Tumorigenesis and Neoplastic Progression

In Situ Analysis of Integrin and Growth Factor Receptor Signaling Pathways in Human Glioblastomas Suggests Overlapping Relationships with Focal Adhesion Kinase Activation

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Deregulated integrin signaling is common in cancers, including glioblastoma. Integrin binding and growth factor receptor signaling activate focal adhesion kinase (FAK) and subsequently up-regulate extracellular regulated kinases (ERK-1/2), leading to cell-cycle progression and cell migration. Most studies of this pathway have used *in vitro* systems or tumor lysate-based approaches. We examined these pathways primarily *in situ* using a panel of 30 glioblastomas and gene expression arrays, immunohistochemistry, and fluorescence *in situ* hybridization, emphasizing the histological distribution of molecular changes. Within individual tumors, increased expression of FAK, p-FAK, paxillin, ERK-1/2, and p-ERK-1/2 occurred in regions of elevated EGFR and/or PDGFRA expression. Moreover, FAK activation levels correlated with EGFR and PDGFRA expression, and p-FAK and EGFR expression co-localized at the single-cell level. In addition, integrin expression was enriched in EGFR/PDGFRA-overexpressing areas but was more regionally confined than FAK, p-FAK, and paxillin. Integrins $\beta 8$ and $\alpha 5 \beta 1$ were most commonly expressed, often in a perinecrotic or perivascular pattern. Taken together, our data suggest that growth factor receptor overexpression facilitates alterations in the integrin signaling pathway. Thus, FAK may act in glioblastoma as a downstream target of growth factor signaling, with integrins enhancing the impact of such signaling in the tumor microenvironment. (*Am J Pathol* 2005, 167:1379–1387)

Glioblastomas are the most common and most malignant primary brain tumors.¹ Despite the best current surgical, radiation, and chemotherapeutic approaches, nearly all patients have tumor recurrences that lead to death, primarily because these tumors invade surrounding tissues, thus making local therapeutic approaches ineffective. Understanding the regulation of signaling pathways involved in glioblastoma migration and invasion is therefore of considerable importance for the development of more efficient and targeted tumor therapies.

As in many other human cancers, glioblastomas commonly demonstrate alterations of the integrin signaling pathway. Integrins are heterodimeric transmembrane receptors that bind to extracellular matrix components. More than 20 integrin heterodimers have been identified to date, comprised of various pairings of 13 α - and 8 β -subunits. Important, functionally defined integrin subtypes include the fibronectin receptor ($\alpha 5 \beta 1$), the vitronectin receptor ($\alpha V \beta 3$), the laminin receptor ($\alpha 3 \beta 1$), and the collagen receptor ($\alpha 2 \beta 1$).² Whereas the normal brain parenchyma contains an amorphous matrix primarily consisting of hyaluronic acid,³ glioma cells can modulate the extracellular matrix via expression of integrin ligands such as fibronectin, laminin, vitronectin, and collagen type IV.^{4–6} Ligand-receptor interactions between extracellular matrix components and integrins activate cytoplasmic tyrosine kinases, such as focal adhesion kinase (FAK, also known as PTK2) and then downstream effector kinases, such as the extracellular signal-regulated kinases 1/2 (ERK1/2). In particular, FAK plays a crucial role in the integrin signaling cascade.^{7,8} FAK activation, via phosphorylation, is mediated by interaction of its C-terminal domain with integrin-associated proteins

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such as paxillin (PXN)^{9,10} and talin.¹¹ Expression levels of FAK have been correlated with invasive properties in a variety of human tumors;¹² specifically, astrocytic gliomas show an association between FAK expression levels and malignancy grade.^{13,14}

Activated growth factor receptors, such as epidermal growth factor receptor (EGFR) and platelet-derived growth factor receptor- α (PDGFRA), are also capable of modulating the tyrosine phosphorylation status of FAK.^{13,15} Notably, activated EGFR and PDGFRA signaling pathways are common in glioblastoma, due to amplification and/or overexpression.¹⁶ *In vitro* studies have proposed a functional connection between EGFR and FAK impacting the migratory potential of glioma cells. Although EGFR activation increases glioma cell migration,^{17–19} glioma cells lacking FAK are refractory to motility signals from EGFR.²⁰ Moreover, FAK co-immunoprecipitates with EGFR and PDGFRA after stimulation with their respective ligands, suggesting recruitment of FAK in an active signaling complex. Thus, FAK likely plays a central role as both a mediator of integrin-dependent cell motility and an effector molecule in growth factor-triggered signaling pathways.^{21,22}

