Upregulation of pro-angiogenic genes in tumor cells that promote an invasive and proliferative phenotype are found in poor responders to bevacizumab therapy.

Roshan Lodha,a,b Candece Gladsonb,\*

aLerner College of Medicine, Cleveland Clinic, 9501 Euclid Avenue, Cleveland, Ohio 44195

bLerner Research Institute, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, Ohio 44195

**Abstract**. Glioblastoma is the most common primary brain tumor in adults with a 15-month median overall-survival. After surgical resection and radio-chemotherapy, tumor recurrence occurs in more than 90% of patients. Therapies at recurrence include bevacizumab, a monoclonal antibody to vascular endothelial growth factor (VEGF) that blocks VEGF from binding to its receptor (VEGFR). While most patients do not show improvement in overall survival, a small percent are good responders to bevacizumab with a longer overall-survival. Given the poor prognosis of glioblastoma, it is important to transition patients from ineffective therapy as soon as possible. To find genetic contributors to ineffective bevacizumab response, we used computational methods to analyze existing RNA-sequencing data of human glioblastoma xenograft tumors propagated in athymic nude mice that were either poor or good responders to bevacizumab. Of note, we hallmarked the angiogenic genes cholinergic receptor nicotinic alpha 7 subunit (*CHRNA7*) to be positively enriched in poor responders’ tumor cells. In endothelial cells, the gene product of *CHRNA7*, α7-nAChR, regulates angiogenesis, whereas in non-brain cancer cells it has been reported to promote proliferation or migration. α7-nAChR expression, along with several other differentially expressed angiogenic factors, is regulated by the early growth response 1 (*EGR1*) transcription factor which is also upregulated in poor responders to bevacizumab, hinting at an epigenetic response to bevacizumab. In aggregate, our data suggest that poor responders to bevacizumab therapy show aberrant expression of endothelial genes that modulate pro-tumorigenic functions.

**Keywords**: bevacizumab, VEGF, *CHRNA7*, *EGR1*, glioblastoma

**\***Candece Gladson**,** E-mail: [gladsoc@ccf.org](mailto:gladsoc@ccf.org)

# 1 Introduction

## Grade IV glioma, or glioblastoma, is the most common primary brain tumor in adults with a median overall survival of just 14-15 months1. Despite primary treatment consisting of surgical resection followed by radiotherapy and chemotherapy, glioblastoma tumors relapse in over 90% of patients2 and within 10 weeks on average3. There is currently no consensus for second-line therapy at recurrence, and the available options include bevacizumab therapy, new combinations of existing agents, clinical trials of new agents, and additional chemotherapy.

## Glioblastoma is known to be a highly vascular, proliferative and invasive tumor3. One second-line option for therapy is to selectively target angiogenesis, with the goal of reducing nutrient supply to tumor cells and potentially inducing downstream death of tumor cells. Due in part to the highly vascular nature of glioblastoma, the U.S. Food and Drug Administration (FDA) has approved single-agent use of bevacizumab as a second-line treatment for recurrent glioblastoma4. Bevacizumab is a humanized monoclonal antibody directed towards vascular endothelial growth factor-A (VEGF) and is a frequently used therapy for patients with recurrent glioblastoma. VEGF binds the VEGF receptors (VEGFR1 and 2) and signals for survival, proliferation and migration. Bevacizumab binds to circulating VEGF, as well as VEGF in the perivascular tumor niche5, competitively preventing VEGF binding/signaling through its receptor (VEGFR), and thereby dampening angiogenesis and in some instances tumor progression6. Unfortunately, bevacizumab therapy alone improves overall patient survival in only a small percentage of patients6, nevertheless bevacizumab therapy is frequently used as a second line therapy as it mitigates brain edema and enhances the quality of life for patients. The mechanisms for resistance to anti-VEGF therapy in glioblastoma are still being identified. Therefore, identifying molecular drivers of poor response to bevacizumab therapy could aid in identifying patients with glioblastoma who would be poor responders, in order to transition such patients to alternative therapies.

