Video Article

Quantitative Immunohistochemistry of the Cellular Microenvironment in Patient Glioblastoma Resections

Jessica X. Yuan[1], Jennifer M. Munson[1]

[1]Biomedical Engineering, University of Virginia

Correspondence to: Jennifer M. Munson at jm4kt@eservices.virginia.edu

URL: https://www.jove.com/video/56025
DOI: doi:10.3791/56025

Keywords: Medicine, Issue 125, Glioblastoma, tumor microenvironment, glial cells, parenchyma, patient samples, immunohistochemistry

Date Published: 7/31/2017

Citation: Yuan, J.X., Munson, J.M. Quantitative Immunohistochemistry of the Cellular Microenvironment in Patient Glioblastoma Resections. *J. Vis. Exp.* (125), e56025, doi:10.3791/56025 (2017).

Abstract

With the growing interest in the tumor microenvironment, we set out to develop a method to specifically determine the microenvironment components within patient samples of glioblastoma, the deadliest and most invasive brain cancer. Not only are quantitative methods beneficial for accurately describing diseased tissues, they can also potentially contribute to more accurate prognosis, diagnosis, and the development of tissue-engineered systems and replacements. In glioblastoma, glial cells, such as microglia and astrocytes, have been independently correlated with poor prognosis based on pathologist grading. However, the state of these cells and other glial cell components has not been well-described quantitatively. This can be difficult due to the large processes that mark these glial cells. Furthermore, most histological analyses focus on the overall tissue sample or only within the bulk of the tumor, as opposed to delineating quantifications based on regions within the highly heterogeneous tissue. Here, we describe a method for identifying and quantitatively analyzing the populations of glial cells within the tumor bulk and adjacent regions of tumor resections from glioblastoma patients. We used chromogenic immunohistochemistry to identify the glial cell populations in patient tumor resections and ImageJ to analyze percent coverage of staining for each glial population. With these techniques we are able to better describe the glial cells throughout regions of the glioma tumor microenvironment.

Video Link

The video component of this article can be found at https://www.jove.com/video/56025/

Introduction

Glioblastoma (GBM), the most common and malignant brain cancer, is characterized by highly diffuse invasion from the primary tumor bulk into the surrounding healthy brain parenchyma[1,2]. This diffuse invasion makes the tumor particularly difficult to resect fully, and the invading cancer cells that remain post-therapy is the most common reason for inevitable recurrence[2,3,4]. Previously, we found inhibiting the diffuse glioma cell invasion to be therapeutically beneficial[5], however little is known about the complex mechanisms contributing to GBM invasion. The tumor microenvironment, or tissue surrounding the cancer, has been implicated in the progression of tumors in multiple cancers[6,7]. The glioblastoma tumor microenvironment, in particular, is relatively under-characterized and is uniquely complex, composing of multiple glial cells, such as astrocytes, microglia, and oligodendrocytes, as well as extracellular matrix, soluble factors, and biophysical factors. Experimentally, astrocytes and microglia have been shown to increase glioma progression and invasion[8,9,10], but the composition of all glial cells in the native human brain microenvironment is unknown.

We previously showed microenvironmental components can predict patient survival by quantitatively analyzing cellular components of the glioblastoma microenvironment and incorporating our analyses into a proportional hazards model[11]. Here, we describe the quantitative analysis method for identifying the populations of glial cells within the tumor bulk and adjacent regions of tumor resections from glioblastoma patients. We used chromogenic immunohistochemistry to identify the glial cell populations and ImageJ to analyze percent coverage of staining for each glial population. Assessing percent coverage creates a simple measurement for determining the morphological differences of cells, particularly those affected by interactions with cancer cells. Previous studies for quantifying histopathological staining use standard staining such as hematoxylin and eosin[12] or Masson's trichrome[13], which do not take advantage of the specificity of antibody-based immunohistochemistry staining. Our method was developed to directly quantify the glial populations within glioblastoma patient tumor resections, which we aim to use to elucidate the complex glioblastoma microenvironment.