Most studies of this pathway in glioblastomas have used *in vitro* systems. In addition, although FAK has been proposed as the central mediator of migration in glioma cells, the exact interactions between the individual molecules within the pathway are not fully understood. Nor is it clear whether growth factor receptors or integrin signaling is the primary upstream driver of FAK activation in glioblastomas. We therefore directly evaluated FAK activation and upstream integrin and growth factor pathways *in situ* in human glioblastoma samples. In addition, because many molecules are only focally overexpressed in glioblastomas, we used this *in situ* approach to investigate topographical relationships between specific activated molecules in these pathways.

Materials and Methods

Materials

Thirty formalin-fixed paraffin-embedded glioblastomas, World Health Organization grade IV,¹ were randomly selected from resection specimens in the Department of Pathology, Massachusetts General Hospital, Boston, MA, after appropriate human studies permission was obtained. Additional frozen material, snap-frozen immediately after operation and stored at -80°C , was available for 11 of these tumors. To ensure that tumor fragments taken for molecular analysis contained a sufficient proportion ($>80\%$) of tumor cells, histological evaluation was performed on each studied specimen. Commercially available total RNA extracted from nonneoplastic cerebral tissue of a male, adult patient (catalog no. T5595-7241; US Biological, Swampscott, MA) was used as an mRNA reference.

Gene Expression Profiling

Tissues were homogenized in guanidinium isothiocyanate, and RNA was isolated using a CsCl gradient. RNA integrity was confirmed by gel electrophoresis. For each sample, 2 μg of total RNA were used to generate cDNAs, which were radioactively labeled with [$\alpha^{32}\text{P}$]dCTP (catalog no. AA0005; Amersham Biosciences, Piscataway, NJ) using the GEArray AmpoLabeling-LPR kit (catalog no. L-03; SuperArray Bioscience Corp., Frederick, MD). Probes were hybridized overnight to GEArray Q series human extracellular matrix and adhesion molecules gene arrays (catalog no. HS-010; SuperArray Bioscience Corp.) containing the following 24 integrin subunits: *ITGA1* (integrin $\alpha 1$), *ITGA2* (integrin $\alpha 2$), *ITGA2B* (integrin $\alpha 2b$), *ITGA3* (integrin $\alpha 3$), *ITGA4* (integrin $\alpha 4$), *ITGA5* (integrin $\alpha 5$), *ITGA6* (integrin $\alpha 6$), *ITGA7* (integrin $\alpha 7$), *ITGA8* (integrin $\alpha 8$), *ITGA9* (integrin $\alpha 9$), *ITGA10* (integrin $\alpha 10$), *ITGA11* (integrin $\alpha 11$), *ITGAL* (integrin αL), *ITGAM* (integrin αM), *ITGAV* (integrin αV), *ITGAX* (integrin αX), *ITGB1* (integrin $\beta 1$), *ITGB2* (integrin $\beta 2$), *ITGB3* (integrin $\beta 3$), *ITGB4* (integrin $\beta 4$), *ITGB5* (integrin $\beta 5$), *ITGB6* (integrin $\beta 6$), *ITGB7* (integrin $\beta 7$), and *ITGB8* (integrin $\beta 8$). After washing, hybridized arrays were exposed to imaging plates (Bio-Rad Laboratories, Hercules, CA) and analyzed using the Personal Molecular Imager FX (Bio-Rad). Densitometric analysis of gene expression values was performed with the Scan Analyze version 2.50 software and data analysis performed with the GEArray Analyzer 1.3 software, both supplied with the arrays. All raw signal intensities were corrected for background by subtracting the minimum value to avoid the appearance of negative numbers and were normalized to the housekeeping gene *GAPDH*.

