Tumors that respond poorly to bevacizumab therapy show upregulation of angiogenesis genes in glioblastoma.

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**Abstract**

Glioblastoma (GBM) is the most common primary brain tumor in adults with a 15-month median survival, despite surgical-resection and radio-chemotherapy, and a recurrence rate of 90%. Despite improving survival in only a small percentage of patients, the common treatment for recurrent-GBM includes bevacizumab, a monoclonal antibody toward vascular endothelial growth factor-A, as it reduces brain edema and improves quality of life. To find predictors of poor response to bevacizumab, we performed RNA-sequencing on multiple GBM patient-derived xenograft (PDX) tumors after orthotopic propagation in athymic nude mice. The study was repeated, and once PDX-tumors were established, mice were treated with bevacizumab or vehicle until euthanasia. PDX-tumors were grouped based on their survival. Bioinformatic analysis of RNA-sequencing data from tumors in untreated mice demonstrated differential gene expression poor responders (decreased survival) relative to good-responders along with upregulation of an angiogenesis gene set. Within this gene set, multiple genes known to be regulated by the early growth response 1 (EGR1) transcription factor were identified; two were selected for further study based on their role in promoting cancer cell migration and proliferation, RAMP3 (accessory-receptor for adrenomedullin) and CHRNA7 (cholinergic-receptor-nicotinic-α7-subunit, or α7-nAChR). Immunostaining/multiplex staining validated the increased expression of EGR1, RAMP3, and α7-nAChR in tumor cells from poor-responder tumors. Data mining revealed a shorter patient survival in GBM with upregulated EGR1 or CHRNA7 mRNA. In summary, PDX-tumors with upregulated expression of an angiogenesis gene set demonstrated a poor response to bevacizumab; upregulation of these genes could potentially be used to predict bevacizumab response.

# 1 Introduction

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

## GBM is known to be a highly vascular, proliferative and invasive tumor3. One option for therapy of recurrent GBM is to target angiogenesis, with the goal of reducing nutrient supply to tumor cells to potentially induce tumor cell death. Due in part to the highly vascular nature of GBM, the U.S. Food and Drug Administration (FDA) approved the single-agent use of the humanized monoclonal antibody to vascular endothelial growth factor-A (VEGF-A), known as bevacizumab, as a second-line treatment for recurrent GBM4. VEGF-A binds the VEGF receptors (VEGFR1 and 2) and signals for survival, proliferation, and migration5. Bevacizumab binds to circulating VEGF-A, as well as VEGF-A in the perivascular tumor niche6, competitively preventing VEGF-A binding and signaling through its receptor (VEGFR) and thereby dampening angiogenesis and in some instances tumor progression7. Unfortunately, bevacizumab therapy alone improves overall-patient survival in only a small percentage of patients7. Nevertheless, it is frequently used as a second line therapy as it mitigates brain edema and enhances the quality of life for patients8. The mechanisms of resistance to bevacizumab therapy in recurrent GBM are still being identified, but they include upregulation of cMet9, upregulation of alternative pro-angiogenic growth factors9, and growth factor starvation-induced autophagy which provides basic building blocks for cancer cell survival6.

## Other pro-angiogenic factors, in addition to VEGF-A, drive angiogenesis in cancer, including adrenomedullin and nicotine in smokers, as well as basic fibroblast growth factor (bFGF), interleukin-6 (IL-6), and other pro-angiogenic factors9. Adrenomedullin signals through its receptors that include calcitonin receptor-like receptor (CALCRL) and the receptor activity modifying proteins – RAMP1, RAMP2 or RAMP3, to promote vascular sprouting, tube formation and vessel maturation in blood vessels10,11. Supporting the importance of adrenomedullin in angiogenesis, the adrenomedullin knockout mouse is embryonic lethal, and adrenomedullin and its receptors have been reported to be upregulated in the vasculature of GBM and other cancers12–14. Of interest is the aberrant expression of the adrenomedullin receptors, CALCRL and RAMP3, in GBM tumor cells, along with tumor cell expression of adrenomedullin13. Blockade of adrenomedullin receptor signaling inhibited the proliferation of GBM cells in vitro and inhibited subcutaneous GBM xenograft tumor growth in the immunocompromised mouse, suggesting aberrant expression of adrenomedullin receptors, along with expression of adrenomedullin, could be a driver of GBM progression13,15. Nicotine has been shown to promote angiogenesis through the α7-nicotinic acetylcholine receptor (α7-nAChR) expressed on endothelial cells16,17. Of interest, the nicotine receptor, α7-nAChR, is aberrantly expressed on some cancer cells including GBM18, and knockdown of α7-nAChR or inhibition with an α7-nAChR antagonist in non-brain cancer cells inhibited migration and proliferation13, 14.

## Transcriptional regulation of genes modulating angiogenesis is known to occur, and the early growth response 1 (EGR1) transcription factor is an example of a transcription factor that regulates multiple angiogenesis genes19,20. The gene for α7-nAChR, CHRNA7, is transcriptionally regulated by EGR1 in endothelial cells and in small cell lung cancer cells21–23. Also, RAMP3 has been inferred to be a transcriptional target of EGR1, based on genome-wide ChiP-X experiments24.