## While computational identification of differentially expressed genes can be used to delineate patients with predicted poor response to bevacizumab, it alone does not improve patient prognosis. In the long term, understanding the molecular mechanisms driving a good and a poor response to bevacizumab could highlight candidate targets for combination therapy, opening a new subset of patients to bevacizumab therapy through synthetic lethality. Thus, finding candidate molecular targets that when targeted would be additive or synergistic in effect with bevacizumab therapy, could improve median overall survival while retaining the anti-symptomatic benefits (enhanced quality of life) for patients with recurrent glioblastoma that are treated with bevacizumab. Here we use bulk RNA-sequencing techniques in tandem with immunohistochemical validation to highlight key genomic differences in differential responders to bevacizumab and propose potential novel therapeutic targets to be used in conjunction with bevacizumab therapy.

# 2 Methods

Human patient-derived xenograft tumors (PDXs) were propagated orthotopically in athymic nude mice. Good and poor responders to bevacizumab therapy were defined by their median overall survival time under bevacizumab treatment, with poor responders having a significantly shorter median overall survival time and good responders having no significant change or a significantly longer median overall survival time relative to the median. Tumor tissues were harvested at euthanasia for immunohistochemical and RNA-sequencing analysis.

## 2.1 Biochemical Methods

FLAG

## 2.2 Computational Analysis

RNA sequencing was done through the Illumina Next-Generation Sequencing (NGS) protocol. Following quality control, reads were processed through sequential pairing, alignment, and mapping. Subsequently processed reads were analyzed in R. Group factoring by PDX is shown in Supplemental Table 1.

## All analysis was done using GalaxyProject (version 2.11.0) and R (version 4.0.3). All plots were generated using ggplot2 (version 3.3.5) and all tables were generated using gt (version 0.3.0).

## 2.2.1 Data loading, FASTQ extraction, and preprocessing

## Data was retrieved in the SRA format from the NCBI directly onto GalaxyProject servers. Using the “Download and Extract Reads in FASTA/Q” workflow, fastq files were generated from the SRA. Reads were aligned to the hg19 reference genome using HISAT2. Specified parameters were unstranded paired-end data from a single interleaved dataset. Sample-level quality control was done through principal component analysis (PCA). Properly clustered points were retained for downstream analysis (Supplemental Figure 1A). A dendrogram was generated using euclidean distances between PCA points to better visualize outliers (Supplemental Figure 1B). Following PCA filtering, transcript-level filtering was done through mean-variance analysis (Supplemental Figure 2). Through hyperparameter optimization, a minimum read count of 350 reads was chosen as the cutoff threshold.

## 2.2.2 Biological analysis

## Gene annotation was carried out in R using the ensembldb (version 2.12.1) package. EntrezID was paired to gene symbol. Differential gene expression analysis (DGE) was carried out using DESeq2 (version 3.13) started with loading samples using DESeqDataSetFromMatrix function. The filter was chosen using hyper-parameter optimization of the mean-variance plot. Samples were normalized using the estimateSizeFactors function. The resulting matrix was calculated using lfcShrink function using ashr (version 1.10.0) from shrinking.

## Differentially expressed genes were sorted in descending order by , where adj(p) represented the Benjamini-Hochberg adjusted p-value and FC represents the fold-change output in RNA levels from differential gene expression analysis. Subsequently, the GSEA function of clusterProfiler (version 3.16.1) was used to perform enrichment analysis of several curated gene sets, including KEGG, GO, and Hallmark. The gene sets were retrieved from msigdbr (version 7.4.1).

# 3 Results

## 3.1 Poor responders to anti-VEGF therapy delineate a distinct subtype of glioblastoma.

Chart

Description automatically generated

Figure 1 Across all plots, points in orange are enriched in poor responders to bevacizumab therapy while points in blue are enriched in good responders. A. Volcano plot of differential gene expression. Log 2 fold change is plotted against negative log-scaled significance. Labeled points indicate the top 20 differentially expressed genes. B. Hallmark gene set enrichment analysis of responders to bevacizumab. Normalized enrichment score is plotted against negative log-scaled significance. The KRAS signaling downregulation gene set is labeled. C. Heatmap of log-scaled raw RNA-expression of differentially expressed angiogenic genes. Points in orange indicate higher overall expression while points in blue indicate lower overall expression.