Array results were confirmed by quantitative reverse transcriptase-polymerase chain reaction (PCR) analyses for *ITGB8* and *ITGA5* on seven glioblastoma cases. B2-microglobulin (*B2MG*) transcript levels were used as a reference. One μg of total RNA from each tumor was reverse-transcribed into cDNA in a total volume of 20 μl using oligo(dT) primers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR conditions, including cycle numbers (33 cycles for *ITGB8* and *ITGA5* and 28 cycles for *B2MG*), MgCl_2 concentration, and annealing temperature were optimized for each PCR reaction. The respective primer sequences are available on request. After electrophoresis on 1% agarose gels, ethidium bromide-stained bands were recorded by the Gel-Doc 2000 system (Bio-Rad). Quantitative analysis of the signal intensities was performed with the Quantity One software (version 4.2.2; Bio-Rad) and the ratio between target mRNA and *B2MG* mRNA signal intensities was calculated for each tumor and normalized to the target mRNA:*B2MG* ratio determined for nonneoplastic brain tissue.

Immunohistochemistry

Immunohistochemistry was performed on 5- μm serial sections that were mounted on poly-L-lysine-coated

Table 1. Primary Antibodies Used for Immunohistochemical Analyses

Protein	Clone	Company	Catalog no.	Raised in	Positive control	Dilution
EGFR	EGFR.113	Novocastra	NCL-L-EGFR	Mouse	Normal skin	1:30
PDGFRA	C-20	Santa Cruz	sc-338	Rabbit	Neurofibroma	1:300
Integrin $\alpha 2\beta 1$	BHA2.1	Chemicon	mAb1998	Mouse	Tonsil	1:300
Integrin $\alpha 3$	P1B5	Santa Cruz	sc-13545	Mouse	Tonsil	1:25
Integrin $\alpha 5\beta 1$	JBS5	Chemicon	mAb1969	Mouse	Tonsil	1:300
Integrin αV	P2W7	Santa Cruz	sc-9969	Mouse	Tonsil	1:50
Integrin $\beta 8$	C-19	Santa Cruz	sc-6638	Goat	Lung carcinoma	1:200
PXN	ZO35	Zymed	03-6100	Mouse	Prostate carcinoma	1:1000
FAK	H-1	Santa Cruz	sc-1688	Mouse	Prostate carcinoma	1:50
p-FAK (Tyr397)	Polyclonal	Biosource	44-624	Rabbit	Prostate carcinoma	1:300
ERK	Polyclonal	Zymed	61-7400	Rabbit	Pituitary gland	1:100
p-ERK (Thr202/Tyr204)	Polyclonal	Cell Signaling	9101	Rabbit	Prostate carcinoma	1:100
Vimentin	V9	BioGenex	MU074-UC	Mouse	Tonsil	1:100

slides and baked at 60°C overnight. After deceration, slides were treated with 0.5% hydrogen peroxide in methanol for 10 minutes at room temperature. Sections were rehydrated and washed with phosphate-buffered saline (PBS), followed by antigen retrieval with 10 mmol/L sodium citrate buffer (pH 6) in a microwave oven for 10 to 20 minutes. After blocking with 10% normal goat or horse serum in PBS/1% bovine serum albumin for 30 minutes at room temperature, the sections were incubated overnight at 4°C with the specific primary antibodies. The 13 primary antibodies as well as respective dilutions and positive control tissues are listed in Table 1. On the following day, biotinylated goat anti-rabbit IgG or biotinylated horse anti-mouse IgG at dilutions from 1:250 to 1:1000 (Vector, Burlingame, CA) were applied for 30 minutes at room temperature, followed by detection of immunoreactivity with an avidin-biotin system (Vector) using either 3,3'-diaminobenzidine tetrahydrochloride or NovaRED (Vector) as a chromogen. Sections were lightly counterstained with Mayer's hematoxylin (Richard-Allan Scientific, Kalamazoo, MI). Negative controls without primary antibodies were performed for all reactions. In addition, negative controls with the respective IgG isotopes were performed using negative control antibodies for mouse IgG_{2a}, mouse IgG₁ and rabbit IgG (LabVision, Fremont, CA) as well as normal goat IgG (R&D Systems, Minneapolis, MN). The controls were run under the same conditions and the same IgG concentrations as were used for the respective primary antibodies.