## Identifying molecular drivers of a poor response to bevacizumab could aid in identifying patients with GBM who would potentially be poor responders to bevacizumab therapy, to treat such patients with alternative therapies. Here we used bulk RNA-sequencing techniques, in tandem with validation by immunostaining and multiplex staining, to identify key genetic differences in GBM PDX tumors that were poor responders to bevacizumab therapy. Specifically, we have identified upregulation of a transcription factor and of multiple angiogenesis genes that it regulates in the tumors that were poor responders to bevacizumab. Targeting the transcription factor or one of the downstream genes it regulates in conjunction with the targeting of VEGF-A may improve the efficacy of bevacizumab therapy in GBM.

# 2 Methods

*2.1 Animal Studies*

Human patient-derived xenograft tumors (PDXs) from GBM biopsy (PDX #64, 76, 80, 85, 108, 115, 12, 39 and 59) were propagated orthotopically in athymic nude mice as described previously(Ref-clg will find). PDX tumor cells (300,000 cells in 3 µl) were injected with stereotactic assistance into the right basal ganglia, tumors were allowed to establish and propagate, and mice were euthanized when moribund, followed by harvesting of tumor for RNA-sequencing. The experiment was repeated, and the tumors were allowed to establish for two weeks, and mice were then randomly assigned to a treatment group, bevacizumab (5 mg/kg i.p. biweekly) or vehicle. Treatment was continued until euthanasia, which was performed when the mice were moribund. Tumors were defined as poor- or good-responders to bevacizumab therapy based on their overall-survival compared to the vehicle group, with poor-responders having no change in median overall-survival as compared to the vehicle treatment group, and good-responders having a significantly longer median overall-survival as compared to the vehicle treatment group. At euthanasia, brains were fixed in 4% paraformaldehyde, followed by 30% buffered sucrose, frozen in OCT and stored at -70o, as described15. All animal experiments were done in accordance with, and with approval of the Institutional Animal Care and Use Committee (IACUC) of the Mayo Clinic in Rochester, MN.

## 2.2 Biochemical Methods

Immunostaining for EGR1 was performed as described previously6. Briefly, frozen sections were blocked for peroxidases, permeabilized with 0.25% Triton X-100 (1 min, 22oC), subjected to antigen retrieval (Tris-EDTA buffer, pH 9.0), blocked with 5% BSA/PBS, reacted with 1.37 µg/ml rabbit anti-EGR1 IgG (ProteinTech, cat #55117-1-AP) overnight at 4oC, washed, reacted with goat anti-rabbit HRP-conjugated secondary antibody (Sigma-ThermoFisher), developed with DAB substrate, the nuclei stained with hematoxylin and the slides coverslipped. Sections were imaged and photographed on a Leica DMRB microscope. Multiplex staining for RAMP3, adrenomedullin, α7-nAChR and EGR1, along with staining for human nuclear antigen (HNA), CD31, and Ki67 was performed as described previously (clg will get Dr. Judy Drazba’s help with protocol details as it is a little different and a reference) (Refs).

## 2.3 Computational Analysis

RNA sequencing was done through the Illumina Next-Generation Sequencing (NGS) protocol {TODO: define exact NGS subprotocol with citation-clg will get from Drs. Sarkaria and Decker. clg has verified that the pipelines used for analysis of the RNAsequencing data were specific for human RNA}. 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). Plots were generated using ggplot2 (version 3.3.5) and tables were generated using sjPlot (version 2.8.9).

## 2.3.1 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 SR3. 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.

## 2.3.2 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), and started with the loading of samples using DESeqDataSetFromMatrix function. Samples were normalized using the estimateSizeFactors function. Log-fold change was shrunk using the ashr (version 1.10.0) lfcShrink function for better visualization.

## 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).

## 2.4 Data mining for gene expression in GBM tumors

## Roshan, could you try to describe your data mining for CHRNA7 and GBM survival analysis, as well as the data mining and analysis done by Dr. Jill Barnholtz-Sloan for the mRNA expression of EGR1 in GBM and overall survival. I have placed the two pieces of data on EGR1 from Jill Barnholtz-Sloan after your figures at the end of the Results. We don’t need to keep the Oncoprint, we could just describe in the figure legend for the overall survival with elevated EGR1 mRNA the number of tumors with elevated EGR1 mRNA levels and the total number of tumors.

# 3 Results

## 3.1 PDX tumors that were poor responders to anti-VEGF-A therapy delineate a distinct population of GBM tumors.

Differential gene expression (DGE) analysis and gene set enrichment analysis (GSEA) using the Hallmark curated gene sets25 revealed global and pathway-specific differences in mRNA expression between PDX tumors that were poor- and good-responders to bevacizumab therapy. Of all protein coding genes, 9.5% were significantly differentially expressed between the two response groups (Figure 1A). The most differentially expressed genes included, *MXRA5*, *FIGNL2*, *DPP10*, *SHD*, *IGLON5*, *SYT13*, *NCAN*, *SIX6*, *SCN3B*, *VGF*, *MMD2*, *B3GAT1*, *NAT16*, *USP43*, *ABCC8*, *ATCAY*, EXTL1, *KCNA6*, *TLX1*, *SCG3*, with the majority of differentially expressed genes being positively enriched in tumors that were poor responders to bevacizumab therapy (Supplemental Table 2). (Roshan, are any of the above 20 most differentially expressed genes in the angiogenesis subset?)

