Analyzing Phospho-signaling in Human Glioblastoma Cell Lines Using *Ex Vivo* Culture

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BRIEF. Phospho-protein activation was highest at the edges of organoids grown from glioblastoma cell lines, which may indicate key locations for potential treatment targets.

ABSTRACT. Glioblastoma (GBM), a Grade IV tumor, is the most common primary malignant brain tumor in adults, and remains resistant to current treatments. It has been proposed that this resistance is due, in part, to the extensive genomic and functional heterogeneity of these tumors. A recently developed organoid culture system was used to investigate this heterogeneity. Organoids are a form of three-dimensional *ex vivo* culture, and are able to mimic many aspects of organs or more complex *in vivo* structures. This study used organoids grown from GBM cell lines to understand how cellular organization and oncogenic signaling correlate in response to stimuli. The organoids were stained for p-S6, p-Akt, and p-NF-κB after acute stimulation and fixation. Phospho-protein activation was found to be higher at the edges of organoids, suggesting that visualizing such areas can ultimately help formulate effective therapeutic strategies. Targeting cells at the edge of a tumor may also help prevent further cancerous invasion into the surrounding healthy brain tissue.

INTRODUCTION.

Glioblastoma, a Grade IV glioma tumor, is the most common primary malignant brain tumor in adults. Glioblastoma is genetically and functionally heterogeneous between and within individual tumors, meaning that all the cells may not carry the same mutations. Genetic changes may or may not result in changes in gene and protein expression. Sequencing the RNA of individual glioblastoma cells from the same tumor suggests that transcript, and thus protein, expression varies between cells [1]. The degree of tumor heterogeneity can also vary from patient to patient. Such heterogeneity can contribute to therapeutic resistance and relapse of tumors.

Currently, treatment for glioblastoma patients involves radiotherapy and temozolomide (chemotherapy), but the treatment has increased the median survival time only to 15 months [2]. The extensive genetic mutations and variable protein expression observed in these tumors have highlighted a variety of potential treatment targets. However, therapies targeting specific alterations, such as those in the Epidermal Growth Factor Receptor (EGFR), have had limited success so far in glioblastoma [3]. Therefore, examining response to stimulation in several key signaling proteins is a major focus of this study.

EGFR is a receptor tyrosine kinase involved in many cellular functions, including stimulating proliferation and cell migration [4]. EGFR is stimulated canonically by the ligand Epidermal Growth Factor (EGF). EGFR mutations often result in the overexpression of EGFR in multiple cancer types [4], including glioblastoma. One type of EGFR genetic alteration is the kinase domain duplication (KDD), in which the receptor has two kinase domains instead of one, as in wild type EGFR. EGFR-KDD is an oncogenic form of EGFR and is responsive to the drug Afatinib [5].

Protein phosphorylation events downstream of EGFR (such as phosphorylation of Akt, Nuclear Factor kappa B (NF-κB), and S6), including phosphorylation of the receptor itself, are thought to drive proliferation in glioblastoma. Akt is involved in anti-apoptotic pathways and promotes cell survival [6]. NF-κB is an anti-cell-death protein which activates anti-apoptotic genes, thereby promoting cell survival [7]. Phosphorylation of S6 affects mRNA binding during translation [8].

Cancer cells are often studied outside of their physiological context *in vitro*, but a recently developed technique involves *ex vivo* culture. In *ex vivo* culture, cells are grown from patient tissue, in an attempt to mimic conditions *in vivo*. Organoid culture is a type of *ex vivo* culture where cells can grow in a larger three-dimensional structure when compared to monolayers or spheres of cells, and are therefore able to better mimic some aspects of organs or more complex structures. This culture technique can be applied to tumor cells [9]. We can study cellular organization within an organoid, especially due to its larger size. For example, spatial pattern of cells in organoids can indicate how glioblastoma cells interact *in vivo*. Organoids allow us to visualize how cells react to treatments in an easy-to-manipulate system.

