Gefitinib selectively inhibits tumor cell migration in EGFR-amplified human glioblastoma

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Background. Tissue invasion is a hallmark of most human cancers and remains a major source of treatment failure in patients with glioblastoma (GBM). Although EGFR amplification has been previously associated with more invasive tumor behavior, existing experimental models have not supported quantitative evaluation of interpatient differences in tumor cell migration or testing of patient-specific responses to therapies targeting invasion. To explore these questions, we optimized an ex vivo organotypic slice culture system allowing for labeling and tracking of tumor cells in human GBM slice cultures.

Methods. With use of time-lapse confocal microscopy of retrovirally labeled tumor cells in slices, baseline differences in migration speed and efficiency were determined and correlated with EGFR amplification in a cohort of patients with GBM. Slices were treated with gefitinib to evaluate anti-invasive effects associated with targeting EGFR.

Results. Migration analysis identified significant patient-to-patient variation at baseline. EGFR amplification was correlated with increased migration speed and efficiency compared with nonamplified tumors. Critically, gefitinib resulted in a selective and significant reduction of tumor cell migration in EGFR-amplified tumors.

Conclusions. These data provide the first identification of patient-to-patient variation in tumor cell migration in living human tumor tissue. We found that EGFR-amplified GBM are inherently more efficient in their migration and can be effectively targeted by gefitinib treatment. These data suggest that stratified clinical trials are needed to evaluate gefitinib as an anti-invasive adjuvant for patients with EGFR-amplified GBM. In addition, these results provide proof of principle that primary slice cultures may be useful for patient-specific screening of agents designed to inhibit tumor invasion.

Keywords: EGFR, gefitinib, glioblastoma, invasion, migration, personalized therapy, slice culture.
The underlying drivers of tumor cell movement are multifactorial. Both intrinsic and extrinsic forces are known to modulate glioma migration in the brain, although the relative contributions of nature (genetic and epigenetic characteristics of individual tumors) and nurture (nutrient supply and factors present in the tumor microenvironment) remain unclear. From a genetic standpoint, signaling through the epidermal growth factor receptor (EGFR) has been implicated as an activator of glioma cell motility. On the environmental side, increasing evidence suggests that treatment-associated effects may alter the microenvironment and modulate the invasive behavior of tumors, as observed in the combined clinical experience with anti-angiogenic agents. However, the a priori propensity for GBM to invade normal brain parenchyma on a patient-specific basis remains an ongoing area of study.

A variety of experimental paradigms have been previously used to study glioma cell migration. Prior studies exploring molecular mechanisms of glioma cell migration have documented differences between cells moving in the native brain extracellular matrix (ECM), compared with those migrating on rigid substrates in vitro, emphasizing the need for model systems that more faithfully recapitulate the in vivo architecture of human brain tissue.

The use of in vitro ECM models, transwell assays, or cells transplanted into normal animal brain slices, have been similarly limited. Human orthotopic xenografts have been proposed to offer a closer representation of the human tumor microenvironment, but these models are complicated by incomplete penetrance for establishment of high-grade lesions, protracted growth periods, and loss of human stromal elements over time.

We hypothesized that a model system using direct ex vivo organotypic slice cultures from freshly resected surgical specimens of human GBM would address these experimental limitations and allow for quantitative exploration of baseline differences in tumor cell migration among patients. Using this system, we evaluated the contribution of EGFR amplification to tumor migration and explored the potential patient-specific benefit of targeting EGFR to limit tissue invasion.