Specific pathways similarly showed perturbations, with 23 gene sets showing significantly altered expression in tumors that were poor responders to bevacizumab therapy (Figure 1B). Notably, only genes Downregulated Under KRAS Pathway Activation showed a positive enrichment in poor responders to bevacizumab. Some of the most downregulated gene sets included Interferon Gamma Response; the mTORC1 Genes; Myc Targets; and Interferon Alpha Response Genes (Supplemental Table 3). Several of these pathways and gene sets have previously been shown to be involved in angiogenesis26,27. RAS mutations have been shown to activate the mTORC1 pathway ultimately leading to angiogenesis via VEGF-A28. The high degree of correlation between the KRAS and angiogenic pathways29,30, along with the upregulation in the poor responders of the set of genes Downregulated Under KRAS Pathway Activation, suggests differential expression of angiogenic pathway genes in the poor-responder tumors may provide a genetic environment for bevacizumab-driven cellular adaptation. Roshan, maybe providing examples here of genes regulating angiogenesis that are upregulated in the poor-responder tumors and are found in the gene set of Downregulated Under KRAS Pathway Activation” would be helpful to a reviewer/reader?

To better understand the impact of altered regulation of angiogenic factors on the response to bevacizumab, the raw expression data of genes annotated under the Gene Ontology Angiogenesis31 pathway was quantified (Figure 1C). Poor-responders to bevacizumab therapy showed differential expression of multiple genes regulating angiogenesis, including *APOD*, *GATA4*, *KDR*, *ACVRL1*, *RAMP3*, *LAMA1*, *ADGRB1*, *JCAD*, *SFRP1*, *TAL1*, *PPP1R16B*, *NGFR*, *EPHB1*, *AMOT*, *CHRNA7*, *NPR1*, *EGR3*, *HOXB13*, and *CAV1* (Table 1). A number of these genes, such as *RAMP3* and *CHRNA7* are transcriptionally regulated by EGR122,23,32. Furthermore, we also showed *EGR1* to be differentially expressed in the tumors of the poor-responders to bevacizumab. In the subsequent studies, we have focused on *EGR1* and the aforementioned two genes in the angiogenesis set reported or inferred to be regulated by *EGR1 -* *CHRNA7* and *RAMP3*.

## Roshan, are any of the genes differentially expressed in the poor-responder tumors from the Hallmark “Angiogenesis Gene Set” also found in the gene set called “Downregulated Under KRAS Pathway Activation”?

## 3.2 EGR1 may drive poor response to bevacizumab in part through regulation of CHRNA7 and RAMP3.

Previous studies have highlighted *RAMP3*, *CHRNA7* and *EGR1* genes as potential players in regulating angiogenesis, as well as in promoting the proliferation and migration of cancer cells11,13,15,20,21,33. EGR1 has been shown to either directly or indirectly regulate several differentially expressed angiogenic genes that we found to be upregulated in the tumors with a poor-response to bevacizumab, including *CHRNA7*12, *AMOT*34, *RAMP3*32, and *ACVRL1*35. Differences in the protein expression of α7-nAChR*,* EGR1 and RAMP3 were quantitated through immunohistochemistry and multiplex staining. The protein expression of EGR1 and α7-nAChR showed upregulation in the tumors that were poor-responders to bevacizumab, as compared to the tumors that were good-responders, respectively (Figure 2A and 2B). Multiplex staining showed ?expression of EGR1 and α7-nAChR in the same tumor cells?, consistent with potential EGR1 transcriptional regulation of α7-nAChR. RAMP3 protein showed a similar \_\_\_\_\_\_\_\_\_\_ by a factor of z (Figure 2C). Multiplex staining demonstrated ?expression of EGR1 and RAMP3 in the same tumor cells?, also consistent with the potential regulation of RAMP3 by EGR1, based on the paper by Lachmann et al (2010) inferring transcriptional regulation of target genes using CHiP-X analysis. These data parallel the RNA-sequencing results that showed a 22-fold upregulation of *CHRNA7*, a 13-fold upregulation of *EGR1*, and an 85-fold upregulation of *RAMP3* in tumors that were poor-responders to bevacizumab therapy as compared to good-responders. The above protein studies validate the upregulated mRNA expression found for *CHRNA7*, *RAMP3* and *EGR1* in the tumors that were poor responders to bevacizumab.

## 3.3 Upregulated expression of EGR1 and of CHRNA7 correlates with a shorter overall survival in GBM.

Following validation of the differential transcription of *EGR1*, *CHRNA7* and *RAMP3* by analyzing the protein expression, the relevance of the mRNA levels of these genes to overall survival in GBM was assessed through analysis of datasets curated through cBioPortal36,37,38. GBM patients with upregulation of *CHRNA7* mRNA in their tumors had a significantly worse prognosis; the median overall-survival time was approximately 4 months (Figure 3). GBM patients with upregulation of *EGR1* mRNA in their tumors also showed a significantly worse prognosis; the median overall-survival time was 5-6 months (Figure 4). However, GBM patients with altered *RAMP3* mRNA in their tumors showed no change in survival (Figure 5).

