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 a small percentage of patients, bevacizumab, a monoclonal antibody toward vascular endothelial growth factor-A, is frequently used to treat recurrent-GBM. 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 and grouped based on survival. Bioinformatic-analysis of RNA-sequencing data demonstrated differential gene expression in tumors that were poor-responders to bevacizumab (no survival-change) as compared to tumors that were good-responders (longer-survival), along with upregulation of an angiogenesis gene set in poor-responders. Within this gene set, multiple genes known to be regulated by the early growth response 1 (EGR1) transcription factor were identified; CHRNA7 (cholinergic-receptor-nicotinic-α7-subunit) was selected for further study based on the reported role in promoting cancer cell migration and proliferation. Results were validated at the protein level using immunohistochemical staining. Additionally, Sry-box transcription factor-10 (SOX10) showed upregulation in poor-responders, potentially driven by impaired proteosome degradation. SOX10 is known to promote mural-cell coverage of neovasculature, cancer cell migration and glioma development when cooperating with PDGFB. In summary, GBM PDX-tumors with upregulated expression of an angiogenesis gene set and of two transcriptional regulators (EGR1 and SOX10) demonstrated a poor response to bevacizumab; upregulation of these genes could 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 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 (Avastin), 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 factors, in addition to VEGF-A, drive angiogenesis in cancer, including adrenomedullin, basic fibroblast growth factor (bFGF), interleukin-6 (IL-6)9. 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. Adrenomedullin and its receptors are upregulated in the vasculature of GBM and other cancers12–14. Aberrant expression of adrenomedullin and its receptors (CALCRL and RAMP3) has been reported in GBM tumor cells, and blockade of receptor signaling inhibited the proliferation of GBM cells in vitro and in vivo in GBM xenografts progression13,15. Nicotine has been shown to promote angiogenesis through the α7-nicotinic acetylcholine receptor (α7-nAChR) expressed on endothelial cells16,17. The α7-nAChR can also be aberrantly expressed on cancer cells including GBM tumor cells18. The downstream signaling from the α7-nAChR is not entirely clear. In non-brain cancer cells knockdown of α7-nAChR or inhibition with an α7-nAChR antagonist inhibited cell migration and proliferation13, 14. More recently, a partial agonist of α7-nAChR, known as GTS-21, was shown to inhibit GBM cell proliferation in vitro, pointing to the complexity of α7-nAChR signaling.

## Finally, SOX10, a master regulator and promotor of vascular development in brain tissue19–22, has been shown to delineate a distinct molecular subtype of glioblastoma. All these findings point to a potentially significant role of SOX10 in regulating response to bevacizumab. 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 genes23,24. The gene for α7-nAChR, CHRNA7, is transcriptionally regulated by EGR1 in endothelial cells and in small cell lung cancer cells25–27. Also, RAMP3 has been inferred to be a transcriptional target of EGR1, based on genome-wide ChiP-X experiments28. SOX10 has similarly been implicated in glioma, as it cooperates with EGR1, EGR2, and EGR3 to regulate myelin genes21.

## 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 in accordance with the guidelines and regulations of the Institutional Animal Care and Use Committee (IACUC) at the Mayo Clinic. 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 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), 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 biotin-conjugated secondary antibody (Sigma-ThermoFisher), reacted with the streptavidin complex and developed with the 3,3’-diaminobenzidine (DAB) substrate. Nuclei were stained with hematoxylin and the slides coverslipped. Rabbit IgG was substituted for the first antibody as a negative control. Sections were stained for α7-nAChR with rat monoclonal anti-α7-nAChR (\_\_ µg/ml, Santa Cruz, #sc-58607) as described above, except that the permeabilization step utilized \_ % Triton X-100 for \_\_\_min. Rat IgG was substituted for the first antibody as a negative control. Sections were stained for SOX10 with rabbit anti-SOX10 IgG (\_\_µg/ml, Sigma-Aldrich, #383A) as described above, except that the permeabilization step utilized 0.25% Triton-X-100 for 3 min. Immunostained sections were imaged and photographed on a Leica DMRB microscope. Multiplex staining for EGR1, RAMP3, adrenomedullin, and α7-nAChR, as well as for beta-microglobulin, CD31, and Ki67, was performed as described previously (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 Differential gene expression 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 Survival analysis based on altered mRNA expression

Survival analysis was carried out using cBioPortal29,30. RNA-sequencing data stored in The Cancer Genome Atlas (TCGA) was used31,32. The high expression group was defined as 1.5 standard deviations above the mean. Data was downloaded from cBioPortal and Kaplan-Meier survival curves were produced in R using ggplot2.

## 2.5 H-scoring analysis

Each slide was assigned a semi-quantitative histochemical score (H-score). The h-score is determined by assigning several sample fields a sub-score from 0 to 3 indicating the staining strength in the field of view33. The overall H-score for an animal is given by a weighted average of the sub-scores pertaining to that animal. Homogeneity was tested per animal and per bevacizumab response group using a chi-squared goodness of fit test34.