Materials and Methods

Human GBM Organotypic Slice Culture Preparation and Retroviral Labeling

Regulatory assurances, patient information, and methods associated with tissue harvesting are outlined in the Supplementary materials. Freshly resected human GBM specimens were embedded in low–melting temperature agarose (Invitrogen) and sliced into 350-μm thick sections with a VT1000S Vibratome (Leica). Tumor-containing agarose blocks were processed while continuously submerged in media equilibrated with 95% O₂ and 5% CO₂. Tumor slices were plated on 0.4-μm pore hydrophilic PTFE inserts (Millipore) and maintained at 37°C in a humidified incubator with 5% CO₂. Inserts were plated on 1 mL of minimal media (Supplementary Materials) that was exchanged every 48 h. We found that the slice culture system, under minimal media conditions, provides sufficient trophic support for long-term culture while maintaining tissue viability, cellular constituency, and histological concordance with the originating tumor tissue (Supplementary Material, Fig. S1). To label tumor cells in GBM slices, we relied on retroviral tropism for dividing cells with use of a ZsGreen-expressing MMLV-based vector (Supplementary Materials). Tumor slices were infected and cultured for 72 h to allow for maximal expression of the fluorescent protein. For a subset of imaging experiments, Isolectin-IB4 (a microglial binding lectin) conjugated to AlexaFluor 647 (Invitrogen) was added to the slice media at the concentration of 5 μg/mL 2 h before imaging.

Evaluation of EGFR Amplification via Fluorescent In Situ Hybridization (FISH)

FISH was undertaken to detect genomic amplification of the EGFR gene locus. Two dual-color chromosome enumeration assays for interphase cells were performed on formalin-fixed, paraffin-embedded tumor tissue that was pretreated with proteinase K and hybridized with a chromosome 7p12 (EGFR)–specific DNA probe and an alpha satellite probe for chromosome 7 centromere (Abbott Laboratories). Tumors were considered to be EGFR amplified if they contained populations of cells with >10 copies of EGFR per cell, based on 2 independent observers scoring 50 cells. All FISH and scoring were performed at the College of American Pathologists–certified Colorado Genetics Laboratory.

Time-Lapse Laser Confocal Microscopic Imaging of Labeled Human GBM Slices

Tumor slices were transferred to non lectin containing media before imaging in 1.5 thickness glass bottom dishes (MatTek). The slices were maintained at 37°C and 5% CO₂ in a sealed incubator (Pecon) on the microscope stage. An LSM 510 (Zeiss) confocal microscope equipped with a 10× air objective (c-Apochromat NA 1.2) was used to image fields spanning a region between the slice edge and the center. The imaging depth varied from 150 to 250 μm, with constant Z-step of 10 μm and imaging interval of 11 min.

Tumor Cell Migration Path Tracking and Analysis

Time-lapse confocal imaging data for each slice culture were preprocessed using Zen software (Zeiss) to make a maximum intensity projection through the depth of imaging, transforming 3-dimensional to 2-dimensional sequences. Manual cell-tracking was performed by one observer (J.J.P.) by marking the visually approximated center point of the ZsGreen-positive cell body (cell body centroid). Cell location was tracked approximately every 53 min. Tracking data were recorded using...
ImageJ (NIH) and MTrackJ. All cells with clearly visualized migration paths in one 10× field were tracked. Microglial cells that were both ZsGreen and Isolectin-IB4 positive or had characteristic morphology were eliminated from subsequent analysis. Migration analysis was limited to those cells tracked at least 7.5 h and not stationary, defined as moving at least 10 μm (the approximate width of a tumor cell body) from their starting location. Cell track data were then analyzed in a defined coordinate system with use of Chemotaxis and Migration Tool V1.01 (Ibidi) to determine cell migration speed (μm/h), total path length (μm), and net path length (μm). Directionality was calculated using the ratio of net path length (μm) to total path length (μm). All calculated distances and subsequent speeds are an underestimate of actual values, which is inherent in the transformation of 3-dimensional images to 2-dimensional images.

**GBM Slice Culture Treatment with Gefitinib**

Slice cultures were imaged for 11 h in vehicle control media (DMSO; 1:1000) and then switched to temperature and CO2 pre-equilibrated media containing gefitinib at a concentration of 10 μM for another 11 h of imaging. Between control and gefitinib imaging periods, the culture insert was briefly removed from the imaging stage incubator, the media were changed, and the same imaging field was relocated. Identical imaging parameters were used for both imaging periods of the experiment, and cell tracking and filtration were performed as described above.

**Statistical Analysis**

All statistical analysis was performed using Prism 5 (GraphPad). Data are presented as mean ± standard deviation unless otherwise noted. Significance was defined as a P value <.05. When significance was not reached, the P value is reported to indicate the strength of the statistic. When 2 groups of means were compared, a 2-tailed t test was used.