Immunofluorescence

Immunofluorescence double labeling was performed to assess EGFR and p-FAK co-localization on a single cell level. Slides were incubated overnight with a mix of the two primary antibodies used for conventional immunohistochemistry (Table 1). The next day fluorochrome-labeled secondary antibodies (Texas Red goat anti-mouse IgG, 1:50 dilution and fluorescein isothiocyanate-conjugated goat anti-rabbit, 1:25 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) were applied for 3 hours at room temperature. After washing with PBS, sections were counterstained with 4,5-diamidino-2-phenylindol.

Fluorescence in Situ Hybridization (FISH)

Gene-specific and control clones were obtained according to the National Center for Biotechnology Information database: CTD-2113A18 (*EGFR*)/RP11-340A14 (7q control); RP11-626H4 (*PDGFRA*)/RP11-732L22 (4p control); RP11-466C17 (*ITGB8*)/RP11-340A14 (7q control). Each probe was confirmed for the presence of gene by PCR with specific primers and by mapping on metaphase control slides. FISH was performed as described previously, with some modification.²³ In brief, DNA probes were labeled with Cy3-dCTP and fluorescein isothiocyanate-dUTP by nick translation. Hybridization was followed by washing and counterstaining with 4,5-diamidino-2-phenylindol. Only an increase of target:control probe ratios of more than fivefold was considered as gene amplification. Target:control probe ratios of twofold to fivefold were referred to as low-level copy number gains. The percentage of cells bearing the respective copy number gains was determined.

Scoring and Interpretation of Immunohistochemistry

Protein expression levels were converted into a numerical score, based on the labeling index (LI) of percentage of positive-stained tumor cells. Labeling indices were grouped into six scores: 0 (no or minimal reactivity, similar to nonneoplastic brain tissue), 1 (1% or less), 2 (1 to 10%), 3 (10 to 50%), 4 (50 to 90%), and 5 (>90%). Because many of the integrins showed a regionally restricted expression pattern, we designed the staining score with a higher resolution for lower percentages. Positive staining of vessels was not included into the expression score. Slides were scored independently by two neuropathologists (M.J.R. and W.M.). Discrepant cases were discussed and agreement was reached to reduce interobserver variability. Complete immunohistochemistry scoring data are available online (Supplementary Table 1 see <http://ajp.amjpathol.org>).

Given that there was considerable intratumoral heterogeneity for expression of these molecules, *in situ* corre-

Figure 1. a: mRNA array expression analysis of integrin subunits in glioblastomas (GBM) with *GAPDH* as a reference gene. Note that the two glioblastoma cases strongly overexpress *ITGB8* (GBM07, 15-fold; GBM15, 30-fold) in comparison to nonneoplastic brain tissue (NB). Slightly increased mRNA levels can be also detected for *ITGB1* and *ITGAV*, whereas *ITGA5* was not overexpressed on the mRNA level. Overexpression of *CD44* was detected in both tumors and served as a positive control for validation of array results. **b:** Protein expression analysis of integrins in the same two glioblastoma cases. $\beta 8$ overexpression is confirmed, but also $\alpha 5\beta 1$ with weak or absent expression of the respective subunits on the mRNA level can be regionally detected by immunohistochemistry. Note that integrin expression is often related to perivascular or perinecrotic tumor regions. N, necrosis; V, vessel. Original magnifications: $\times 400$; $\times 200$ ($\alpha 5\beta 1$ immunohistochemistry in GBM07).

lations between specific molecules were evaluated by recording topographical distributions of expressed proteins within individual tumors. In this regard, blocks had been selected from the central parts of the tumors to compare areas of solid tumor tissue. For each case, regions were defined by maximum and minimum expression of the growth factor receptors EGFR and/or PDGFRA (GFRmax and GFRmin). Additionally, within the GFRmax regions, we selected regions with high (ITGmax) and low (ITGmin) expression of integrins. Intact antigenicity in these regions was also scrutinized by staining with a control antibody against vimentin. Slides were then scored blindly for expression and activation of the components of the integrin signaling pathway in the respective regions.

Statistical Analyses

Spearman correlation coefficients were used to assess the correlation between any two ordinal scores. The bootstrap with 2000 repetitions was used to calculate 95% confidence intervals for the correlation coefficients. Dependent correlation coefficients were compared using Stieger's (1980) test statistic, and its significance assessed using the bootstrap (2000 repetitions). Adjustment for multiple comparisons was accomplished with Bonferroni corrections and adjusted *P* values are reported. Associations in differentials between max and min regions were assessed using both the actual differ-

ences (max-min) and the normalized differences [(max-min)/max] to adjust for the differences in scale due to different maximum levels. The exact Wilcoxon signed rank test was used to compare max and min regions within tumors.