Protocol

This protocol identifies cellular components in formalin-fixed paraffin embedded (FFPE) samples, as is typical for banked clinical patient samples. Paraffin embedding allows for the best maintenance of cellular and tissue morphology as well as has better longevity of sections. The samples used for this analysis were accessed through the University of Virginia Biorepository and Tissue Research Facility. Patient samples

were selected by a neuropathologist based on a definitive diagnosis of glioblastoma (astrocytoma, WHO grade IV) who had completed tumor resections at the University of Virginia between 2010 and 2013, and were de-identified prior to this analysis[11].

1. FFPE Sample Deparaffinization and Rehydration

NOTE: This portion of the protocol is specific to FFPE samples. While paraffin-embedded samples can be more useful for this analysis because of the preservation of cellular and tissue morphology, this analysis can also be done with frozen sections. If using frozen sections, this portion can be omitted and proceed directly to chromogenic immunohistochemistry.

1. Perform the following washes for 5 min each: Xylene, Xylene, 100% Ethanol, 100% Ethanol, 95% Ethanol, 95% Ethanol, 70% Ethanol, Deionized water, Deionized water

2. Antigen Retrieval

NOTE: This portion of the protocol is necessary to break methylene bridges formed during formalin fixation of FFPE samples and expose antigen sites for antibodies to bind.

1. Dilute Tris-based high pH antigen unmasking solution at manufacturer recommendation in distilled water.
2. Perform heat-mediated antigen retrieval using a microwave. Other forms of heat-mediated retrieval (such as pressure cooker, vegetable steamer, or boiling water) would also suffice.
    1. Add the diluted unmasking solution into a non-sealed microwaveable vessel. Place slides into vessel. Place slides into microwave.
    2. Boil for 20 min at high power. Monitor liquid levels for evaporation, and replenish with distilled water as necessary.
3. Allow samples to cool in solution for 1 h at room temperature.

3. Chromogenic Immunohistochemistry

1. Outline tissue sample with a hydrophobic pen to minimize volume of reagents necessary to cover sample.
   NOTE: Be sure to keep tissue samples hydrated and do not let them dry out as this will affect staining efficacy.
2. Pipet enough permeabilization solution (Tris-buffered saline (TBS) + 0.01% Triton-X) to cover tissue sample (typically about 100 - 200 µL).
3. Remove and discard solution and repeat step 3.2.
4. Incubate samples at room temperature with blocking solution (2.5% horse serum + permeabilization solution).
5. Incubate samples overnight in 4 ºC with primary antibodies diluted in blocking solution.
   NOTE: All primary antibodies used here are diluted at 1:200, but optimal dilutions can be determined using serial dilutions starting with manufacturer recommendation. Detailed information on antibodies used in this protocol was previously published[11].
6. Detect primary antibodies using a horseradish peroxidase polymer reagent corresponding with the primary antibody host animal, following manufacturer protocol.
7. Pipet enough permeabilization solution to cover tissue sample and incubate for 5 min.
8. Remove and discard solution and repeat Step 3.7.
9. Incubate slides in 0.3% H_2O_2 in 1x TBS for 15 min.
10. Develop samples with a peroxidase diaminobenzidine (DAB) substrate for 2 - 10 min until desired stain intensity is achieved.
11. Counterstain samples to identify cell nuclei, such as with hematoxylin, following manufacturer protocol.
12. Dehydrate samples with 100% ethanol and xylene.
13. Mount samples permanently with mounting media.

4. Regions of Interest Identification

1. Image slides under brightfield microscopy capable of high resolution images.
   NOTE: Use imaging cellular components at a minimum of 20x resolution.
2. Move camera to specific regions of interest throughout tissue samples.
3. Save images as TIFF files for quantification.

5. Image Analysis

1. Open images in ImageJ for quantification of percent coverage.
2. Use the Threshold Colour plugin to remove purple color from hematoxylin stained nuclei.
3. Convert image to 8-bit.
4. Add threshold without dark background.
5. Process image using one of the 17 pre-loaded ImageJ threshold filters (*i.e.* MaxEntropy) so only DAB stained portions are included in the threshold.
   NOTE: Select the optimal pre-loaded threshold filter that minimizes inclusion of background staining. This can depend on the quality of staining and specificity of antibody. Use the same threshold for all technical replicates within each patient sample.
6. Apply threshold.
7. Measure percent area of thresholded image.
8. Average percent area coverage for multiple regions within each sample.