In this study, the human glioblastoma cell lines U87, A172, U118, T98G, and LN229 were grown as organoids. We observed the responses of the organoids to EGF, Neuregulin-1 (NRG-1), and Rapamycin. EGF stimulates EGFR, which stimulates a series of protein signaling cascades. NRG-1 is a growth factor that binds an EGFR-related receptor and influences cell differentiation, proliferation, and migration, especially in the nervous system [10]. Rapamycin inhibits the expression of S6 [11]. Understanding the activation of downstream signaling proteins in cell lines can be useful as a point of comparison to patient samples. The process of stimulating and fixing organoids can be optimized in cell lines, and applied to patient samples. Variations between cells or patients in response to stimulation may indicate specific targets to pursue in treatment.

We hypothesized that cell lines with differing EGFR mutational status would have varying growth rates and patterns of protein phosphorylation in organoid culture in response to EGF, NRG-1, and Rapamycin. We suspected that organoid size and shape would affect patterns of protein activation upon stimulation, with a gradient of protein activation throughout the organoid having the highest activation near the edges. We further hypothesized that the mutational status of EGFR might have an effect on the amounts of downstream proteins that are phosphorylated.

Based on preliminary data gathered using traditionally cultured glioblastoma cells, different cell lines have varied responses to stimuli. The spatial response to stimuli was not examined for these data, but we hypothesized that the use of organoids could increase understanding of the impact of a cell's location on its ability to respond to stimuli. This study aimed to investigate the importance of three-dimensional spatial organization, access to resources, and cell-cell interactions for protein phosphorylation after acute stimulation.

We will gain an understanding of the effects of an EGFR-KDD and the importance of cellular organization within an organoid by investigating basal and stimulated EGFR signaling in glioblastoma cell lines. This research contributes to the understanding of an important oncogenic signaling pathway in an *ex vivo* model of glioblastoma. Additionally, understanding how the EGFR signaling pathway operates in different cell lines can be used as a comparison point for a study of patient samples, which are likely to be more heterogeneous. Understanding how cellular organization and oncogenic signaling correlate in organoids will help identify where therapy-resistant cells may reside in large tumors.

MATERIALS AND METHODS.

Mammalian Cell Culture.

Cells from the U87, A172, U118, T98G, and LN229 cell lines were thawed and cultured as monolayers and neurospheres. The monolayers were grown to be analyzed via Western blot that would identify if any of the cell lines had an EGFR-KDD. The neurospheres were grown to be implemented in organoid culture.

Monolayers were grown in tissue culture treated flasks. The U87, A172, U118, and T98G cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) + 10% Fetal Bovine Serum (FBS) media and the LN229 cells were cultured in Minimum Essential Medium (MEM) + 5% FBS media. All cell lines were given gentamycin as an antibiotic. The monolayers were cultured for one week before being harvested for Western blotting.

Neurospheres were cultured in serum-free media, so that they grew non-adherently in suspension, as described by Reynolds and Weiss [12].

Organoid Culture.

Organoids were generated from all five cell lines in a procedure derived from Hubert *et al* [9]. In brief, small indentions were made in a sheet of parafilm by placing the parafilm on an empty tip box and rubbing across the parafilm. One neurosphere and liquid Matrigel were placed in each indention. Once the Matrigel containing the cells solidified (now called an organoid), it transferred to an individual well in a 6-well plate containing EGF-rich, serum-free neurosphere media. The media was changed every week. Halfway during the week, the media was supplemented with an additional 0.5 mL of media to sustain the organoids until the media was completely changed. Up to 1 mL of media might be supplemented if the organoids were quickly consuming media, which was indicated by the change in color of the pH indicator in the media.

Acute Stimulation and Fixation of Organoids.

The organoids were stimulated using 4 different conditions for each cell line: Unstimulated (control), EGF, NRG-1, and Rapamycin. One organoid from each cell line was used for each condition. Each treatment was administered in a separate well. The EGF and NRG-1 stimuli were added at concentrations of 100 ng/mL and 20 ng/mL respectively, and incubated for 15 minutes at 37°C. The Rapamycin was added at a concentration of 30μM and incubated for 4 hours at 37°C. The organoids were then immediately fixed with 16% paraformaldehyde for 10 minutes. The fixed organoids were transferred directly to phosphate-buffered saline (PBS).