In aggregate, our data suggests that GBM tumors with a poor-response to bevacizumab have a distinct molecular phenotype - upregulation in tumor cells of angiogenesis genes found within the Angiogenesis Pathway gene set.

Chart

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Figure 1. Differential gene expression in GBM PDX-tumors that were poor-responders to bevacizumab. Across all plots, points in orange are enriched in tumors that were poor-responders to bevacizumab therapy, while points in blue are enriched in tumors that were good-responders to bevacizumab therapy. 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 genes regulating angiogenesis. Points in orange indicate higher overall expression while points in blue indicate lower overall expression.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Angiogenesis Differential Gene Expression** | | | | |
| *Gene* | *Fold.Change* | *P-value* | *Adjusted P-value* | *Mean Expression* |
| APOD | 227.46 | 0.00 | 0.00 | 9745.38 |
| GATA4 | 160.41 | 0.00 | 0.00 | 682.76 |
| KDR | 0.01 | 0.00 | 0.00 | 3358.70 |
| ACVRL1 | 103.49 | 0.00 | 0.00 | 184.83 |
| RAMP3 | 84.64 | 0.00 | 0.00 | 40.72 |
| LAMA1 | 72.39 | 0.00 | 0.00 | 1030.16 |
| ADGRB1 | 53.71 | 0.00 | 0.00 | 19203.15 |
| JCAD | 52.25 | 0.00 | 0.00 | 723.10 |
| SFRP1 | 46.57 | 0.00 | 0.00 | 1223.68 |
| TAL1 | 41.14 | 0.00 | 0.00 | 184.06 |
| PPP1R16B | 36.06 | 0.00 | 0.00 | 1187.25 |
| NGFR | 36.04 | 0.00 | 0.00 | 23550.01 |
| EPHB1 | 31.78 | 0.00 | 0.00 | 3240.27 |
| AMOT | 30.63 | 0.00 | 0.00 | 2750.02 |
| CHRNA7 | 21.96 | 0.00 | 0.00 | 64.28 |
| NPR1 | 18.30 | 0.00 | 0.00 | 1308.78 |
| EGR3 | 18.02 | 0.00 | 0.00 | 2077.38 |
| HOXB13 | 17.86 | 0.00 | 0.00 | 2183.89 |
| CAV1 | 0.06 | 0.00 | 0.00 | 6744.06 |

Table 1. List of differentially expressed genes regulating angiogenesis. Fold-change represents the ratio of expression in tumors that were poor-responders to bevacizumab therapy as compared to tumors that were good-responders to bevacizumab therapy.

**Figure 2**. **Protein expression and analysis of EGR1 and α7-nAChR in the tumors that were poor-responders to bevacizumab**.

A. violin plot CHRNA7 proteomic data B. violin plot EGR1 proteomic data C. violin plot ACVRL1 proteomic data D. Picture of actual slide for CHRNA7 E. Picture of actual slide for EGR1.

Chart, line chart

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Figure 3. Kaplan Meier survival curve of GBM patients with altered levels of *CHRNA7* mRNA. Log-rank survival analysis shows a significant difference in median overall survival.

Overall-Survival Analysis for elevated expression of EGR1 mRNA in GBM from Dr. Barnholtz-Sloan



Figure 4. Kaplan Meier survival curve of GBM patients with altered levels of *EGR1* mRNA. Log-rank survival analysis shows a significant difference in median overall survival.

# 4 Discussion

*4.1. Key findings*

*4.2. Discuss EGR1 regulation of angiogenesis genes in relation to your findings*

List all the genes in the differentially upregulated angiogenesis genes (Table 1) found in the tumors that were poor responders to bevacizumab that EGR1 regulates or is inferred to regulate. Roshan, you could mention here several of the well-known transcription factors that regulate angiogenesis. I don’t think that EGR1 will be the first transcription factor to come to the minds of most investigators in regard to the regulation of angiogenesis.

*4.3. Discuss RAMP3 and adrenomedullin findings in relation to cancer cell migration and proliferation, and what is known regarding EGR1 regulation of RAMP3*

We cite literature in the Introduction that adrenomedullin promotes proliferation of GBM cells, but we need to mention here that adrenomedullin is also mitogenic for some cancer cell lines (Refs).

*4.4. Discuss α7-nAChR findings in relation to cancer cell migration and proliferation and in relation to EGR1.*

There may be bi-directional crosstalk between EGR1 and α7-nAChR in some cells or in specific microenvironments, as in the retina choroidal neovascularization and an upregulation of α7-nAChR expression are induced with laser treatment21 and nicotine treatment of this model induced α7-nAChr signaling that resulted in increased EGR1 transcription of FGF-2, promoting angiogenesis21.