Representative Results

For this analysis, two regions of interests within our tumor resections - the primary tumor bulk and the adjacent regions, primarily composed of healthy tissue with diffuse invading cancer cells (**Figure 1A**, **1B**) - were identified by collaborating neuropathologists on hematoxylin and eosin stained patient samples. Within each patient sample, positive staining for astrocytes (**Figure 1C**), microglia (**Figure 1D**), and oligodendrocytes (**Figure 1E**) via chromogenic immunohistochemistry was identified. These populations were identified using primary antibodies ALDH1L1, Iba1, and Oligodendrocyte-Specific Protein (OSP1) respectively[11]. Optimization of antibody dilutions is recommended, as well as consultation with manufacturer protocols to confirm if staining is accurate and positive (**Figure 2**). For example, we performed staining with no primary antibody for a negative control (**Figure 2A**), the manufacturer recommended (**Figure 2B**), and further dilutions (**Figure 2C**, **2D**) before settling on the 1:200 dilution (**Figure 2C**) due to optimal positive staining of cell body and processes with minimizing background for the anti-ALDH1L1 antibody.

Using ImageJ, the percent coverage of OSP1+ oligodendrocyte staining was quantified to eventually compare differences in our regions of interest (**Figure 3A**). We removed the counterstained nuclei from the image using the Threshold Colour plugin in order to solely assess the positive DAB staining (**Figure 3B**). After converting the image to 8bit (**Figure 3C**), we then applied the MaxEntropy default threshold to the remaining positive DAB staining (**Figure 3D**). Here, we used the MaxEntropy threshold default because this particular default threshold best captured our positive stain while minimizing background, but different thresholds can be used to ensure analysis of the population of interest. Once the appropriate threshold has been applied to the image (**Figure 3E**), the percent coverage from the threshold can be measured (**Figure 3F**) and compared for multiple patients. Here, we quantified 3 - 5 areas within each region of interest, depending on the total area of the region of interest, and compared groups using paired t-tests with GraphPad Prism software. We also compared our GBM analyses with corresponding staining found in healthy brain tissue in the Protein Atlas database (**Figure 3F**). In our previous publication, we used this analysis for multiple microenvrionmental components (astrocytes, microglia, oligodendrocytes, and blood vessels) across 33 patients to develop a proportional hazards model for predicting overall patient survival[11].