Differential gene expression (DGE) analysis and gene set enrichment analysis (GSEA) using the Hallmark curated gene sets7 revealed global and pathway-specific genomic differences between poor responders and good responders to bevacizumab therapy, respectively. 9.5% of all protein coding genes were significantly differentially expressed between the two response groups (Figure 1A, Table 1). Specific pathways similarly showed perturbations, with dozens of gene sets being downregulated in poor responders to bevacizumab therapy (Figure 1B). Some downregulated gene sets included FLAG (Figure 1B). Notably, only genes downregulated with KRAS activation showed positive enrichment in poor responders to bevacizumab (Figure 1B). The high degree of synteny between the KRAS and angiogenic pathways8,9 suggested differential expression of angiogenic factors as a method of cellular bevacizumab evasion. To better understand the role of differential expression of angiogenic factors on bevacizumab therapy, we looked at raw expression data of genes annotated under the Gene Ontology Angiogenesis pathway FLAG. Poor responders to bevacizumab therapy showed upregulation of several angiogenic factors (Figure 1C), including FLAG.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Differential Gene Expression** | | | | |
| *Gene* | *Fold.Change* | *p.value* | *Adjusted.p.value* | *Mean.Expression* |
| MXRA5 | 1868.27 | 0.00 | 0.00 | 1759.89 |
| FIGNL2 | 1569.38 | 0.00 | 0.00 | 436.46 |
| DPP10 | 1492.07 | 0.00 | 0.00 | 2903.70 |
| SHD | 1490.51 | 0.00 | 0.00 | 3325.96 |
| IGLON5 | 1353.83 | 0.00 | 0.00 | 2933.21 |
| SYT13 | 1308.00 | 0.00 | 0.00 | 925.22 |
| NCAN | 1187.78 | 0.00 | 0.00 | 38401.23 |
| SIX6 | 1050.67 | 0.00 | 0.00 | 646.75 |
| SCN3B | 923.04 | 0.00 | 0.00 | 1313.92 |
| VGF | 878.53 | 0.00 | 0.00 | 27375.38 |
| MMD2 | 784.09 | 0.00 | 0.00 | 300.90 |
| B3GAT1 | 733.52 | 0.00 | 0.00 | 9828.08 |
| NAT16 | 699.09 | 0.00 | 0.00 | 1335.63 |
| USP43 | 683.91 | 0.00 | 0.00 | 816.73 |
| ABCC8 | 630.66 | 0.00 | 0.00 | 2770.25 |
| ATCAY | 624.96 | 0.00 | 0.00 | 10365.40 |
| EXTL1 | 621.35 | 0.00 | 0.00 | 622.66 |
| KCNA6 | 619.83 | 0.00 | 0.00 | 1845.29 |
| TLX1 | 570.41 | 0.00 | 0.00 | 795.25 |
| SCG3 | 567.91 | 0.00 | 0.00 | 10645.30 |

Table 1 List of top 20 differentially expressed genes identified through differential gene expression analysis.

## 3.2 EGR1 may drive poor response to bevacizumab in part through regulation of CHRNA7.

Literature revolving around these angiogenic genes highlighted *CHRNA7* and *EGR1* as potential key players in the poor response to bevacizumab therapy, stemming from the upregulation of these two genes in the poor responder group to bevacizumab treatment (Figure 2). *CHRNA7* has been shown to promote angiogenesis or tumor cell proliferation and migration through an *EGR1* dependent mechanism10,11. Specifically, prior research has demonstrated a mitigation of tumor cell migration following antagonization of the *CHRNA7* protein product α7 Nicotinic Acetylcholine Receptor subunit12. *EGR1* serves as a transcription factor for *CHRNA7* expression, positively driving its transcription. Moreover, *EGR1* has been shown to regulate several of the other differentially expressed angiogenic genes, including *AMOT*, *RAMP3*, and *ACVRL1*, FLAG (Figure 1C).