## 4.5 Clinical Significance

Currently, there is limited literature regarding genetic biomarkers that predict response to bevacizumab therapy in patients with GBM, with little knowledge about the effect of various treatments on distinct molecular subtypes of glioblastoma39,40. Existing biomarkers are largely MRI biomarkers of the tumor microenvironment41. Identification of the upregulation of specific genes as predictive biomarkers for a poor response to bevacizumab therapy may aid in the identification of patients that would be unresponsive, allowing for the selection of alternative therapies. Given the poor prognosis of GBM, early identification of this patient population could improve median overall-survival. Moreover, despite bevacizumab’s inability to improve overall-survival in most patients, its ability to reduce brain edema through vascular normalization enhances the quality of life for patients. Thus, retaining bevacizumab’s anti-symptomatic effects while recovering its tumor-specific potency remains a tantalizing prospect. Our data highlights the potential for use of individualized tumor-specific gene expression characteristics in selecting patient therapy for GBM.

## 4.6 Future Directions

While computational identification of differentially expressed genes can be used to potentially delineate patients with a predicted poor-response to bevacizumab, it alone does not improve patient prognosis. In the long term, a better understanding of the molecular mechanisms driving a poor- and good-response to bevacizumab will highlight the best candidate targets for combination therapy. Thus, finding candidate genes or proteins that when targeted would be additive or synergistic in effect when combined with bevacizumab therapy, could improve median overall-survival while retaining the anti-symptomatic benefits (enhanced quality of life) for patients with recurrent GBM upon treatment with bevacizumab. Better understanding the role of *CHRNA7* and *RAMP3* in GBM tumor cell proliferation and migration should be undertaken through *CHRNA7* and *RAMP3* knockout studies in GBM models, and this will be crucial to understanding how upregulation of these genes promotes tumor progression. These studies should also aim to uncover the role of *EGR1* in regulating the expression of α7-nAChR and RAMP3 in GBM through both knockout studies and luciferase reporter assays. Beyond *RAMP3*, *CHRNA7* and *EGR1*, other genes involved in the differentially expressed angiogenesis pathway, have potential as therapeutic targets in combination with bevacizumab and could be the topic of future research.

## 4.7. Limitations

While our study did identify significant transcriptomic changes that were validated at the level of the protein as potential contributors to a poor-response to bevacizumab therapy in GBM tumors, the relatively small number of GBM PDX-tumors and the heterogeneity amongst GBM PDX-tumors are limitations of the study. Due to the infrequent biopsy of recurrent GBM it is difficult to obtain a significant number of biopsies from patients with recurrent tumor to assess the poor- and good-response to bevacizumab, and this has resulted in bevacizumab response frequently being expressed as a change or the absence of a change in median overall-survival. Also, genetic and biologic differences between recurrent and primary GBM tumors are known to occur and this is an additional layer of complexity in identifying predictive biomarkers of response to bevacizumab from the primary GBM tumor42.

**Acknowledgements**:

# 5 Appendix

## 5.1 Supplemental Figures



Supplemental Figure 1. Analysis of GBM samples before filtering. 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

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

## 5.2 Supplemental Tables

**Supplemental Table 1**. **Glioblastoma patient derived xenograft samples reference table**.

|  |  |  |
| --- | --- | --- |
| **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 2**. **List of the top 20 differentially expressed genes identified through differential gene expression analysis**. Fold change represents the ratio of expression in poor responders to good responders to bevacizumab treatment. Roshan, are any of these genes in the gene set called “Downregulated Under KRAS Pathway Activation” or in the “Angiogenesis Gene Set” that was upregulated?

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **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 |

**Supplemental Table 3.** **List of the top 10 differentially expressed gene sets identified through gene set enrichment analysis**. Positive normalized enrichment indicates upregulation in poor responders to bevacizumab. Genes listed in core enrichment are sorted in decreasing order of differential expression. Roshan, I wonder if you could highlight any genes in the gene set from “Downregulated Under KRAS Pathway Activation” to help a reviewer/reader see the connection between this gene set and angiogenesis? I see FGFR3 and COL2A1 in this gene set that I think one can easily connect to angiogenesis, but there must be others? Is VEGF-A in this gene set? I don’t see it, but maybe all of the genes in the gene set are not included here.