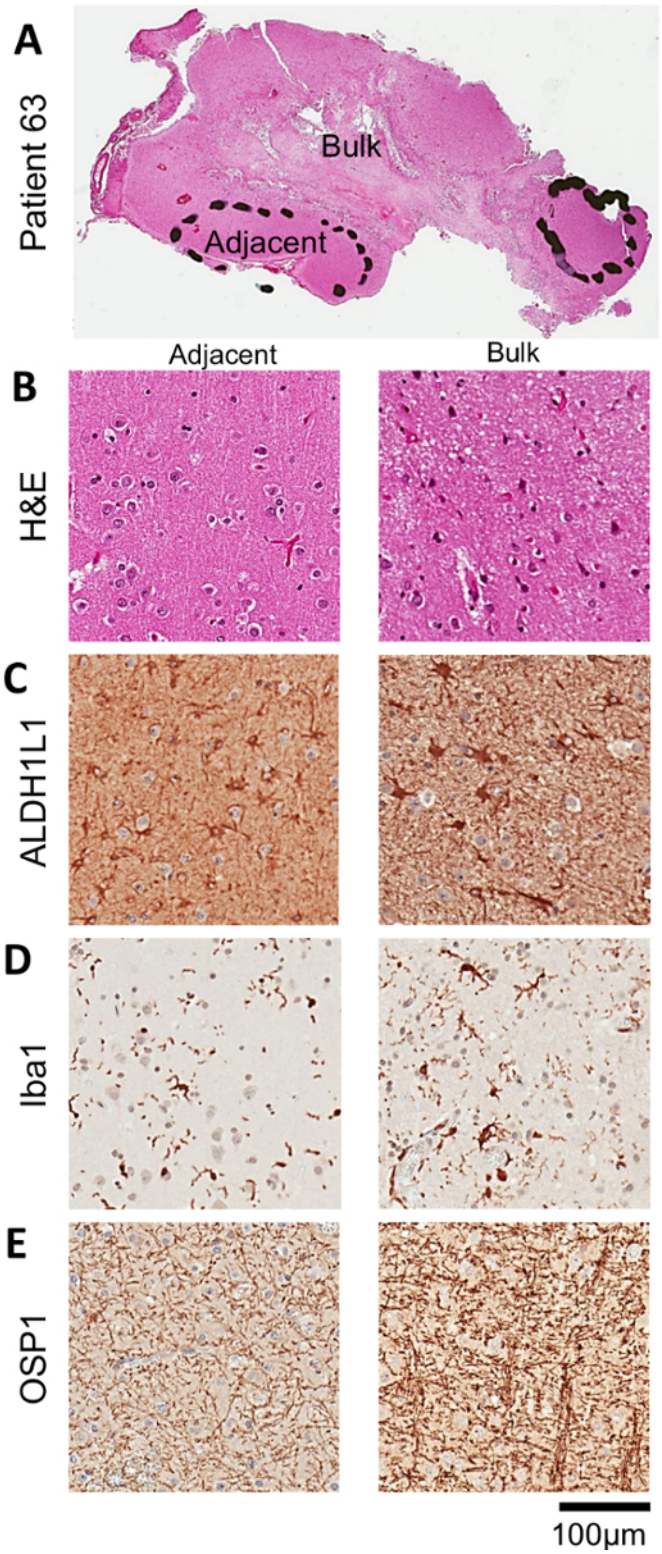

Figure 1: Comparison of Tumor Bulk and Tumor Adjacent Regions of Interest for Glial Cells.
A) Whole tumor sample from Patient 63 stained with hematoxylin and eosin with bulk and adjacent regions indicated. **B)** Hematoxylin and eosin staining, **C)** ALDH1L1+ astrocytes, **D)** Iba1+ microglia, and **E)** Oligodendrocyte Specific Protein-1 (OSP1)+ oligodendrocytes. Scale bar = 100 µm. Please click here to view a larger version of this figure.

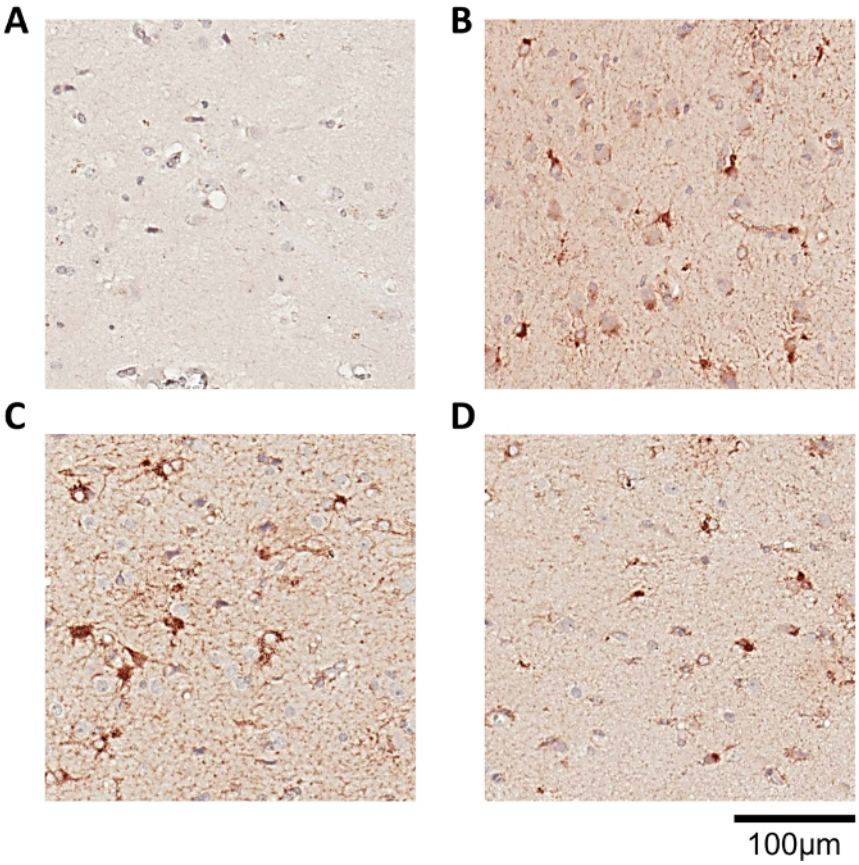

Figure 2: Example Titration of Anti-ALDH1L1 Antibody.
A) Negative control, **B)** 1:70 manufacturer recommendation dilution from stock, **C)** 1:200 dilution from stock, and D) 1:400 dilution from stock. 1:200 dilution was used for staining and analysis due to optimal positive staining of cell body and processes with minimizing background. Scale bar= 100 µm. Please click here to view a larger version of this figure.