Results

ITGB8 Is the Most Frequently Up-Regulated Integrin Subunit in Glioblastoma

To screen for expression of integrin subunits, we performed mRNA array analysis in a subset of 11 glioblastomas for which snap-frozen tissue was available. None of the 24 investigated integrin types showed expression in nonneoplastic brain tissue, except for *ITGB8*, which was weakly expressed. In tumors, *ITGB8* was by far the most frequently overexpressed integrin (8 of 11 investigated glioblastoma cases) with fold changes ranging from 3-fold to >30 -fold (GBM15; Figure 1a) in comparison to nonneoplastic brain tissue. Other integrin subtypes were less commonly up-regulated; moderately elevated expression levels in comparison to the reference tissue ($0.3 < \times < 0.6$ after minimal value subtraction and normalization) were found for *ITGA2*, *ITGA10*, and *ITGAX* in one case and for *ITAV*, *ITGB1*, and *ITGB5* in two cases, respectively (Figure 1a), whereas the other integrin subunits showed even lower expression levels ($\times < 0.3$ after

minimal value subtraction and normalization), approaching expression in nonneoplastic brain tissue.

For validation purposes, seven glioblastomas were also investigated by quantitative reverse transcription-PCR analysis for *ITGB8* and *ITGA5* transcripts. In concordance with the array results, *ITGB8* was overexpressed in six of seven glioblastomas (fold changes ranging from 3-fold to 10-fold for GBM15) and showed minimal expression in nonneoplastic brain tissue (Supplementary Figure 1a see <http://ajp.amjpathol.org>). *ITGA5* was only weakly expressed in some of the tumor cases and had no detectable expression in nonneoplastic brain tissue.

Expression of Various Integrin Subtypes Is Regionally Restricted and Frequently Related to Morphological Structures Such as Necrosis or Pathological Vessels

Given the above findings, we used immunohistochemistry to study the entire series of 30 glioblastomas for integrin $\beta 8$ as well as for the functionally defined integrins $\alpha 5\beta 1$, αV , $\alpha 3$, and $\alpha 2\beta 1$. Integrin expression in most cases was regionally confined and frequently related to perinecrotic or perivascular areas (Figure 1b). Confirming the mRNA screen, we found the highest median expression scores for integrin $\beta 8$ (median, 3; range, 0 to 4), but integrin $\alpha 5\beta 1$ (median, 2.5; range, 1 to 3) was also expressed in a majority of the cases. Integrin αV (median, 1; range, 0 to 3), integrin $\alpha 3$ (median, 0; range, 0 to 3), and integrin $\alpha 2\beta 1$ (median, 0; range, 0 to 2) expression occurred in a much lower number of cases and usually just involved a small number of tumor cells.

p-FAK Expression Co-Localizes Significantly to Regions of Growth Factor Receptor Expression but Not Integrin Expression

We correlated expression of the different integrin subtypes with expression scores for the growth factor receptors EGFR (median, 3; range, 0 to 5) and PDGFRA (median, 4; range, 1 to 5) and the downstream molecules FAK (median, 4; range, 2 to 5), PXN (median, 4; range, 3 to 5) and ERK1/2 (median, 3; range, 2 to 5). We also assessed the phosphorylation status of FAK (median, 4; range, 2 to 5) and ERK1/2 (median, 3; range, 1 to 4) using phospho-specific antibodies.

Protein expression showed considerable variability between the 30 cases as well as within individual tumor samples. We first compared the estimate of the total percentage of positive cells across the specimen. FAK and p-FAK ($P < 0.001$; 95% CI, 0.51 and 0.85), as well as FAK and PXN ($P = 0.012$; 95% CI, 0.19 and 0.77) were significantly co-localized. Phosphorylated-FAK and PXN were marginally significantly co-localized ($P = 0.066$; 95% CI, 0.11 and 0.65). In addition, p-FAK-expressing cells co-localized significantly with overall areas of EGFR and/or PDGFRA expression ($P = 0.0006$; 95% CI, 0.42 and 0.82), whereas there was no significant co-localization between overall p-FAK ex-