The fixed organoids were embedded and frozen in Optimal Cutting Temperature (OCT) compound and stored at -80°C. All samples were sectioned at the institution's pathology core facility. The core facility made 3 microscope slides per sample, with three sections (10 μm thick) on each slide.

Immunostaining.

The sectioned organoids were stained for phospho-Akt (p-Akt), p-S6, and p-NF-κB with antibodies conjugated to fluorophores. The p-Akt antibody was conjugated to Alexa 647 (Cell Signaling Technology, D9E), p-S6 to Alexa 594 (Cell Signaling Technology, D57.2.2E), p-NF-κB to Alexa 488 (BD Biosciences, K10-895-12-50). The p-Akt, p-S6, and p-NF-κB antibodies were diluted at the ratios 1:100, 1:200, and 1:20 respectively in blocking buffer. The solution was added to each of the slides at room temperature for 1 hour. The slides were then washed with PBS twice and dried. The sections were mounted in Mowiol mounting media (Sigma-Aldrich). Images of the stained sections were acquired with a Zeiss LSM710 confocal microscope.

Western Blotting.

To identify if any of the cell lines had an EGFR-KDD, a Western blot was run using monolayers from all 5 cell lines. The cells were lysed with Radioimmunoprecipitation Assay (RIPA) buffer, and equal amounts of total protein from each cell line were determined using a traditional Bradford Assay.

Sodium Dodecyl Sulfate (SDS) was used to mask the charges of the isolated proteins in linearized form, causing the protein bands of the Western blot to separate based on molecular mass. The protein bands from the gel were then transferred to a hydrophobic membrane. Primary antibodies for EGFR (Cell Signaling Technology, #4267), p-EGFR (Cell Signaling Technology, #2234), and actin (Sigma-Aldrich, #A2066) were diluted at the ratios 1:2000, 1:1000, and 1:5000 respectively in 5% Bovine Serum Albumin (BSA) in Tris-buffered saline-Tween-20 (TBST) and added to the membrane. The membrane was incubated with the antibodies at 4°C overnight.

The membrane was then washed in TBST. Anti-mouse (Cell-Signaling Technology, #7076) and anti-rabbit (Cell-Signaling Technology, #7074) secondary antibodies conjugated to horseradish peroxidase were diluted at the ratio 1:5000 in 5% BSA in TBST and added to the membrane. The membrane was incubated at room temperature for 2 hours and was washed in TBST again before incubation with a developing solution. Film exposure of the membrane was performed in a dark room.

RESULTS.

In order to observe cells in a three-dimensional context, organoids were grown from 5 cell lines. The organoids from the U87 cell line were started earlier, and were therefore allowed to grow for a longer time period. The cells initially grew in a cluster and began to expand into the Matrigel pearls by day 6 (Figure 1). For the next 2-3 weeks, the organoids were very fragile, as evidenced by a tendency to tear when manipulated. The cells continued to grow for two months, during which the organoids demonstrated increased structural integrity and could be manipulated more easily without tearing.

Images of the organoids were acquired after media changes (Figure 1A). The cells grew throughout the Matrigel and assumed a three-dimensional shape in the 4th week. The rate of expansion and the size of the final organoids varied widely between cell lines (Figure 1C). The average rate of expansion for each cell line was 24369 pixels per day for LN229, 16272 pixels per day for T98G, -302 pixels per day for A172, 9303 pixels per day for U118, and -2470 pixels per day for U87. The final organoid size, on average, for each cell line was 481938 pixels for LN229, 332738 pixels for T98G, 24441 pixels for A172, 287078 pixels for U118, and 217306 pixels for U87.

We performed a Western blot to identify whether any of the 5 cell lines used in this study had an EGFR-KDD mutation. The results (Figure 2) indicated that all cell lines tested had readily detectable EGFR except for LN229. The T98G cell line had EGFR, but the receptor was not phosphorylated. The A172 cell line had an EGFR-KDD. The U118 cell line appeared to have an EGFR that migrated below 150 kDa instead of to 170 kDa.

