surface expression of integrins was measured using the CellQuest software.

Clonogenic assay

Clonogenic survival was determined as previously described (9).

Immunofluorescence

A total of 20,000 cells were seeded onto IBIDI μ -dishes coated with 10 $\mu\text{g}/\text{mL}$ of poly-L-lysine. Cells were treated with nutlin-3a (10 $\mu\text{mol}/\text{L}$) or with solvent during 24 hours before fixation with 4% paraformaldehyde (10 minutes at room temperature) and then processed for $\alpha 5$ immunodetection (IIA1 antibody; 1:300). Confocal images were taken with a confocal microscope (BioRad 1024) equipped with a water immersion $\times 60$ objective. Images were collected using the Laser-Sharp 2000 software.

Human biopsies

This study was conducted on 115 adult brain biopsies, 95 brain tumors (22 grade II, 38 grade III, and 35 grade IV) and 20 nontumoral brain tissues collected retrospectively from archival material stored at the Centre de Ressources Biologiques et Tumorotheque (Hopitaux Universitaires de Strasbourg, Strasbourg, France). The patient characteristics have been described elsewhere (17). Each sample was histologically analyzed by a pathologist to specify the tumor grade and the percentage of tumor cells. Only samples with at least 50% of tumoral cells (>50% of samples were >70% tumoral cells) have been included in the study. Control tissues were obtained from epileptic surgery. The study was conducted in accordance with the Declaration of Helsinki. Real-time qPCR was carried out as described previously (17). The threshold cycle (C_t) values for each gene were normalized to expression level of *cyclophilin* used as the housekeeping gene. Values were normalized relatively to the value obtained for one nontumoral control brain tissue, which was included in each qPCR run. Immunologic analysis of $\alpha 5$ protein expression was conducted as shown previously (17).

Human brain tumor data sets

Glioma gene expression data sets from 2 other cohorts were downloaded from the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>; accession numbers GSE4271 and GSE4412). Microarray raw data were processed using R (version 2.10.1; <http://cran.r-project.org/>), implemented with the BioConductor package (<http://www.bioconductor.org>). Estimates of the survival curves were computed using the Kaplan-Meier method. Univariate survival comparisons between the patients, according to low or high $\alpha 5$ integrin expression levels, were conducted using a log-rank test.

Human brain tumor xenografts

TCG4, TCG9, and TCG17 glioma xenograft models were obtained as previously described (18). Subcutaneous tumor growth was followed by measuring, 3 times per week, 2 perpendicular diameters with a caliper. Treatments began when tumors reached a volume of approximately $250 \pm 50 \text{ mm}^3$. Temozolomide was administered orally at the dose of 50 mg/kg/d for 5 days. Mice were sacrificed when the tumor volumes reached 4 times their initial volume (V_0). For each mouse, the time between the treatment onset and the animal sacrifice was defined as the "survival time." *TP53* status of each xenograft was determined by the yeast functional assay (16).

Statistical analysis

Data are represented as the mean \pm SEM, and n is the number of independent experiments. Statistical analyses were conducted using the Student t test or the Mann–Whitney test with the GraphPad Prism program. $P < 0.05$ was considered significant.

Results

$\alpha 5 \beta 1$ integrin impedes temozolomide-induced p53-wt activity

We compared the effect of temozolomide in U87MG cells depleted in (shRNA $_{\alpha 5}$) or overexpressing (pcDNA $_{\alpha 5}$) $\alpha 5$ integrin versus control cells (shRNA $_{ns}$ and pcDNA $_{ctrl}$, respectively). Because p53 is largely involved in chemotherapeutic drug effects and we showed previously that $\alpha 5 \beta 1$ integrin antagonists modulate the p53 pathway (10), we focused on temozolomide-induced p53 activation. Temozolomide caused an increase in p53 protein in all cell lines but not significantly in pcDNA $_{\alpha 5}$ cells (when normalized to GAPDH: Fig. 1A). Interestingly, a significant increase in p53 protein was already observed in untreated shRNA $_{\alpha 5}$ cells versus shRNA $_{ctrl}$ cells (Fig. 1A). After temozolomide treatment, an increase in p53^{pser15} was detectable in pcDNA $_{ctrl}$ and shRNA $_{ns}$ cells, which was significantly more pronounced in shRNA $_{\alpha 5}$ (Fig. 1A). In contrast, in pcDNA $_{\alpha 5}$ cells, significantly less p53^{pser15} was measured after temozolomide treatment. Transcriptional activity of p53 was higher in shRNA $_{\alpha 5}$ cells and lower in pcDNA $_{\alpha 5}$ cells than in temozolomide-treated control cells (Fig. 1B). Taken together, these results indicate that $\alpha 5 \beta 1$ integrin modulates p53 activity and that high expression of this integrin inhibits temozolomide-induced p53 stimulation. Modulation of p53 activity was related to cell survival, as pcDNA $_{\alpha 5}$ cells are significantly more resistant at high temozolomide concentration whereas shRNA $_{\alpha 5}$ cells appear more sensitive than their control counterparts (Fig. 1C). $\alpha 5$ integrin overexpression did not modulate p53, nor clonogenic survival in U373 and LN18 cells expressing a p53-mutant (Supplementary Fig. S1). In addition, repression of $\alpha 5$ integrin in p53-deficient LNZ308 cells did not sensitize cells to temozolomide (Supplementary Fig. S1). From these data, we concluded that $\alpha 5 \beta 1$ integrin-induced temozolomide resistance requires a functional p53.