**Results**

**Significant Patient-to-Patient Variation Exists in the Invasive Behavior of GBM**

To explore intrinsic baseline differences in tumor cell migration in human GBM, we evaluated tumor cell migration parameters in organotypic slice cultures from a cohort of patients (Supplementary Material, Table S1). We limited sampling variability among patients by selectively obtaining tissue from the contrast-enhancing locations in the tumor (verified by intraoperative navigation). Slice viability at the time of imaging was confirmed through histopathological analysis, which demonstrated a general lack of necrosis or the appearance of pyknotic bodies (as determined by the senior neuropathologist on the study, B.K.D.). In addition, staining for Ki67 at the time of imaging confirmed significant levels of ongoing cellular proliferation (mean, 28%; range, 5.5%–45%) across our cohort. Finally, minimal apoptosis was noted in late-stage slices as visualized by IHC for cleaved caspase-3 (Supplementary Material, Fig. S1).

For each tumor, individual migration paths were manually tracked in a single microscope field to generate a cell track map (Fig. 1A and B, Supplementary Material, Movie S1). Because the extent of brain invasion may be correlated on a macroscopic level with the relative burden of actively migrating cells, we initially generated a motility index for each patient specimen allowing for comparison of overall migratory activity among tumors. These ratios were calculated by comparing the number of cells actively migrating during the imaging period (moving at least a cell body’s width, allowing for filtering of potential artifact-related micro-movement of the cell body throughout the imaging period) to the total number of fluorescent tumor cells in the imaging field. Calculated motility indices varied from 33% to 96% (mean ± standard deviation, 76% ± 18%) (Fig. 1C). Because the majority of tumors demonstrated motility indices >70%, baseline migratory activity on a cell-to-cell basis in most patients with GBM appeared to be fairly high.

To ascertain any tendency for unidirectional movement in slice cultures (ie, tropism, or the potential for all cells to migrate in a uniform direction) and to visualize cell dispersal, we centered the cell paths at a common starting point to create a displacement map (Fig. 1D). As expected, we observed no preferential directional movement of tumor cells in slices.

We next used time-lapse data to calculate cellular metrics of migration that would predictably contribute to the clinical behavior of tumors. Fundamentally, the migration of a cell is modified by 2 parameters: speed (distance travelled over time) and directionality (of efficiency, the dedication of a cell to a linear path during migration). To initiate the comparative analysis among tumors, migration speeds were obtained by averaging the distance traveled over time for individually tracked tumor cells. Mean migration speeds varied over a nearly 3-fold range in our cohort, spanning from 2.8 ± 1.9 μm/h to 7.8 ± 4.7 μm/h (Fig. 2A). We detected limited variability in cell migration speeds between separate slices from the same patient sample, confirming that differences observed between tumors were more likely related to intrinsic tumor-specific factors rather than conditions of culture.

The overall distribution of tumor cell speed in slice cultures displayed varying patterns on an inter-patient basis. Although most slices contained cells that were migrating faster than 5 μm/h (ranging from 7.4% to 78.3% across the cohort) (Fig. 2B), fewer tumors contributed slices with significant numbers of cells migrating faster than 10 μm/h (ranging from 1.1% to 28.6%) (Fig. 2C). Furthermore, as the mean speed for each tumor increased, the distribution of the cell speeds widened, as demonstrated by the positive correlation between mean speed and standard deviation (R² = 0.76; P < .0001). Together,
these data indicate that tumors with higher mean speed harbor a subpopulation of fast tumor cells that are rare in tumors of lower mean speed and that the overall increase in mean speed is attributable to the presence of the faster population.

We next evaluated potential differences in the efficiency of tumor cell invasion among patients. Efficient migration (calculated as the migration directionality) requires that tumor cells demonstrate some level of persistence in their overall vector of movement (Fig. 2D). This biological metric may differ intrinsically among patient tumor cell populations and is predicted to exert significant effects on the overall rate of tumor cell invasion into the brain. To explore this hypothesis, we calculated and compared mean directionality quotients of labeled tumor cells in slices from our cohort of patients with GBM. As predicted, calculated values of mean tumor cell directionality varied significantly from patient to patient, ranging from 0.43 (±0.23) to 0.81 (±0.16) (mean, 0.67 ± 0.12) (Fig. 2E).