All the organoids were immunostained to identify locations of protein phosphorylation. Due to time constraints, we were able to collect images only for the LN229 cell line (Figure 3). In the stimulated LN229 organoids, p-NF-κB had the most intense signal, followed by p-S6 and then p-Akt. In the LN229 organoids stimulated by EGF and NRG-1, there was a relative increase in phosphorylation of NF-κB and S6 at the edges of the organoids when compared to the Rapamycin treatment, as determined by fluorophore intensity in ImageJ. p-Akt activation appeared higher in the LN229 organoid stimulated by EGF than in the organoids treated with NRG-1 or Rapamycin . The increase in protein phosphorylation at the edges of organoids may be used as a location for a treatment target.
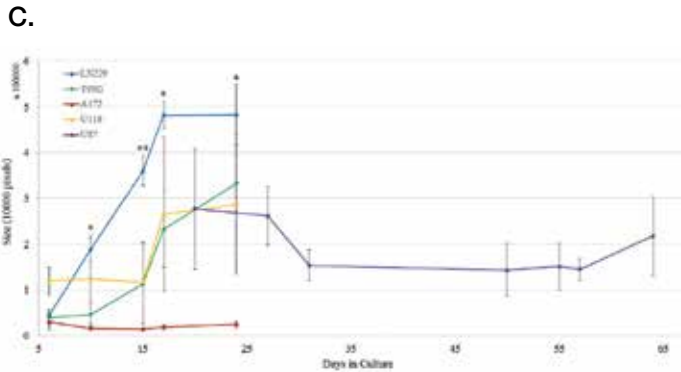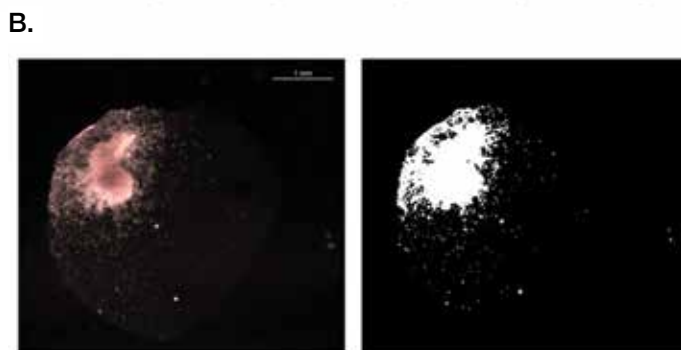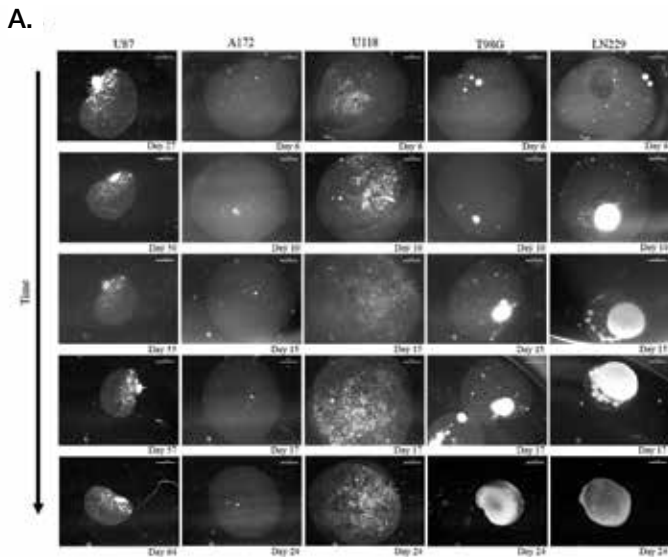
Figure 1. Organoids from Different Cell Lines Develop at Various Rates Over Time **A)** The cell lines are listed across the top. The number of days since the organoid was first embedded is listed in the bottom right corner of each image. One representative organoid is depicted for each cell line. The cells in all the organoids became more densely packed, and the LN229 organoids even assumed an easily discernable three-dimensional shape over time. Images were acquired using a Leica dissection microscope. Scale bars represent 1 mm. **B)** All images were processed in ZEN. The images were converted to binary (black and white), where only the cells are white. The number of white pixels were counted to determine organoid size (1C). **C)** Organoid size was quantified. The LN229 cell line had the largest organoids and the A172 cell line had the smallest organoids. Days in culture is depicted on the x-axis. The number of white pixels (quantification of cells) is on the y-axis. The sizes of the LN229 and A172 organoids were significantly different at the times indicated by an * or **. A two-tailed t-test was performed for this
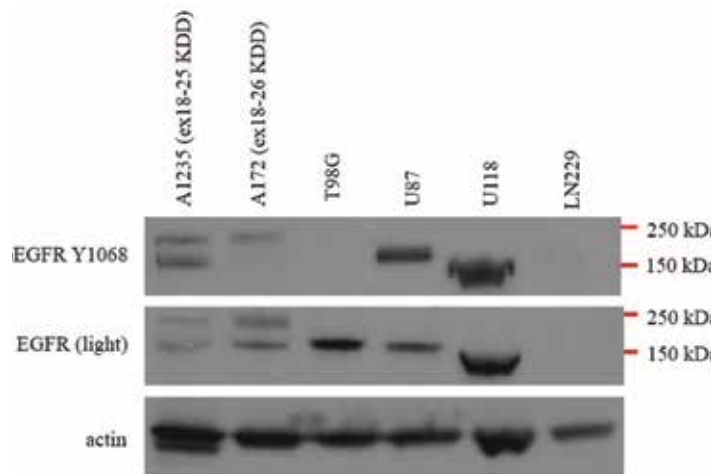