|  |  |  |  |
| --- | --- | --- | --- |
| **Gene Set Enrichment** | | | |
| *Genset* | *Normalized Enrichment* | *Adjusted P-value* | *Core Enrichment* | |
| HALLMARK INTERFERON GAMMA RESPONSE | -2.34 | 0.00 | IRF9/CMPK2/BPGM/CXCL11/IFIH1/PNPT1/USP18/JAK2/HLA-A/CASP3/IL7/MX2/MYD88/STAT1/NLRC5/CASP4/IFI44L/SPPL2A/PARP14/ST8SIA4/RTP4/MT2A/NOD1/IFI35/CASP8/EPSTI1/PTPN2/HERC6/PML/HIF1A/CASP7/IFIT2/PLSCR1/IRF1/PARP12/PTPN1/LGALS3BP/IFI44/RSAD2/UPP1/LAP3/SP110/NCOA3/ICAM1/EIF2AK2/IFITM2/DDX58/ZNFX1/RIPK1/PSMB10/IFITM3/IL15/OAS2/PSME1/PSMA2/MX1/NMI/IFIT3/VAMP5/FAS/NAMPT/PSMA3/IFI30/OASL/SOD2/PNP/ISG20/PSMB2/B2M/NFKB1/IFIT1/ISG15/NFKBIA/GBP4/MVP/IFI27/PSME2/TNFAIP2 | |
| HALLMARK MTORC1 SIGNALING | -2.45 | 0.00 | RDH11/CCT6A/NIBAN1/STIP1/PITPNB/COPS5/LGMN/EEF1E1/EDEM1/HMBS/CACYBP/SLC9A3R1/BHLHE40/CYP51A1/USO1/RAB1A/DDX39A/CYB5B/PDK1/MTHFD2L/PLK1/BTG2/HSPA5/PSMA4/HSP90B1/SDF2L1/RPN1/SQLE/M6PR/GPI/FKBP2/G6PD/AK4/BUB1/GLRX/PSMD13/ERO1A/STC1/PSMG1/MAP2K3/ARPC5L/TBK1/EGLN3/POLR3G/ATP6V1D/SEC11A/GMPS/PRDX1/SSR1/PPIA/HSPA4/ENO1/HSPD1/GBE1/P4HA1/ELOVL5/ACTR3/PNO1/CALR/IMMT/GAPDH/LDLR/UBE2D3/PSMC2/GTF2H1/HSPE1/ETF1/STARD4/CD9/PSMB5/EIF2S2/EBP/PSMD12/PSMC4/NAMPT/PSMA3/IFI30/TPI1/CCNG1/ACTR2/PSMC6/GLA/PNP/SQSTM1/HPRT1/AURKA/RIT1/LDHA/ELOVL6/ALDOA/SLC2A1/PSMD14/PGK1 | |
| HALLMARK MYC TARGETS V1 | -2.54 | 0.00 | ORC2/EIF3B/DEK/DDX21/CNBP/TXNL4A/SRPK1/NME1/NOP56/CANX/RPL22/PRDX3/RPL14/SYNCRIP/COPS5/RPS10/EIF3D/VBP1/APEX1/HNRNPA2B1/TYMS/EIF1AX/RPS5/PTGES3/PCBP1/G3BP1/RPS2/EEF1B2/ILF2/POLE3/KARS1/PRPF31/TCP1/KPNA2/PWP1/YWHAE/EIF2S1/SSBP1/GOT2/PSMA4/RPLP0/SRSF1/TARDBP/EIF3J/UBA2/TRA2B/CUL1/AIMP2/SERBP1/RPL34/PCNA/NPM1/YWHAQ/RPS6/ERH/HNRNPC/DHX15/RACK1/VDAC1/SNRPD3/MRPL9/SSB/HSP90AB1/HDDC2/ACP1/UBE2L3/SNRPB2/PPM1G/PPIA/CDC20/EIF4E/LSM7/SLC25A3/HSPD1/PRPS2/RPL18/SRSF3/NOP16/CCT4/NDUFAB1/CCNA2/CCT7/PSMA2/HSPE1/SNRPD1/PSMD7/PSMD1/ETF1/PSMA1/GLO1/VDAC3/ABCE1/AP3S1/XRCC6/SRSF7/EIF4G2/EIF2S2/MAD2L1/H2AZ1/PSMC4/PSMD8/SNRPG/PSMC6/SNRPD2/PSMB3/PSMB2/HPRT1/LDHA/SNRPA1/PSMA6/PRDX4/PSMA7/PSMD14/PGK1 | |
| HALLMARK OXIDATIVE PHOSPHORYLATION | -2.47 | 0.00 | NDUFA2/MRPS22/COX11/NDUFC2/PDHX/ATP6V0B/PRDX3/MRPL35/DLD/ATP5MC3/ACAT1/SDHC/BAX/NDUFB2/SUCLG1/ISCA1/COX7C/DLST/NDUFS4/NDUFS6/NDUFB5/MRPL11/NDUFB8/ATP6V1F/NDUFB3/ATP5MG/NDUFS1/GOT2/COX5B/ATP6V1E1/TIMM50/GPI/NDUFA8/MRPS12/MDH2/ATP5F1C/NDUFS8/VDAC2/DLAT/MRPS30/CASP7/ECHS1/TIMM8B/MGST3/ATP5ME/ATP5MF/UQCRC2/VDAC1/NDUFA9/ATP6AP1/HADHA/NDUFA3/AFG3L2/NDUFA7/UQCR10/CYCS/CPT1A/ATP6V1D/TIMM9/NDUFB4/ATP5PB/ATP5F1A/NDUFA4/NDUFB7/NDUFA6/UQCRFS1/SLC25A3/COX7A2/NDUFC1/TOMM22/ETFDH/COX4I1/HSD17B10/IMMT/SLC25A5/NDUFB1/OXA1L/NDUFAB1/TIMM17A/CYB5A/UQCRQ/GPX4/ETFA/ABCB7/PDP1/MDH1/ATP5PD/DECR1/COX6C/NDUFV2/PDHA1/UQCRH/GRPEL1/ATP6V0E1/UQCR11/AIFM1/FH/VDAC3/MRPL15/NQO2/COX6B1/NDUFA1/IDH3G/ECH1/ACAA2/HCCS/MPC1/NDUFB6/COX7B/COX7A2L/MRPL34/SDHB/ATP5F1E/LDHA/MRPS15 | |
| HALLMARK INTERFERON ALPHA RESPONSE | -2.23 | 0.00 | WARS1/SAMD9L/TRIM25/IRF2/CD47/CXCL10/TRIM21/IRF9/CMPK2/CXCL11/IFIH1/PNPT1/USP18/SAMD9/IL7/PARP9/IFI44L/PARP14/CSF1/RTP4/IFI35/CASP8/EPSTI1/ELF1/HERC6/IFIT2/PLSCR1/IRF1/PARP12/LGALS3BP/IFI44/RSAD2/LAP3/SP110/EIF2AK2/IFITM2/GMPR/IFITM3/IL15/PSME1/MX1/NMI/IFIT3/PROCR/PSMA3/IFI30/OASL/ISG20/B2M/ISG15/IFITM1/GBP2/GBP4/IFI27/PSME2 | |
| HALLMARK PROTEIN SECRETION | -2.18 | 0.00 | ANP32E/OCRL/GALC/TSG101/AP3B1/ARFGAP3/IGF2R/ARCN1/SCAMP3/BET1/PAM/USO1/GOLGA4/SEC31A/CLTC/RER1/M6PR/BNIP3/TMED10/ZW10/SOD1/COPB1/VPS4B/NAPA/SEC22B/YIPF6/RAB22A/ARF1/TMED2/LMAN1/SEC24D/ADAM10/CD63/ARFGEF2/TMX1/COPE/SNX2/ARFIP1/CLTA/AP2S1/RAB9A/AP3S1/ERGIC3/KRT18/GLA/LAMP2 | |
| HALLMARK FATTY ACID METABOLISM | -2.01 | 0.00 | IDI1/SERINC1/RETSAT/NBN/SMS/DLD/RDH11/SDHC/SUCLG1/NTHL1/HSP90AA1/APEX1/DLST/ERP29/MIF/HMGCL/HSD17B11/HSD17B7/MDH2/GRHPR/G0S2/ACSL1/ACOT8/ECHS1/HADH/ACAT2/CRYZ/PTS/YWHAH/METAP1/CPT1A/EPHX1/ETFDH/ELOVL5/HSD17B10/ADSL/UROD/MDH1/PSME1/DECR1/RAP1GDS1/PDHA1/FH/IDH3G/ECH1/ACAA2/HCCS/H2AZ1/NSDHL/PRDX6/OSTC/LGALS1/S100A10/ACSL4/LDHA/ALDOA/ECI2 | |
| HALLMARK PI3K AKT MTOR SIGNALING | -2.00 | 0.00 | PDK1/CLTC/HSP90B1/NOD1/ATF1/RALB/PPP1CA/MAP2K3/TBK1/MKNK1/EIF4E/ARF1/ARPC3/RIPK1/ACTR3/CALR/UBE2D3/YWHAB/ACTR2/SQSTM1/PPP2R1B/RIT1/SLC2A1/PFN1 | |
| HALLMARK KRAS SIGNALING DN | 1.81 | 0.00 | SOX10/CLSTN3/CAMK1D/TLX1/THRB/MACROH2A2/SLC29A3/GDNF/YBX2/MYH7/RYR1/KCNN1/GPR19/ARHGDIG/RGS11/COL2A1/THNSL2/ACTC1/YPEL1/RIBC2/ALOX12B/HSD11B2/TEX15/KCND1/FGFR3/MYO15A/PDE6B/SLC30A3/GAMT/SPTBN2/C5/NR4A2/DLK2/NRIP2/PDK2/ARPP21/SKIL/STAG3/TGM1/SYNPO/CPA2/WNT16/IDUA/MAST3/TFAP2B/SNCB/CHST2/DCC/EFHD1/PRODH/CPEB3 | |