As a first approach to confirm the role of the $\alpha 5 \beta 1$ integrin in temozolomide chemoresistance *in vivo*, we used subcutaneous xenografted human brain tumors in nude mice. We selected 3 xenografts that exhibited a wild-type p53 and different levels of the $\alpha 5$ subunit. Kaplan–Meier analysis of the mouse survival suggests a relationship between $\alpha 5$ integrin level and resistance to temozolomide (Fig. 1D), providing some evidence for a role of the $\alpha 5$ integrin in the chemoresistance of p53-wt-expressing tumors *in vivo*.

Activation of p53 by nutlin-3a overrides the $\alpha 5$ integrin effects

We next investigated whether high $\alpha 5$ also impacts on p53 activation by a non-genotoxic p53 activator in glioma cells. U87MG cells were treated with nutlin-3a. In contrast

to the effects of temozolomide, nutlin-3a stabilized p53, markedly increased the p53^{pser15} and the transactivation of p53 target genes in pcDNA $_{ctrl}$ and in pcDNA $_{\alpha 5}$ cells (Fig. 2A and B). Addition of temozolomide to nutlin-3a does not further increase these effects (Fig. 2A). In clonogenic assays, the $\alpha 5 \beta 1$ integrin did not efficiently protect the cells from death when p53 was activated by 10 μ mol/L nutlin-3a (Fig. 2C). Survival of LNZ308 cells (p53 KO) or U373 cells (p53-mutant) was less affected after treatment with nutlin-3a (Fig. 2C).

Activation of p53 by nutlin-3a markedly decreases the $\alpha 5$ expression level in glioma cells

U87MG cells treated with nutlin-3a rounded up and detached from the wells. This effect was lost when p53 expression was inhibited with specific siRNA (Fig. 3A, left). In contrast, LNZ308 cells did not exhibit any morphologic alterations after a 10 μ mol/L nutlin-3a treatment unless p53 was reexpressed in the cells (Fig. 3A, right). Interestingly, cell treatment with nutlin-3a decreased the expression of the $\alpha 5$ integrin at the protein level in U87MG-pcDNA $_{ctrl}$ and U87MG-pcDNA $_{\alpha 5}$ cells, an effect not observed after treatment with temozolomide (Fig. 3B). The decrease in $\alpha 5$ protein expression after nutlin-3a treatment was confirmed by specific immunostaining of the $\alpha 5$ subunit in U87MG-pcDNA $_{ctrl}$ and U87MG-pcDNA $_{\alpha 5}$ cells and by flow cytometric analysis of the $\alpha 5$ subunit at the cell membrane (Fig. 3B). A significant decrease in the $\alpha 5$ mRNA level was measured in U87MG pcDNA $_{ctrl}$ but not in pcDNA $_{\alpha 5}$ cells, suggesting that nutlin-3a affected the $\alpha 5$ subunit at translational and posttranslational levels (Fig. 3C). Nutlin-3a also decreased $\beta 1$ at the protein and mRNA level in the U87MG cells, suggesting that both subunits of the $\alpha 5 \beta 1$ integrin are processed similarly after nutlin-3a treatment (Supplementary Fig. S2). However, no effect on the $\beta 1$ subunit expressed at the cell membrane could be detected after nutlin-3a treatment (Supplementary Fig. S2 and Supplementary Table S1).