Figure 2. Boxplots for the relative differences in protein expression scores comparing GFRmax and GFRmin regions on a tumor-by-tumor basis in the panel of 30 glioblastomas. **Top** of box is 75th percentile, **bottom** is 25th percentile, whiskers are data points not more than 75th percentile + 1.5 * interquartile range (IQR) and not less than 25th percentile - 1.5 * IQR. Note that expression of Int $\alpha 5\beta 1$, Int $\beta 8$, PXN, p-FAK, and p-ERK is clearly elevated in GFRmax in comparison to GFRmin regions (boxplots centering in the positive range), whereas no differences between the two regions can be detected for the VIM control.

pression and areas of integrin expression ($P = 0.312$; 95% CI, 0.02 and 0.62).

FAK, p-FAK, PXN, ERK-1/2, p-ERK-1/2, and Integrin Expression Are Dependent on Growth Factor Receptor Expression

We then focused on evaluating protein expression in specific histological regions on a tumor-by-tumor basis. For each case, we compared regions defined by high (GFRmax) and low (GFRmin) expression scores for EGFR and/or PDGFRA. Within individual tumors, significantly increased expression levels of FAK, p-FAK, PXN, ERK-1/2, and p-ERK-1/2 were found in regions defined by elevated growth factor receptor expression (Wilcoxon signed rank test, $P < 0.001$; Figures 2 and 3b). Moreover, the level of FAK activation was not only increased in GFRmax areas but there was also a significant correlation between p-FAK and both EGFR ($P = 0.006$; 95% CI, 0.24 and 0.84) and PDGFRA ($P < 0.001$; 95% CI, 0.42 and 0.92) expression levels using the normalized measure of difference between GFRmax and GFRmin. Interestingly, integrin expression was also enriched in GFRmax areas (Wilcoxon signed rank test, P value for sum of integrins < 0.0001) but was more regionally confined than FAK, p-FAK, and PXN expression (Figures 2 and 3b).

Within the GFRmax regions, we also compared regions with high (ITGmax) and low (ITGmin) integrin expression scores. Eight cases showed slightly increased FAK activation levels in the ITGmax areas, whereas the rest of the cases demonstrated no differences in expression scores between the two regions. Differences in FAK expression between such regions were significant (Wilcoxon signed rank test, $P = 0.006$), but there was no significant corre-

Figure 3. Evaluation of the topological distribution of molecular changes at a histological level by comparing regions of high (GFRmax) and low (GFRmin) GFR expression within the same tumors. Note that expression of integrins, PXN, and activated FAK and ERK1/2 is associated with GFR (EGFR and/or PDGFRA) expression. **b:** VIM serves as a positive control to demonstrate intact antigenicity of both regions. GBM18 has *EGFR* gene amplification on FISH and shows normal copy numbers for *PDGFRA*, whereas in GBM12 amplification of neither genes could be detected by FISH analysis (red, target probe; green, control probe). Also note that GFR overexpression can occur without corresponding gene amplification, but nonetheless be associated with increased expression and activation of components of the integrin signaling pathway (**a** and **b**). Original magnifications: $\times 400$; $\times 1000$ (FISH images).

Figure 4. Demonstration of EGFR and p-FAK co-localization by immunofluorescence double labeling on a single cell level in a glioblastoma with EGFR amplification (GBM24). EGFR is labeled with Texas Red giving a red signal, p-FAK with fluorescein isothiocyanate providing a green signal. The large, central, pleomorphic cell has both EGFR and p-FAK co-expression (indicated by a yellow signal) and shows matching intracellular distribution patterns of both molecules, with highest co-expression near the cell membrane, including in short cell processes, P. N, nucleus; C, cytoplasm; M, membrane. Original magnifications, $\times 1000$.

lation between integrin and p-FAK expression levels ($P = 0.09$; 95% CI, -0.08 and 0.64).

FAK Activation Co-Localizes to EGFR Expression on a Single Cell Level

To further investigate the relationship between FAK activation and growth factor receptor expression, we investigated EGFR and p-FAK co-expression at the single cell level using immunofluorescence double labeling. High FAK activation levels were found in larger cells expressing EGFR and were particularly localized near the cell membrane, including in short cell processes, directly overlapping with EGFR expression (Figure 4).