Because both migration speed and efficiency contribute to the overall biological behavior of tumor cells, we next used a metric including both aspects of tumor cell movement to compare inter-patient differences in invasion. The resulting effective migration speed, calculated as the accumulated average for the product of migration speed and directionality for each tumor cell, was determined for each patient in our cohort. Critically, mean effective migration speeds varied over a 7-fold range in this analysis (from 0.92 μm/h to 6.7 μm/h) (Fig. 2F), confirming significant heterogeneity in effective tumor cell migration among patients and providing a possible physiologically relevant in vitro correlate for observed clinical differences in tumor invasion.

**EGFR Amplification Is Associated with Augmented Migration in GBM**

Amplification of the *EGFR* gene is present in ~40% of primary human GBM and has been previously associated with infiltrative behavior.⁷,⁹,²¹–²³ Although prior work has suggested that the invasive front of tumor growth is enriched with *EGFR*-amplified cells²² and comparative MRI analysis has implied that *EGFR*-amplified tumors exhibit increased tumor invasion,²⁴ a direct correlation between *EGFR* amplification and increased metrics of migration on a cellular basis has not been shown with human
GBM. We therefore attempted to directly correlate EGFR amplification in donor tumor tissue specimens with migration characteristics of tumor cells in organotypic slices. Tumor cells in slices from EGFR-amplified patients trended strongly toward greater mean speed (6.1 ± 1.5 μm/h) than did tumor cells from nonamplified patients (4.8 ± 1.6 μm/h; P = .14) (Fig. 3A), and EGFR amplification was significantly associated with increased mean directionality (0.73 ± 0.07), compared with nonamplified tumors (0.61 ± 0.11; P < .05) (Fig. 3B). Perhaps of most importance, effective migration speed was significantly increased in EGFR-amplified tumors (4.6 ± 1.4 μm/h), compared with nonamplified tumors (3.0 ± 1.1 μm/h; P < .05) (Fig. 3C). Together, these data provide critical and physiologically relevant support for the hypothesis that EGFR amplification is associated with augmented cell migration in human GBM.

Targeting EGFR with Gefitinib Selectively Decreases Tumor Cell Migration in EGFR-Amplified Tumors

Given confirmatory data that EGFR-amplification is associated with increased intrinsic migratory behavior in human GBM, we hypothesized that targeting the activity of this receptor would selectively affect tumor cell movement in receptor-amplified tumors. We chose to use the small molecule tyrosine kinase inhibitor gefitinib for these purposes, because this drug has been previously tested in early trials of GBM and is widely used on a clinical basis for other systemic cancers.25–27 Control and gefitinib-treated time windows were generated from slices for each tested patient. To visualize the effect of gefitinib on cell migration across tumor slices on a macroscopic level, we again created displacement maps showing the effective dispersal distance of each tracked cell in the population. A substantial qualitative decrease was observed in cell dispersion in EGFR-amplified tumors after treatment with gefitinib, whereas no differences were noted in the EGFR-nonamplified tumors (Fig. 4D, E, and Supplementary Material, Movie S2).

On a quantitative level, treatment of EGFR-amplified tumors resulted in a mean decrease in mean speed of 2.3 μm/h (6.1 ± 1.6 μm/h to 3.8 ± 0.7 μm/h), whereas mean speed in nonamplified tumors displayed a negligible mean change of 0.3 μm/h (4.6 ± 1.3 μm/h to 4.3 ± 1.3 μm/h) (Fig. 4A). Minimal effects were noted in either population with regard to tumor cell directionality after gefitinib treatment (Fig. 4B). However, treatment of EGFR-amplified tumors resulted in a
significant decrease in effective migration speed of $2.1 \, \mu m/h$ ($4.8 \pm 1.3 \, \mu m/h$ to $2.7 \pm 0.7 \, \mu m/h$), whereas negligible changes were seen in nonamplified tumors (Fig. 4C).