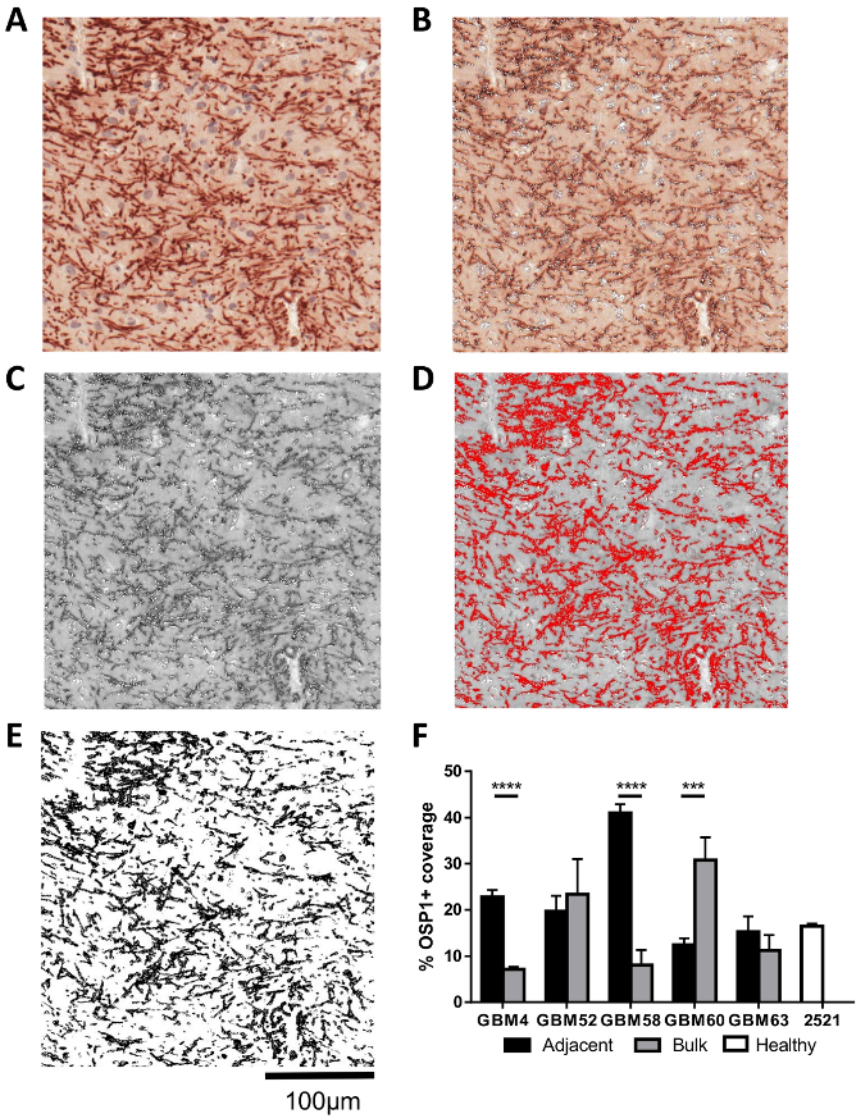

Figure 3: ImageJ Analysis and Quantification of Area Coverage for Oligodendrocytes.
A) Original OSP1+ staining of Patient 52. **B)** Removal of purple nuclei using Threshold_Colour plugin. **C)** Conversion to 8bit. **D)** MaxEntropy threshold captured and **E)** applied to minimize background staining. **F)** Comparison of adjacent and bulk regions in five patient samples, as well as healthy brain tissue through the Protein Atlas, for OSP1+ percent coverage. ***p< 0.001, ****p< 0.0001 via paired t-tests. Scale bar= 100 µm.
Please click here to view a larger version of this figure.