Nutlin-3a did not affect the endogenous $\alpha 5$ protein in p53-knockout LNZ308 cells unless p53 was reexpressed (Fig. 3D). Nutlin-3a had no effect on $\alpha 5$ expression in U373 cells (Supplementary Fig. S3). Altogether, these data suggest that nutlin-3a requires a functional p53 to decrease $\alpha 5$ expression, which in turn make the cells susceptible to the nutlin-3a-induced cell death.

High $\alpha 5 \beta 1$ integrin expression is associated with worse clinical outcome in high-grade glioma

To our knowledge, no studies have associated $\alpha 5 \beta 1$ integrin with clinical outcome in patients with glioma. To investigate first whether integrin expression is associated with the grade of brain tumors, gene expression of the $\alpha 5$ and $\beta 1$ subunits were examined by qPCR in 95 human brain tumors of different grades and compared with 20 nontumor brain samples. The data revealed that $\alpha 5$ subunit gene expression was increased with increasing tumor grade, although the $\beta 1$ subunit was equally overexpressed in the 3 tumoral grades compared with control tissue (Fig. 4A). Data were confirmed at the protein level (Fig. 4B). Because the $\alpha 5$ subunit only dimerizes with $\beta 1$,

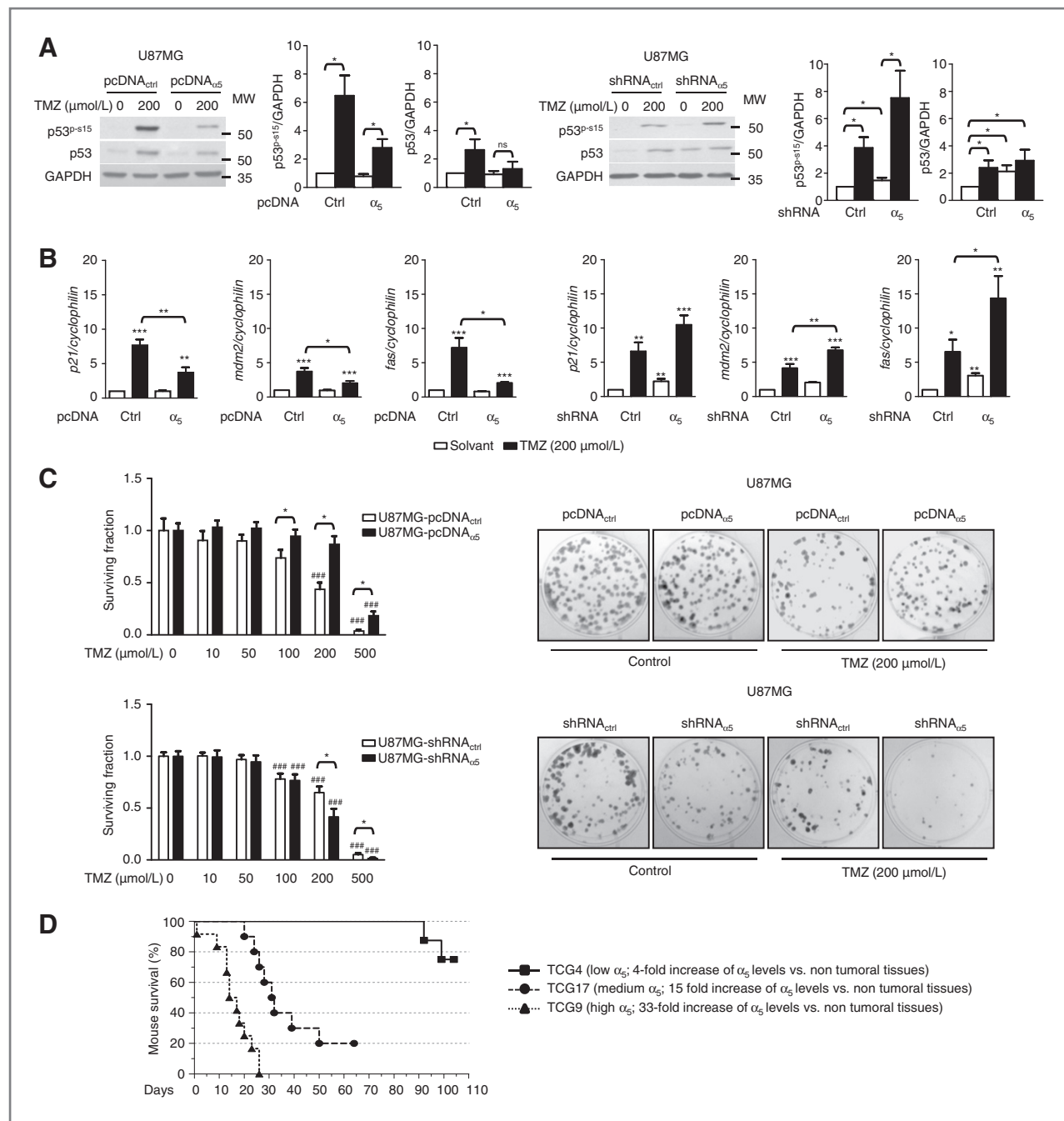
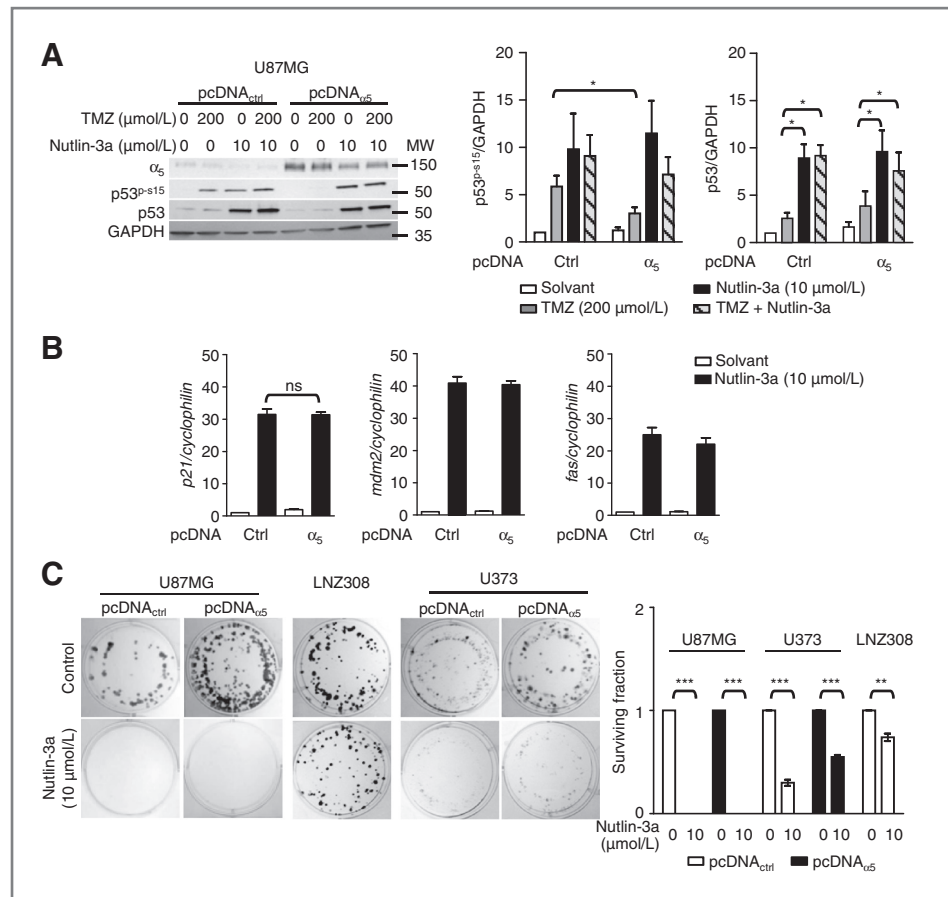