To validate transcriptome level changes, protein-level differences in expression of *CHRNA7* and *EGR1* were measured through immunohistochemistry. *CHRNA7* and *EGR1* showed upregulation in poor responders to bevacizumab by factors of x (Figure 2A) and y (Figure 2B), respectively. Another pro-angiogenic target of *EGR1*, w, showed a similar upregulation by a factor of z (Figure 2C), further solidifying the role of *EGR1* in driving angiogenic response. These data parallel RNA-sequencing level results showing upregulation of *CHRNA7* by x, *EGR1* by y, and w by z (Figure 1C) FLAG.

## 3.3 Differential expression of both CHRNA7 correlates with poor prognosis.

Following validation of differential transcription and translation of *CHRNA7*, its pertinence to survival in glioblastoma was measured through analysis of clinical datasets curated through cBioPortal. Patients with alterations in *CHRNA7* in glioblastoma showed significantly worse median overall survival, with *CHRNA7* alteration-marked patients having a median overall survival of around 4 months (Figure 3).

Chart, line chart

Description automatically generated

# 4 Discussion

## 4.1 Clinical Significance

Currently, there is limited literature regarding genetic biomarkers predicting response to bevacizumab for treatment of recurrent glioblastoma. Existing biomarkers are relegated to MRI’s in the tumor microenvironment13. In the short term, identification of specific genes as biomarkers for poor responders to bevacizumab therapy can help determine potentially responsive or unresponsive patients, allowing them to transition to alternative therapies. Given the poor prognosis of glioblastoma, early identification of these patient populations can significantly improve median overall survival. Moreover, despite bevacizumab’s inability to improve median overall survival in most patients, its ability to reduce brain edema through vascular normalization makes it a popular choice. Thus, retaining bevacizumab’s anti-symptomatic effects while recovering its tumor-specific potency remains a tantalizing prospect. Beyond *CHRNA7*, genes involved in the differentially expressed pathways (including angiogenesis), have potential as therapeutic targets in combination with bevacizumab and can be the topic of future research.

## 4.2 Future Directions

While computational identification of differentially expressed genes can be used to delineate patients with predicted poor response to bevacizumab, it alone does not improve patient prognosis. In the long term, understanding the molecular mechanisms driving a good and a poor response to bevacizumab could highlight candidate targets for combination therapy, opening a new subset of patients to bevacizumab therapy through synthetic lethality. Thus, finding candidate molecular targets that when targeted would be additive or synergistic in effect with bevacizumab therapy, could improve median overall survival while retaining the anti-symptomatic benefits (enhanced quality of life) for patients with recurrent glioblastoma that are treated with bevacizumab. First, we hope to understand the role of *CHRNA7* in glioblastoma tumor cell proliferation and migration using a *CHRNA7* knockout model generated via CRISPR-Cas9 *in vitro*. Based on successful validation studies (i.e., inhibition of proliferation and/or migration with knockdown of CHRNA7), the impact of *CHRNA7* knockout on bevacizumab response can be quantified *in vivo*.

While *CHRNA7* shows promise as a potential target to recover response to bevacizumab, its transcription factor, *EGR1*, may play a more central role in regulating response. Thus, repeating the aforementioned experiments to validate *EGR1*’s role through a CRISPR interference experiment can similarly lead to development of downstream therapeutic targets.

## 4.3 Limitations

While our analysis did identify significant transcriptomic and proteomic level changes including potential contributors to bevacizumab response, limited sample size and variability amongst xenografts may confound our results. Moreover, due to current insurance practices, it is difficult to obtain recurrent glioblastoma tumors with differential response to bevacizumab, forcing artificial replication of bevacizumab response through changes in median overall survival. Additionally, biological differences between recurrent and primary tumors do exist14, further confounding our results.

# 5 Appendix

## 5.1 Supplemental Figures

Chart

Description automatically generated

Supplemental Figure 1 A. The first two principal components of each mouse are plotted with color indicating the response group. Samples GBM44\_poor and GBM5\_good do not cluster in their respective group. B. The same data is shown as a dendrogram.