## 5.3 Reproducible scripts

<https://github.com/roshanlodha/bevacizumab-response>

## 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. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J Off Publ Fed Am Soc Exp Biol 1999;13(1):9–22.

6. 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.

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

8. Shen G, Wang Y-J, Guan Y-J, et al. Relief Effect of Bevacizumab on Severe Edema Induced by Re-irradiation in Brain Tumor Patients. Chin Med J (Engl) 2015;128(15):2126–9.

9. Haibe Y, Kreidieh M, El Hajj H, et al. Resistance Mechanisms to Anti-angiogenic Therapies in Cancer. Front Oncol [Internet] 2020 [cited 2022 Mar 5];10. Available from: https://www.frontiersin.org/article/10.3389/fonc.2020.00221

10. Eto T. A review of the biological properties and clinical implications of adrenomedullin and proadrenomedullin N-terminal 20 peptide (PAMP), hypotensive and vasodilating peptides. Peptides 2001;22(11):1693–711.

11. Kuwasako K, Kitamura K, Nagata S, Hikosaka T, Takei Y, Kato J. Shared and separate functions of the RAMP-based adrenomedullin receptors. Peptides 2011;32(7):1540–50.

12. Shindo T, Kurihara Y, Nishimatsu H, et al. Vascular abnormalities and elevated blood pressure in mice lacking adrenomedullin gene. Circulation 2001;104(16):1964–71.