Discussion

Our method proposed here is a quantitative approach to analyzing histological samples stained using traditional chromogenic immunohistochemistry. Current methodology for this type of analysis includes similar staining protocols followed by grading by independent pathologists. This method has been reliable, yet for a number of applications, a more precise understanding of the cellular make-up is required, such as better understanding of the heterogeneity associated with tumors and accurate recapitulation of tumors for in vitro studies.

This protocol demonstrates a straightforward technique for quantitatively analyzing the populations of glial cells within the brain tumor microenvironment. This technique is widely adaptable and can be expanded to identify many other components of the microenvironment, such as blood vessels, chemokines, and more, as well as other cancers, such as breast or pancreatic, where the tumor resections can have larger parenchymal regions. Furthermore, this technique can be adapted to acquire other measurements, such as counts, and perimeter of cells to assess cell shape, as well as more complex morphology outcomes isotropy, clustering, orientation, and more. These techniques are also not limited to cancer, in that these staining and quantification methodologies can be used for any brain analyses, as demonstrated in our representative Protein Atlas quantification of healthy tissue, and our previously published comparison with epileptic brain tissue[11].

Critical steps within this protocol include proper incubation and development of the chromogenic secondary. Care should be taken such that the chromogenic dye does not over-develop. This will make it difficult to differentiate between positive staining and background. The development time varies depending on the substrate reagent used and the affinity of the primary antibody. We use bright field microscopy to

monitor development to ensure optimal staining while minimizing background staining. After staining, proper thresholding during analysis can help differentiate background from positive staining. A major limitation for this method is the inability to stain multiple components within the same tissue sample due to the use of chromogenic staining development. Immunofluorescence analysis could be conducted in a similar manner, but requires modification of the tools discussed in this method.

Specific to glioblastoma, there are a number of markers and cellular stains that have been used to analyze patient samples. These include IDH1[14,15], EGFRvIII[16,17] as prognostic markers, as well as CD11b and CD45 for microglia and macrophages[18,19]. Our method expands this type of analysis to the cellular microenvironment, an often overlooked component of the tumor microenvironment in glioblastoma. Studies have looked at microglia presence and density in tumors[20,21,22] as well as contributions of astrocytes[9,23,24]. Here we developed a protocol examining all of the glia - astrocytes, microglia, and oligodendrocytes - in the brain tumor microenvironment and to differentiate the adjacent or invasive regions of the tumor from the tumor bulk, which is where markers are usually identified in these tumors. By doing this, we believe we can more accurately assess the patient situation because we determined intra-patient heterogeneity and our previous publication showed that analysis of these locations and inclusion in statistical models is stronger at predicting outcomes than either location alone or overall samples[11]. Furthermore, since glioblastoma invasion is so diffuse, making it difficult to resect completely[1,2], we believe the tissue in the adjacent regions is more representative of the invasive tissue left behind after resection, which contributes to inevitable recurrence. Therefore, better understanding of the adjacent regions may give more information about disease recurrence and patient treatment planning, as opposed to the traditionally studied bulk tissue that is resected prior to therapy in glioblastoma patients.

Disclosures

None.

Acknowledgements

The authors thank Drs. Fahad Bafakih and Jim Mandell for acquisition and identification of patient samples, Garrett F. Beeghly for assistance with immunohistochemistry, and the Biorepository and Tissue Research Facility, the Cardiovascular Research Center Histology Core, and the Biomolecular Analysis Facility at the University of Virginia for assistance with sample acquisition, immunohistochemistry, and imaging.