Figure 2. EGFR Mutational Status Varies Among Glioblastoma Cell Lines. The cell lines are labeled at the top. The conditions are listed on the left. Approximate protein size (based on loaded ladder control) is shown at the right. The A1235 cell line was a positive control for the EGFR-KDD. The A172 cell line was the only cell line tested that was positive for the EGFR-KDD. The LN229 cell line did not seem to have EGFR. The T98G cell line has EGRF, but the receptor was not phosphorylated. The U118 cell line appeared to have an EGFR that migrated below 150 kDa instead of at 170 kDa. Actin was used as the loading control (bottom).
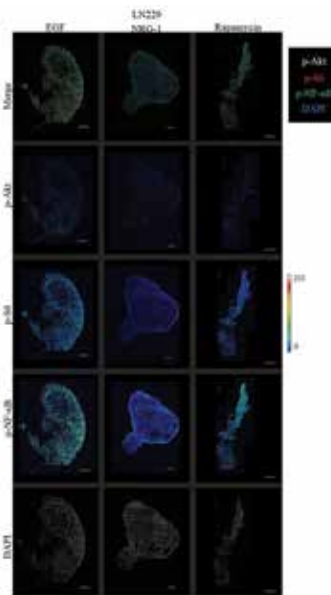

Figure 3. Immunostained Orgnaoids Show Differences in Phosphorylated Protein Expression Depending on the Treatment Condition. The cell lines are labeled at the top. Each column depicts a different treatment. The merged image is shown first. Colors in the merged images represent the fluorophores: p-Akt is white, p-S6 is red, p-NF-κB is green, and DAPI is blue (shown in legend). Individual phosphorylated proteins (labeled on left as follows: p-AKT, p-S6, p-NF-κB) are displayed using a pseudocolor gradient from blue (low) to white (high) indicating fluorophore intensity. The images for DAPI are in grayscale (bottom). Images were acquired using a Zeiss LSM710 confocal microscope at 20x. The scale bars in the EGF column represent 200 μm, in the NRG-1 column represent 100 μm, and in the Rapamycin column represent 500 μm.

DISCUSSION.