Figure 1. Elevated α_5 integrin expression impairs temozolomide (TMZ)-induced p53-wt signalling and triggers TMZ resistance. **A**, stability and p53 phosphorylation on Ser15 are affected by the α_5 integrin expression level. Western blot analysis for p53 and p53^{pser15} from total cell lysates with and without TMZ (200 μ mol/L) treatment during 24 hours in control (pcDNA_{ctrl}) and α_5 integrin-overexpressing (pcDNA_{α5}) U87MG cells (top) or control (shRNA_{ctrl}) and α_5 integrin-downregulated (shRNA_{α5}) U87MG cells (bottom). Histograms represent the mean \pm SEM of 6 to 8 independent experiments. **B**, qPCR quantification of p53 target genes. mRNA of target genes are differentially affected by upmodulated (top) or downmodulated (bottom) α_5 integrin after TMZ treatment in U87MG cells. **C**, TMZ dose response of clonogenic survival in U87MG cells overexpressing α_5 integrin (U87-pcDNA_{α5}) compared with control cells (U87-pcDNA_{ctrl}). pcDNA_{α5} cells are 2 times more resistant than control cells at 200 μ mol/L TMZ (top) or U87MG cells underexpressing α_5 integrin (U87-shRNA_{α5}) compared with control cells (U87-shRNA_{ctrl}). shRNA_{α5} are 1.5 times more sensitive than control cells at 200 μ mol/L TMZ (bottom). Representative images of colonies obtained with and without 200 μ mol/L TMZ are shown. Statistical analysis: ##, $P < 0.01$; ###, $P < 0.001$ for treated cells versus nontreated cells; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for genetically manipulated cells versus corresponding control cells. **D**, TMZ antitumor effect on human malignant glioma xenografts in nude mice. Three glioma xenografts expressing p53-wt were analyzed for α_5 mRNA expression (TCG9, TCG17, and TCG4 with 33-, 15-, and 4.5-fold more α_5 mRNA, respectively, compared with human nontumor brain tissue) and used to evaluate the tumor response to TMZ (orally daily 50 mg/kg \times 5 days). Results are expressed as Kaplan–Meier plots, considering the percentage of tumors that reached four V_0 as the survival end point. Ctrl, control; ns, not significant.

Figure 2. Activation of p53 by nutlin-3a overrides the inhibitory effect of the $\alpha 5$ integrin. A, Western blot analysis of the $\alpha 5$ integrin, p53, and p53^{Ser15} in pcDNA_{ctrl} and pcDNA _{$\alpha 5$} -transfected U87MG cells. U87MG cells were treated with nutlin-3a (10 $\mu\text{mol/L}$), temozolomide (TMZ; 200 $\mu\text{mol/L}$), or both for 24 hours. The histograms display the mean \pm SEM of 5 independent experiments. GAPDH was used as the loading control. B, qPCR analysis of p53 target genes. The histograms represent the fold increase of mRNA in pcDNA_{ctrl} and pcDNA _{$\alpha 5$} -transfected U87MG cells before and after nutlin-3a (10 $\mu\text{mol/L}$) treatment over 24 hours. ns, nonsignificant. C, clonogenic survival of pcDNA_{ctrl}-U87MG and pcDNA _{$\alpha 5$} -U87MG cells (left), LN2308 cells (middle), and pcDNA_{ctrl}-U373 and pcDNA _{$\alpha 5$} -U373 cells (right) after nutlin-3a treatment (10 $\mu\text{mol/L}$). Histograms represent the mean \pm SEM. *, $P < 0.05$ of 3 independent experiments. **, $P < 0.01$; ***, $P < 0.001$ in nutlin-3a-treated versus nontreated cells.



the data point toward a particular role for $\alpha 5\beta 1$ integrin in glioma.

We analyzed the clinical data of grade III and grade IV patients. Log-rank analysis of the Kaplan–Meier survival curves showed a significant survival advantage for patients with low $\alpha 5$ -expressing glioma compared with high $\alpha 5$ -expressing glioma. These results were validated in 2 independent public data sets (2, 19). Considering all 3 cohorts together, the group of high $\alpha 5$ -expressing tumors included 39% of grade III and 81% of grade IV tumors (Fig. 4C; Supplementary Table S2). Finally, we evaluated the relationship between $\alpha 5$ integrin level and the status of p53 in 56 human biopsies (grade III and IV) and in 17 human tumor xenografts. A clear tendency toward a higher level of $\alpha 5$ in p53-wt versus p53-mutant tumors was found in biopsies and in xenografts (Fig. 4D; Supplementary Table S3).

Discussion

The data summarized here document the impact of the $\alpha 5\beta 1$ integrin on the high-grade glioma resistance to temozolomide therapy. When the $\alpha 5$ integrin subunit is overexpressed, the p53-mediated responses to genotoxic damage are compromised. When the $\alpha 5$ integrin level is low or suppressed, p53 is stabilized and fully functional. An inverse relationship between the $\alpha 5$ integrin level and p53 has been revealed through the use of the p53 activator, nutlin-3a. These results

may have clinical relevance in light of the clear advantage reported here for prolonged survival of patients with high-grade glioma with low $\alpha 5$ integrin subunit expression.