Chart, scatter chart

Description automatically generated

Supplemental Figure 2 Mean-variance trends before (left) and after (right) transcript filtering based on hyperparameter optimization.

## 5.2 Supplemental Tables

|  |  |  |
| --- | --- | --- |
| **Study Design** | | |
| *Sample* | *Group* | *SRA* |
| GBM64\_poor | poor | SRR9294073.1 |
| GBM76\_poor | poor | SRR9294072.1 |
| GBM80\_poor | poor | SRR9294077.1 |
| GBM85\_poor | poor | SRR9294060.1 |
| GBM108\_poor | poor | SRR9294041.1 |
| GBM115\_poor | poor | SRR9294043.1 |
| GBM12\_good | good | SRR9294075.1 |
| GBM39\_good | good | SRR9294069.1 |
| GBM59\_good | good | SRR9294032.1 |

Supplemental Table 1 Glioblastoma patient derived xenograft samples reference table.

## 5.3 Acknowledgements

## 5.4 References

1. Gil-Gil MJ, Mesia C, Rey M, Bruna J. Bevacizumab for the Treatment of Glioblastoma. Clin Med Insights Oncol 2013;7:123–35.

2. Weller M, Cloughesy T, Perry JR, Wick W. Standards of care for treatment of recurrent glioblastoma--are we there yet? Neuro-Oncol 2013;15(1):4–27.

3. Chamberlain MC. Bevacizumab for the Treatment of Recurrent Glioblastoma. Clin Med Insights Oncol 2011;5:117–29.

4. Cohen MH, Shen YL, Keegan P, Pazdur R. FDA drug approval summary: bevacizumab (Avastin) as treatment of recurrent glioblastoma multiforme. The Oncologist 2009;14(11):1131–8.

5. Müller-Greven G, Carlin CR, Burgett ME, et al. Macropinocytosis of Bevacizumab by Glioblastoma Cells in the Perivascular Niche Affects their Survival. Clin Cancer Res 2017;23(22):7059–71.

6. Kazazi-Hyseni F, Beijnen JH, Schellens JHM. Bevacizumab. The Oncologist 2010;15(8):819–25.

7. Liberzon A, Birger C, Thorvaldsdóttir H, Ghandi M, Mesirov JP, Tamayo P. The Molecular Signatures Database Hallmark Gene Set Collection. Cell Syst 2015;1(6):417–25.

8. Matsuo Y, Campbell PM, Brekken RA, et al. K-Ras Promotes Angiogenesis Mediated by Immortalized Human Pancreatic Epithelial Cells through Mitogen-Activated Protein Kinase Signaling Pathways. Mol Cancer Res MCR 2009;7(6):799–808.

9. Hamarsheh S, Groß O, Brummer T, Zeiser R. Immune modulatory effects of oncogenic KRAS in cancer. Nat Commun 2020;11(1):5439.

10. Brown KC, Lau JK, Dom AM, et al. MG624, an α7-nAChR antagonist, inhibits angiogenesis via the Egr-1/FGF2 pathway. Angiogenesis 2012;15(1):99–114.

11. Chen S, Kang X, Liu G, Zhang B, Hu X, Feng Y. α7-Nicotinic Acetylcholine Receptor Promotes Cholangiocarcinoma Progression and Epithelial-Mesenchymal Transition Process. Dig Dis Sci 2019;64(10):2843–53.

12. Pepper C, Tu H, Morrill P, Garcia-Rates S, Fegan C, Greenfield S. Tumor cell migration is inhibited by a novel therapeutic strategy antagonizing the alpha-7 receptor. Oncotarget 2017;8(7):11414–24.

13. Stadlbauer A, Roessler K, Zimmermann M, et al. Predicting Glioblastoma Response to Bevacizumab Through MRI Biomarkers of the Tumor Microenvironment. Mol Imaging Biol 2019;21(4):747–57.

14. Maher EA, Brennan C, Wen PY, et al. Marked Genomic Differences Characterize Primary and Secondary Glioblastoma Subtypes and Identify Two Distinct Molecular and Clinical Secondary Glioblastoma Entities. Cancer Res 2006;66(23):11502–13.