Organoid culture can be used to observe the spatial organization of cells [9]. Organoids from 5 glioblastoma cell lines were grown to observe phospho-protein activation in a three-dimensional context. The organoids grew at different rates (Figure 1C). Some organoids grew rapidly only during the first few days of culture, with subsequently slower growth rates. Others grew steadily throughout the entire culture time. The LN229 organoids grew to be significantly larger than the A172 organoids (Figure 1C), demonstrating that organoids grown from different cell lines develop at variable rates. This difference in final size resembles the various growth rates of glioblastoma tumors in different patients.

One contributing factor to glioblastoma heterogeneity is EGFR mutational status. Our data show that, the A172 cell line has the EGFR-KDD mutation (Figure 2). The EGFR-KDD mutation results in a reduction in high-affinity binding sites [13], and is likely to result in a slower cellular growth rate. Therefore, the small size of the A172 organoids is reasonable. Our data indi-

cated that the LN229 cell line does not seem to have readily detectable EGFR, despite the fact that previous studies have demonstrated that the LN229 cell line does express wild type EGFR [14, 15]. All cell lines in this study were grown in monolayers for the same length of time, but the LN229 cells were more densely packed. The LN229 cells might have downregulated EGFR to prevent additional growth, and therefore not have readily detectable levels of EGFR in the Western blot. In the future, controlling for cell density on the plate would be advised before measuring growth factor receptors. The U118 cell line seems to have a smaller form of EGFR (Figure 2). This quickly migrating protein band suggests the cell line has an EGFR variant that is smaller, possibly due to truncating mutations or deletions in the DNA sequence itself. The T98G cell line expresses EGFR, but the receptor was not phosphorylated. Our data indicate that the phosphorylation of EGFR varies among cell lines, which is also indicative of the heterogeneity in glioblastoma.

Among the stimulated organoids that were imaged, p-NF-κB in the LN229 cell line had the most intense signal in response to EGF treatment, especially at the edges of the organoid (Figure 3). Cells at the edges of an organoid have easier access to nutrients and oxygen, which could explain why there are more DAPI positive cells at the edges of the organoids than in the middle. In the LN229 organoids stimulated by EGF and NRG-1, increased activation of p-NF-κB and p-S6 were seen at the edges (Figure 3). This pattern is evidenced by the degree of availability of stimulant, which is more abundant at the edges. Interestingly, the LN229 organoids respond to EGF by phosphorylating proteins downstream of EGFR, even though the LN229 cell line does not appear to express detectable levels of EGFR (Figure 2). In addition, p-S6 activation was higher in the Rapamycin treatment than the NRG-1 treatment, even though Rapamycin inhibits the phosphorylation of S6. Phospho-protein activation in the LN229 organoids with the Rapamycin treatment also was higher on one end of the organoid than the other, unlike the organoids in the EGF and NRG-1 treatments. There were distinct niches in the middle of the stimulated organoids that had lower protein phosphorylation as well (Figure 3). These results suggest that the stimulation of the organoids should be repeated in additional biological repeats and, ultimately, the data can be compared to that of organoids derived from intraoperative samples.

Ongoing studies include further characterization of protein expression in organoids derived from different cell lines in response to treatments. For future studies, organoids from the same 5 cell lines can be grown for longer periods of time and then immunostained for the same phospho-proteins. Time in culture may affect the distribution of protein phosphorylation. In addition, cell lines are more likely to behave uniformly than patient samples, regardless of how many times they are grown. Therefore, the optimization of this culture system in cell lines may be used as a model for future studies conducted using patient samples. Organoids grown from glioblastoma patient samples can also be compared to the original tumor in the patient as another assessment of an organoid's ability to mimic *in vivo* conditions.

CONCLUSION.

Overall, phospho-protein activation was the highest at the edges of organoids, with the interior having lower expression in the EGF and NRG-1 treated organoids. This could be due to differences in cellularity or access to nutrients. Such differences in phospho-protein localization demonstrate the functional heterogeneity of glioblastoma, strengthening the validity of organoids as a model for glioblastoma. Additionally, the high signal of phospho-proteins observed at the edges can be used as locations for potential treatment targets. Targeting the edges of a tumor may not only restrict tumor growth, but also help prevent further invasion into the surrounding area.

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