In agreement with our data, it was reported that the $\alpha 5\beta 1$ integrin is overexpressed at the protein level in a significant proportion of human glioblastoma biopsies (20). Here, we show for the first time that in glioma, the $\alpha 5$ mRNA level is negatively correlated to survival in 3 different cohorts of patients, which adds brain tumors to the growing list of cancers in which the $\alpha 5\beta 1$ integrin should be considered as a therapeutic target. The role of p53 in temozolomide resistance is far from being understood. Although several groups reported that p53 status is not predictive of response to chemotherapy with alkylating agents (18, 21), more recent works suggest that the absence of a functional p53 increases temozolomide sensitivity in glioma cell lines (22) and in intracranial glioblastoma xenografts (23). A trend toward an increased temozolomide sensitivity in patients with p53 mutations was also suggested (24). We propose that overexpression of the $\alpha 5\beta 1$ integrin in GBM represents an alternative mechanism, aside from p53 deletion/mutation, to inactivate the tumor-suppressive function of the p53 pathway. We are currently investigating the molecular mechanisms involved in the integrin–p53 cross-talk by addressing the role of $\alpha 5\beta 1$ integrin in transcriptional and nontranscriptional effects of p53. Activation of the p53 pathway by nutlin-3a led to downregulation of

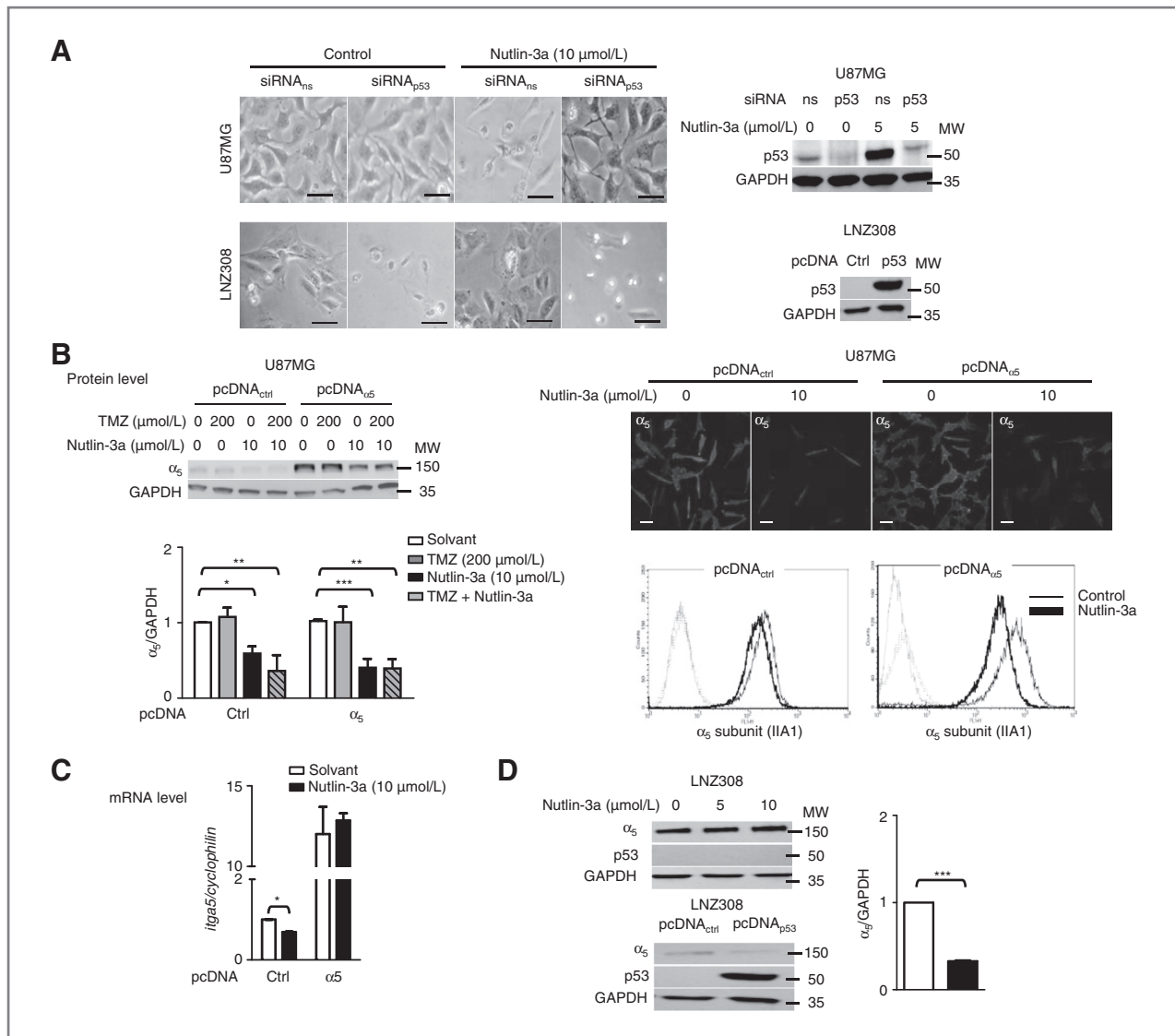


Figure 3. Activation of p53 by nutlin-3a affects the $\alpha 5\beta 1$ integrin expression in U87MG cells. **A**, U87MG and LNZ308 cell morphology after 24 hours of nutlin-3a treatment. U87MG cells were transfected either with control siRNA_{ns} or with siRNA_{p53} and treated with nutlin-3a (5 μmol/L) for 24 hours. Silencing of the p53 protein was verified by immunoblotting. LNZ308 cells were transfected with pcDNA_{ctrl} or pcDNA_{p53} and treated with nutlin-3a (10 μmol/L). Expression of p53 was verified by immunoblotting. Scale bars, 50 μm. **B**, top, Western blot analysis of the $\alpha 5$ integrin protein expression in pcDNA_{ctrl}- and pcDNA_{α5}-transfected U87MG cells. Cells were treated with nutlin-3a (10 μmol/L), TMZ (200 μmol/L), or both drugs for 24 hours. Histograms show the fold increase in the protein expression normalized to GAPDH levels (mean ± SEM of 3–4 independent experiments). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for treated cells versus nontreated cells. Middle, representative fluorescence images with specific anti- $\alpha 5$ integrin antibodies of untreated and nutlin-3a-treated pcDNA_{ctrl}- and pcDNA_{α5}-transfected U87MG cells. Scale bars, 20 μm. Bottom, flow cytometric analysis of the $\alpha 5$ (IIA1 antibody) integrin subunit at the cell membrane of pcDNA_{ctrl}- and pcDNA_{α5}-transfected U87MG cells before and after nutlin-3a (10 μmol/L) treatment for 24 hours. **C**, histograms represent the fold increase of the $\alpha 5$ mRNA in pcDNA_{ctrl}- and pcDNA_{α5}-transfected U87MG cells after nutlin-3a (10 μmol/L) treatment. *, $P < 0.05$ for treated cells versus nontreated cells. **D**, left, Western blot analysis of $\alpha 5$ and p53 protein expression in p53-null LNZ308 cells treated with nutlin-3a (5 and 10 μmol/L) for 24 hours. A representative blot of 3 is shown. Right, LNZ308 cells were transfected with p53-wt, and $\alpha 5$ integrin expression was detected by Western blot analysis. Histogram represents the mean ± SEM of 3 independent experiments. GAPDH was used as the loading control. Ctrl, control; ns, not significant.

the $\alpha 5$ integrin subunit in glioma cells. On the basis of our data, there seems to be a cross-antagonistic interaction between the $\alpha 5$ integrin and p53 that was only revealed by nutlin-3a, which may explain why this drug overcomes the prosurvival activity of the integrin. Our results are similar to recent data showing that nutlin-3a downregulates the oncogene DEK or overcomes

the antiapoptotic Bcl2 overexpression, thus leading to cell apoptosis (25, 26).

In summary, we have shown for the first time that $\alpha 5\beta 1$ integrin plays a critical role in resistance to temozolomide therapy by interfering with the p53 pathway in high-grade glioma. In addition, we have shown that activation of p53 by