Because EGFR amplification occurs in a subset of cells from each tumor, we hypothesized that the fast-moving populations in amplified tumors represent the individual cells with EGFR amplification. Therefore, we predicted that gefitinib would selectively target the subset of faster migrating cells in slices from amplified tumors. To explore this possibility, we stratified individual cell populations in slices into 5 \, \mu m/h bins. Analysis of cell speed distribution demonstrated that gefitinib treatment decreased the number of cells migrating faster than 5 \, \mu m/h in EGFR-amplified tumors by a mean of 33\% (shifting the majority of remaining migrating cells below 5 \, \mu m/h), whereas a minimal change was seen in nonamplified tumors (Fig. 4D and E), confirming that the drug may have a selective effect on the subpopulation of faster migrating tumor cells. Critically, gefitinib treatment did not completely ablate tumor cell migration in any of the EGFR-amplified tumors, but rather reduced mean migration speeds to levels commensurate with the nonamplified tumors ($3.8 \pm 0.7 \, \mu m/h$ vs $4.6 \pm 1.5 \, \mu m/h$).

**Discussion**

Future success in the treatment of GBM will likely derive from disruption of tumor infiltration into previously uninvolved brain tissue. Because the invasive behavior of GBM is known to be markedly heterogeneous,\textsuperscript{3,5,28} exploration of baseline characteristics of tumor cell migration on a patient-to-patient basis will be critical for determining prognosis, predicting response to treatment, and testing efficacy of various targeted therapies. In addition, the ability to disrupt migration of tumor cells will be particularly relevant in combination with locally aggressive or anti-angiogenic therapy, because these...
manipulations have been recently suggested to augment the invasive behavior of targeted tumor cells.\textsuperscript{10,11,29–31}

Although prior experimentation has explored a range of factors associated with increased tumor invasion in GBM,\textsuperscript{13,17,32–34} these studies have been somewhat limited in providing physiological relevance to the human system. The organotypic slice culture system offers an ex vivo approach allowing for relative maintenance of the tumor microenvironment in an in vitro setting. Of note, external nurture elements can be held constant or manipulated at will (through the controlled application of oxygen, glucose, and other nutrients). Using this system, we have confirmed that characteristics of tumor cell migration differ significantly on a patient-to-patient basis under matched macroenvironmental conditions. These results provide in vitro support for the observed heterogeneity in GBM invasion and the contribution of intrinsic nature-based factors (ie, genetic or epigenetic), unique ratios of microenvironmental cellular constituencies, or tumor-specific alterations of extracellular matrix in driving these differences.

A number of investigators have previously explored the role of \textit{EGFR} as a potential driver of migratory behavior in GBM. Aghi et al. noted that patients with \textit{EGFR}-amplified GBM exhibit a significantly higher ratio of T2/T1 signal on MRI (a surrogate for diffuse tumor invasion) compared with nonamplified patients.\textsuperscript{24} These observations were further supported through studies documenting the increased prevalence of \textit{EGFR}-amplified cells at the infiltrative edge of human GBM, compared with the hypercellular core of the tumor mass.\textsuperscript{21,22} Prior in vitro studies have suggested that \textit{EGFR} signaling increases directional persistence of glioma cell migration,\textsuperscript{8,35} and forced overexpression of \textit{EGFR} via retroviral transduction of white matter progenitors in the rodent brain leads to increased numbers of highly migratory cells.\textsuperscript{36} Using the slice culture system, we have for the first time, to our knowledge, provided direct quantitative correlation between \textit{EGFR} amplification and increased tumor cell migration in human GBM. Because GBM slices with higher mean migration speed and efficiency appear to harbor subpopulations of faster cells that are responsible for driving the overall mean migration behavior upward, we hypothesize that this phenomenon is related to the known heterogeneity of \textit{EGFR} amplification on an intra-patient basis and that faster migrating cells are, in fact, the subpopulation of \textit{EGFR}-amplified cells. Although live-imaging conditions required for migration analysis in the slice model have prohibited attempts to confirm this hypothesis using...
formal immunohistochemical methods, observations from the gefitinib treatment experiments provide indirect support. Of note, treatment of EGFR-amplified slices with gefitinib results in a selective dropout of the faster migrating cells, reducing the overall migration characteristics of tumor cells in these slices closer to those recorded from nonamplified specimens.