GFR Amplification Is Not Directly Related to Expression or Activation of Components of the Integrin Signaling Pathway

We screened all 30 tumors with FISH for *EGFR* and *PDGFRA* gene amplification. Copy number gains for *EGFR* were detected in 19 of the 30 cases (15 cases demonstrating high-level gene amplification in the majority of the investigated tumor cells and two additional cases each with regionally restricted *EGFR*-amplified cells or low-level copy number gains in less than 50% of tumor cells). For *PDGFRA*, two cases showed high-level amplification involving the majority of tumor cells, whereas six cases just showed amplification in a small subset of less than 10% of cells. Two additional tumors demonstrated regionally restricted low-level copy number gains for *PDGFRA* in less than 50% of the tumor cells.

When present, *EGFR* and *PDGFRA* gene amplification was associated with high expression of the respective protein as well as overexpression and phosphorylation of molecules in the integrin signaling pathway. However, alterations in the integrin signaling pathway were not significantly associated with *EGFR* or *PDGFRA* gene amplification because growth factor receptor overexpression alone (ie, without gene amplification) was also associated with increased expression and activation of components of the integrin signaling pathway (Figure 3, a and b).

Finally, to evaluate if *ITGB8* gene amplification drove high expression of *ITGB8* independently of GFR expression, we performed FISH analysis for *ITGB8* copy number on the eight cases showing overexpression of *ITGB8* transcripts by array analysis. No cases had amplification of the *ITGB8* locus (Supplementary Figure 1b see <http://ajp.amjpathol.org>).

Discussion

In many types of human cancers, integrin signaling is a key pathway related to tumor cell invasion and migration.²⁴ We analyzed the interrelationships between the integrin signaling and growth factor receptor signaling pathways directly in human glioblastoma samples, with an emphasis on correlating expression of individual molecules across topographical regions of the tumors. We felt that this approach would allow direct observational validation of functional relationships between individual pathway components that had been suggested by experimental data *in vitro*.

Of the 24 different integrin subtypes investigated, *ITGB8*/ $\beta 8$ was the most frequently up-regulated integrin on both the mRNA and protein level, whereas other integrins, such as integrin $\alpha 5 \beta 1$ (fibronectin-receptor) and (in fewer cases) integrin αV (vitronectin-receptor), integrin $\alpha 3$ (laminin-receptor) and integrin $\alpha 2 \beta 1$ (collagen-receptor) were only expressed in small subsets of cells, as shown by immunohistochemistry. That the *in situ* analysis detected expression of integrin subtypes that did not show up in the array analysis may reflect greater sensitivity of an *in situ* approach for detecting expression in scattered cells, but may also represent discordance between mRNA and protein expression levels.²⁵ Our findings are in line with previous reports of increased expression of the integrin subunits $\alpha 3$, $\alpha 5$, αV , and $\beta 1$ in neoplastic astrocytes.²⁶ Expression of the $\beta 8$ subunit, however, has not been studied in human glioblastomas and our results in this regard are therefore novel. $\beta 8$ has been shown to pair exclusively with the αV subunit in the central nervous system to form the $\alpha V \beta 8$ heterodimer, which acts as a functional receptor for vitronectin, and $\beta 8$ expression has been described in synaptic and glial sites

in mouse and rat brain.^{27,28} The functional role of $\beta 8$ expression in human tumors, however, has yet to be elucidated. Some authors postulate a role for $\alpha V\beta 8$ as a negative regulator of epithelial cell growth,²⁹ but our findings argue rather for a potential oncogenic function.

We then focused on delineating correlations between the expression patterns of growth factor receptors, integrins, and downstream kinases involved in both signaling pathways. We first assessed the overall protein expression levels on a tumor-by-tumor basis. Interestingly, areas containing p-FAK-expressing cells co-localized significantly with the areas of growth factor receptor (EGFR and/or PDGFRA) expression, whereas integrin expression was far more regionally confined than growth factor receptor or p-FAK expression. Given that both growth factor receptors and integrins activate FAK, as demonstrated by *in vitro* studies,^{7,30} it is tempting to speculate that the present data provide insight on how these molecules interact *in vivo* in primary human glioblastomas. Indeed, the relative topographical overlap between growth factor receptor and p-FAK expression strongly suggests a close biological relationship between these two events, or at least raises the possibility of a closer relationship between growth factor receptor and p-FAK activation in glioblastomas than between integrin and p-FAK activation. Notably in this regard, in addition to the regional expression relationships, we demonstrated that FAK activation and EGFR expression co-localize on a single cell level, with high FAK expression especially near the cell membrane and in some short cellular processes, consistent with a role in cellular migration. Overall, these single cell findings support the hypothesis that FAK activation in glioblastoma is closely related to growth factor receptor expression.