References

1. Claes, A., Idema, A. J., & Wesseling, P. Diffuse glioma growth: a guerilla war. *Acta Neuropathol.* **114** (5), 443-458 (2007).
2. Holland, E. C. Glioblastoma multiforme: the terminator. *Proc. Natl. Acad. Sci. U. S. A.* **97** (12), 6242-4 (2000).
3. Wild-Bode, C., Weller, M., Rimner, A., Dichgans, J., & Wick, W. Sublethal Irradiation Promotes Migration and Invasiveness of Glioma Cells. *Cancer Res.* **61** (6) (2001).
4. Tuettenberg, J., *et al.* Recurrence pattern in glioblastoma multiforme patients treated with anti-angiogenic chemotherapy. *J. Cancer Res. Clin. Oncol.* **135** (9), 1239-1244 (2009).
5. Munson, J. M., *et al.* Anti-invasive adjuvant therapy with imipramine blue enhances chemotherapeutic efficacy against glioma. *Sci. Transl. Med.* **4** (127), 127ra36 (2012).
6. Correia, A. L., & Bissell, M. J. The tumor microenvironment is a dominant force in multidrug resistance. *Drug Resist. Updat.* **15** (1-2), 39-49 (2012).
7. Rubin, J. B. Only in congenial soil: the microenvironment in brain tumorigenesis. *Brain Pathol.* **19** (1), 144-9 (2009).
8. Bettinger, I., Thanos, S., & Paulus, W. Microglia promote glioma migration. *Acta Neuropathol.* **103** (4), 351-355 (2002).
9. Le, D. M., *et al.* Exploitation of astrocytes by glioma cells to facilitate invasiveness: a mechanism involving matrix metalloproteinase-2 and the urokinase-type plasminogen activator-plasmin cascade. *J. Neurosci.* **23** (10), 4034-43 (2003).
10. Ye, X., *et al.* Tumor-associated microglia/macrophages enhance the invasion of glioma stem-like cells via TGF-β1 signaling pathway. *J. Immunol.* **189**, 444-53 (2012).
11. Yuan, J. X., Bafakih, F. F., Mandell, J. W., Horton, B. J., & Munson, J. M. Quantitative Analysis of the Cellular Microenvironment of Glioblastoma to Develop Predictive Statistical Models of Overall Survival. *J. Neuropathol. Exp. Neurol.* (2016).
12. Yuan, Y., *et al.* Quantitative Image Analysis of Cellular Heterogeneity in Breast Tumors Complements Genomic Profiling. *Sci. Transl. Med.* **4** (157) (2012).
13. Yi, E.S., et al. Distribution of Obstructive Intimal Lesions and Their Cellular Phenotypes in Chronic Pulmonary Hypertension. *Am. J. Respir. Crit. Care Med.* **162** (4), 1577-1586 (2000).
14. Turcan, S., *et al.* IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature.* **483** (7390), 479-483 (2012).
15. Songtao, Q., *et al.* IDH mutations predict longer survival and response to temozolomide in secondary glioblastoma. *Cancer Sci.* **103** (2), 269-273 (2012).
16. Shinojima, N., *et al.* Prognostic Value of Epidermal Growth Factor Receptor in Patients with Glioblastoma Multiforme. *Cancer Res.* **63**, 6962-6970 (2003).
17. Karpel-Massler, G., Schmidt, U., Unterberg, A., & Halatsch, M.-E. Therapeutic inhibition of the epidermal growth factor receptor in high-grade gliomas: where do we stand? *Mol. Cancer Res.* **7** (7), 1000-1012 (2009).
18. Badie, B., & Schartner, J. Role of microglia in glioma biology. *Microsc. Res. Tech.* **54** (2), 106-113 (2001).
19. Watters, J. J., Schartner, J. M., & Badie, B. Microglia function in brain tumors. *J. Neurosci. Res.* **81** (3), 447-455 (2005).
20. Alves, T. R., *et al.* Glioblastoma cells: A heterogeneous and fatal tumor interacting with the parenchyma. *Life Sci.* **89** (15), 532-539 (2011).
21. Hambardzumyan, D., Gutmann, D. H., & Kettenmann, H. The role of microglia and macrophages in glioma maintenance and progression. *Nat. Neurosci.* **19** (1), 20-27 (2015).
22. Zhai, H., Heppner, F. L., & Tsirka, S. E. Microglia/macrophages promote glioma progression. *Glia.* **59** (3), 472-485 (2011).
23. Placone, A. L., Quiñones-Hinojosa, A., & Searson, P. C. The role of astrocytes in the progression of brain cancer: complicating the picture of the tumor microenvironment. *Tumor Biol.* **37** (1), 61-69 (2016).

24. Rath, B. H., *et al.* Astrocytes Enhance the Invasion Potential of Glioblastoma Stem-Like Cells. *PLoS One*. **8** (1), e54752 (2013).