13. Ouafik L, Sauze S, Boudouresque F, et al. Neutralization of adrenomedullin inhibits the growth of human glioblastoma cell lines in vitro and suppresses tumor xenograft growth in vivo. Am J Pathol 2002;160(4):1279–92.

14. Ribatti D, Nico B, Spinazzi R, Vacca A, Nussdorfer GG. The role of adrenomedullin in angiogenesis. Peptides 2005;26(9):1670–5.

15. Kaafarani I, Fernandez-Sauze S, Berenguer C, et al. Targeting adrenomedullin receptors with systemic delivery of neutralizing antibodies inhibits tumor angiogenesis and suppresses growth of human tumor xenografts in mice. FASEB J 2009;23(10):3424–35.

16. Lee J, Cooke JP. Nicotine and Pathological Angiogenesis. Life Sci 2012;91(0):1058–64.

17. Davis SJ, Lyzogubov VV, Tytarenko RG, Safar AN, Bora NS, Bora PS. The effect of nicotine on anti-vascular endothelial growth factor therapy in a mouse model of neovascular age-related macular degeneration. Retina Phila Pa 2012;32(6):1171–80.

18. Kolodziej MA, Gött H, Kopischke B, et al. Antiproliferative effect of GTS-21 in glioblastoma cells. Oncol Lett 2021;22(5):759.

19. Fahmy RG, Dass CR, Sun L-Q, Chesterman CN, Khachigian LM. Transcription factor Egr-1 supports FGF-dependent angiogenesis during neovascularization and tumor growth. Nat Med 2003;9(8):1026–32.

20. Wang B, Guo H, Yu H, Chen Y, Xu H, Zhao G. The Role of the Transcription Factor EGR1 in Cancer. Front Oncol [Internet] 2021 [cited 2022 Mar 31];11. Available from: https://www.frontiersin.org/article/10.3389/fonc.2021.642547

21. 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.

22. Mathelier A, Zhao X, Zhang AW, et al. JASPAR 2014: an extensively expanded and updated open-access database of transcription factor binding profiles. Nucleic Acids Res 2014;42(Database issue):D142-147.

23. Sandelin A, Alkema W, Engström P, Wasserman WW, Lenhard B. JASPAR: an open-access database for eukaryotic transcription factor binding profiles. Nucleic Acids Res 2004;32(Database issue):D91-94.

24. Lachmann A, Xu H, Krishnan J, Berger SI, Mazloom AR, Ma’ayan A. ChEA: transcription factor regulation inferred from integrating genome-wide ChIP-X experiments. Bioinforma Oxf Engl 2010;26(19):2438–44.

25. 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.

26. Baudino TA, McKay C, Pendeville-Samain H, et al. c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. Genes Dev 2002;16(19):2530–43.

27. Indraccolo S. Interferon-alpha as angiogenesis inhibitor: learning from tumor models. Autoimmunity 2010;43(3):244–7.

28. Karar J, Maity A. PI3K/AKT/mTOR Pathway in Angiogenesis. Front Mol Neurosci 2011;4:51.

29. 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.

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

31. angiogenesis Gene Ontology Term (GO:0001525) [Internet]. [cited 2021 Sep 23];Available from: http://www.informatics.jax.org/vocab/gene\_ontology/GO:0001525

32. Lee JY, Kim JH, Bang H, et al. EGR1 as a potential marker of prognosis in extranodal NK/T-cell lymphoma. Sci Rep 2021;11(1):10342.

33. Caron KM, Smithies O. Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional Adrenomedullin gene. Proc Natl Acad Sci U S A 2001;98(2):615–9.

34. Scheicher R, Hoelbl-Kovacic A, Bellutti F, et al. CDK6 as a key regulator of hematopoietic and leukemic stem cell activation. Blood 2015;125(1):90–101.

35. Gonzalez CR, Vallcaneras SS, Calandra RS, Gonzalez Calvar SI. Involvement of KLF14 and egr-1 in the TGF-beta1 action on Leydig cell proliferation. Cytokine 2013;61(2):670–5.

36. Cancer Genome Atlas Research Network. Comprehensive genomic characterization defines human glioblastoma genes and core pathways. Nature 2008;455(7216):1061–8.

37. Brennan CW, Verhaak RGW, McKenna A, et al. The somatic genomic landscape of glioblastoma. Cell 2013;155(2):462–77.

38. Zhao J, Chen AX, Gartrell RD, et al. Immune and genomic correlates of response to anti-PD-1 immunotherapy in glioblastoma. Nat Med 2019;25(3):462–9.

39. Rigakos G, Kyriazoglou A, Vernadou A, et al. Bevacizumab in high grade glioma: Is there a subgroup that benefits? Hematol Med Oncol [Internet] 2017 [cited 2022 Mar 31];2(4). Available from: http://www.oatext.com/bevacizumab-in-high-grade-glioma-is-there-a-subgroup-that-benefits.php

40. Hovinga KE, McCrea HJ, Brennan C, et al. EGFR amplification and classical subtype are associated with a poor response to bevacizumab in recurrent glioblastoma. J Neurooncol 2019;142(2):337–45.

41. 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.

42. 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.