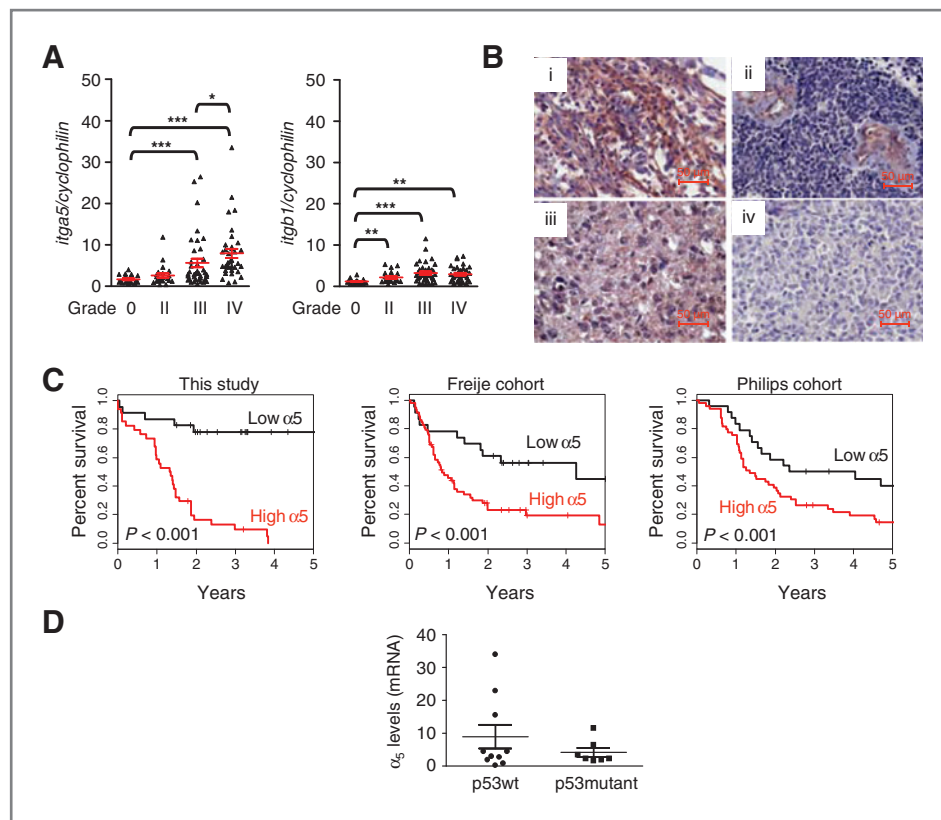


Figure 4. Elevated $\alpha 5$ -integrin gene expression is associated with high-grade glioma and predicts decreased survival rates. **A**, gene expression levels of $\alpha 5$ and $\beta 1$ integrin subunits were quantified with specific primers by qPCR in 20 nontumor brain tissues (G0), 22 grade 2 (GII), 38 grade 3 (GIII), and 35 glioblastoma (GIV) samples. Only the $\alpha 5$ integrin subunit level is associated with the tumor grade. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ as compared with nontumoral brain tissue (Mann-Whitney test). **B**, immunohistochemical analysis of $\alpha 5$ protein in tumoral cells of human high-grade glioma. Representative slides of $\alpha 5$ high intensity staining in GBM (i), $\alpha 5$ -negative staining in GBM (ii), $\alpha 5$ high intensity staining in grade III tumor (iii), and $\alpha 5$ -negative staining in grade III tumors (iv). Scale bars, 50 μ m. **C**, elevated $\alpha 5$ integrin gene expression is associated with significantly decreased long-term survival in patients with high-grade glioma. Kaplan-Meier survival analysis of 3 patient cohorts is shown. Left, cohort from this study; middle, data from Freije and colleagues (2); right, data from Phillips and colleagues (19). **D**, the $\alpha 5$ mRNA expression level and p53 status in human brain tumor xenografts in nude mice. Ten xenografts expressed a wild-type p53 and 7 xenografts had a mutant p53, as determined by the FASAY assay. The $\alpha 5$ mRNA levels were determined in at least 3 different grafts of the same tumor, and the mean levels were plotted according to p53 status. The mean values \pm SEM of the $\alpha 5$ mRNA level in p53-wt and mutant p53 tumors were 8.9 ± 3.6 and 4.1 ± 1.3 , respectively. Although not significant, this difference shows a trend toward an increased level of $\alpha 5$ in p53-wt tumors.

nutlin-3a represses the $\alpha 5\beta 1$ integrin, and we propose that such downregulation is an important mediator of nutlin-3a cytotoxic activity. The relevance of our results is emphasized by the finding that $\alpha 5$ integrin gene overexpression is associated with decreased survival in patients with high-grade glioma. Our data provide the rationale for a preclinical evaluation of p53 activators and/or $\alpha 5\beta 1$ integrin antagonists in a subset of high-grade glioma that expresses a functional p53 and high levels of $\alpha 5\beta 1$ integrin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Maglott, D.Y. Leger, C. Bossert, F. Noulet, E. Guerin, S. Pinel, F. Plenat, N. Entz-Werle, J. Lehmann-Che

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Janouskova, A. Maglott, F. Noulet, P. Chastagner, F. Plenat, J. Godet, M. Dontenwill

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Guenot, N. Entz-Werle, S. Martin
Study supervision: D. Guenot, P. Chastagner, M. Dontenwill

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