To address this question in further detail, we compared regions of high and low growth factor receptor expression on a tumor-by-tumor basis. Strikingly, all components of the integrin signaling pathway, including the integrins themselves, are more likely to be expressed in the same regions in which growth factor receptors are overexpressed. This raises the possibility that growth factor receptors, in addition to direct stimulatory effects, may also act indirectly via up-regulation of cell adhesion molecules. For instance, the combination of growth factor receptor and integrin signaling could result in synergistic or even additive FAK and ERK1/2 activation, particularly given that FAK acts as a bridging molecule, linking growth factor receptor and integrin signaling pathways. Whereas EGFR and PDGFRA associate with the N-terminal domain of FAK, binding sites for integrins predominantly map to the C-terminal domain of the protein.²¹ Furthermore, intense crosstalk has been described between integrins and growth factor receptor pathways.^{31,32} Growth factors can regulate integrin-mediated events such as cell adhesion, cell spreading, and cell migration through alterations in integrin localization and activation.³³ Conversely, signals generated by integrins are required for full activation of growth factor signaling pathways. For example, extracellular matrix-mediated adhesion is required for growth factor-induced cell cycle progression.²⁴

Figure 5. Schematic of the integrin signaling pathway in glioblastoma. Overall FAK activation may be primarily accomplished by direct GFR signaling. However, in certain morphologically defined regions (perivascular, perinecrotic, tumor infiltration zone), in which extracellular matrix components (ECM) are present, integrins are up-regulated in a GFR-overexpressed background and might enhance FAK and ERK1/2 phosphorylation, potentially resulting in increased motility and transcription.

We also investigated the additional effect of integrin expression on FAK activation in growth factor-overexpressing regions. There were significant differences in p-FAK expression when comparing areas defined by integrin and growth factor receptor co-expression to areas defined only by growth factor receptor expression. However, only eight cases demonstrated slightly stronger FAK expression in the regions where integrins were co-expressed, whereas the majority of cases showed no differences between such regions at all. In addition, there was no significant correlation between integrin and p-FAK expression, as described for integrin and EGFR/PDGFR expression. This also argues for a predominant role of growth factor receptor expression in FAK activation. Nonetheless, although it is recognized that there are limitations to precise quantitation in immunohistochemical studies, these findings at least raise the possibility that integrins could enhance the effects of growth factor receptor signaling in particular areas of the tumor micro-environment (Figure 5).

This is of interest in regions such as the edges of tumors, in which migration may be up-regulated; perivascular zones, in which there may be interfaces with the vascular-associated extracellular matrix molecules; and in perinecrotic zones, in which there may be considerable up-regulation of many genes. This hypothesis is in keeping with our finding of integrin expression frequently related to perivascular or perinecrotic areas and with observations in the literature that integrins are highly expressed by endothelial cells associated with new blood vessels³⁴ and at the invasive edges of tumors.^{6,35} Although this study was designed to primarily focus on growth factor receptor and integrin signaling interrela-

tionships and therefore necessarily depended on studying large areas of the cellular tumor center, the findings encourage subsequent studies of other microenvironmental regions. One such region is the invading edge of glioblastoma, as the infiltrating tumor cells overexpress integrins and also select for EGFR-amplified cells.²³

In summary, there are topographical relationships between integrin signaling and growth factor receptor signaling components in primary human glioblastomas. The signaling relationships suggested on the basis of *in vitro* studies appear operative in primary glioblastomas. Moreover, FAK activation may be more closely related to growth factor receptor expression than previous *in vitro* studies would suggest. Integrins may additionally enhance the effects of growth factor receptor signaling in regions defined by the tumor microenvironment. As a result, drugs targeting such pathways in glioblastoma will need to address these activated molecular cascades in a comprehensive manner.

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