The small group of clinical trials targeting EGFR in GBM have generally documented limited therapeutic benefit. In 3 of the largest phase II protocols using gefitinib in either primary or recurrent tumors, a lack of significant survival advantage was seen when compared with historical controls.\(^3^5\)\(^{-2^7}\) In all trials with gefitinib treatment, EGFR amplification did not correlate with clinical response to gefitinib in post-hoc analysis,\(^2^5\)\(^{-2^6}\) although in each of these settings, a small fraction of long-term responders were noted (which may be related to PTEN status\(^3^7\)). Critically, therapeutic efficacy of targeting EGFR in these trials has specifically focused on traditional outcome measures, such as progression-free or overall survival. Because EGFR amplification is not thought to be an oncogenic driver of human GBM, it remains unclear what therapeutic benefit should be expected by targeting this pathway in affected patients. On the basis of data from the slice model, we would propose that specific targeting of patients with EGFR-amplified tumors and the use of alternate outcome measures (eg, effects on expansion of nonenhancing infiltrative tumor) may expose potential benefits of EGFR inhibitors that were not seen in prior clinical trials. We conjecture that combination therapy with more traditional antineoplastic agents could decrease tumor invasion into normal brain tissue, which is a primary source of treatment failure in many patients. Of note, effective penetration of drugs into tumor tissue remains an area of concern. Prior work in mouse models of glioma has indicated that distribution of gefitinib into the tumor and associated brain parenchyma may be limited by several mechanisms, including cellular drug efflux pumps.\(^3^8\) Studies of human GBM tissue from patients receiving gefitinib demonstrate at least a 10-fold drug concentration according to tumor to plasma ratio.\(^2^5\)\(^{-3^9}\) However, in a study of drugs concentrations in tissue from patients undergoing treatment, the mean concentration of intratumoral gefitinib was \(\sim 1.7\) \(\mu\)M (assuming a tumor density of 1 g per mL), which is within a comparable pharmacological range to the concentration used in the current study.\(^3^9\)

Although the organotypic slice model offers many opportunities for physiologically relevant study of tumor cell migration in human GBM, there remain several limitations with this experimental approach. Slice cultures retain many critical elements of the tumor microenvironment; however, the system does not provide for a continuous blood supply, maintenance of macro-level 3-dimensional architecture of the human brain (including long-range white matter tracts, grey-white matter boundaries, or large caliber vessels), or the continually shifting nutrient, growth factor, and chemokine milieu that would be present in the living human brain. Because of constraints associated with live imaging of slices, we have not yet developed a technical approach allowing for histochemical or genetic analysis of individual cells determined to be moving faster or slower in slices. Such analysis will be critical for further direct definition of genetic or protein expression patterns associated with increased migration in the heterogeneous background of GBM. Furthermore, it is well known that regional differences in GBM genetics occur on an intra-tumoral basis, indicating that cellular heterogeneity may contribute to observed differences in cell migration rates.\(^2^3\) Perhaps most importantly from a translational perspective, our experiments do not yet provide a direct causative link between increased ex vivo cell migration speed and/or directionality seen in tissue slices and the extent of tumor invasion and clinical outcome in human GBM. Ongoing studies using an increased number of slices with more detailed protein and genetic analysis will help to further define these relationships.

At present, there remain no clinically available agents designed to target tumor invasion in human GBM. Although a number of candidates have been proposed, the ability to test these agents in a patient-based and physiologically relevant system will be critical for establishing overall efficacy and, more importantly, allowing for personalization of therapy in this highly heterogeneous disease. The organotypic slice model potentially offers an ideal platform for these purposes because drugs can be tested in relatively rapid fashion after initial tumor resection (<2 weeks). Future studies using the slice model for testing anti-invasive agents and correlation with targeted clinical trials will be instrumental in further defining the potential translational use of this model.

**Supplementary Material**

Supplementary material is available at Neuro-Oncology Journal online (http://neuro-oncology.oxfordjournals.org/).

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