# 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 sets35 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). Importantly,

In examining specific pathways, perturbations were also found 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 angiogenesis36,37. For example, RAS mutations have been shown to activate the mTORC1 pathway ultimately leading to angiogenesis via VEGF-A38. The high degree of correlation between the KRAS and angiogenic pathways39,40, along with the upregulation in the poor responders of the set of genes Downregulated Under KRAS Pathway Activation40, 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. The most upregulated gene in this pathway Sry-box 10 (*SOX10*), has been previously shown to contribute to vascular development in the brain, and is known to cooperate with PDGFB in promoting glioma development19,41.

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 Angiogenesis42 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 EGR126,27,43. Furthermore, *EGR1* was also differentially expressed in the tumors of the poor-responders. In the subsequent studies, we have focused on three differentially expressed genes that regulate angiogenesis and cancer cell migration, *EGR1* and the *CHRNA7* gene reported to be regulated by *EGR1,* as well as *SOX10*.

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

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 cells11,13,15,24,25,44. 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*45, *RAMP3*43, and *ACVRL1*46. 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 increased expression of EGR1 and α7-nAChR within tumor cells consistent with potential EGR1 transcriptional regulation of α7-nAChR. RAMP3 protein showed a similar increase by a factor of z (Figure 2C). Moreover, multiplex staining demonstrated consistent with the reported transcriptional regulation of α7-nAChR by EGR1 in endothelial cells and small cell lung cancer cells25–28. Our immunohistochemical staining data parallel the RNA-sequencing results that showed a 22-fold upregulation of *CHRNA7* and a 13-fold upregulation of *EGR1* in tumors that were poor-responders to bevacizumab therapy as compared to good-responders and validate the upregulated mRNA expression found for *EGR1* and *CHRNA7* genes in the poor-responders. Evaluation of the heterogeneity between response groups found that both poor and good responders to bevacizumab displayed no significant difference in homogeneity between poor and good responders indicating that cell-cycle differences did not contribute to differences in gene expression.

## 3.3 Downregulation of the proteosome degradation pathway may underpin response to bevacizumab through SOX10.

Wu et. al. highlighted the characterized the role of *SOX10* as “a master regulator of the RTK I subtype, with both tumor cell-intrinsic and microenvironmental effects” in 202020. Additionally, *SOX10* was previously shown to increase vascular development in brain tissue, implying a relationship between *SOX10* and angiogenesis19. Moreover, *SOX10* has been further implicated in glioma, as *SOX10* cooperates with *EGR1*, *EGR2*, and *EGR3* to regulate myelin genes21. The aggregated, prior literature suggests that the *SOX10* transcription factor contributes to glioma development and progression by synergizing with PDGFB, and by promoting tumor cell migration and invasion41,47,48. In tumors that responded poorly to bevacizumab, we found 512-fold upregulation of *SOX10* (p < 0.001). KEGG pathway analysis to identified potential causes of *SOX10* upregulation, showing that poor responders to bevacizumab had significant downregulation of the proteosome degradation pathway (Table 2), which is the primary regulator of *SOX10* activity22. Protein level results were validated using immunohistochemical staining. We found a \_\_ increase in SOX10 protein expression in poor-responder (Figure 4). The increased expression of SOX10 in tumor cells of the poor-responders may help to drive increased tumor cell migration and invasion, and thus a poor response to bevacizumab.

## 3.4 Collagen genes showed upregulated in poor-responders to bevacizumab.

As angiogenesis is known to dictate increased deposition of collagen in the basement membrane around the neo-vasculature49, we examined whether there was differential expression of collagen genes in poor- versus good-responders to bevacizumab. We found altered expression of eight collagen genes in the poor-responders as compared to the good-responders, and 6 of the 8 collagen genes showed an upregulation in the poor-responder tumors (Table 3). Furthermore, 5 of the 8 collagen genes that were upregulated in poor-responder tumors are predicted to be transcriptionally regulated by *EGR1*28, and *EGR1* is reported to broadly upregulate collagen genes23.

## 3.5 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 cBioPortal32,31,50. GBM patients with upregulation of *CHRNA7* mRNA in their tumors had a significantly worse prognosis; the median overall-survival time was approximately 4 months (Supplemental Figure 5). 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 5). However, GBM patients with altered *RAMP3* mRNA in their tumors showed no change in survival (Supplemental Figure 6). Similarly, upregulated expression of *SOX10* mRNA did not impact survival (Supplemental Figure 7).

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.

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| --- | --- | --- | --- | --- |
| **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.

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Description automatically generatedFigure 2**. **Protein expression and analysis of EGR1 and α7-nAChR in the tumors that were poor-responders to bevacizumab**. A. Box and dot plot showing nuclear EGR1 expression of good and poor responders to bevacizumab. Animals are labeled by colored points and significance was measures by Wilcoxon rank-sum (p = 5.3e-9). B. Raw staining data from which H-scores were drawn of poor responders to bevacizumab. C. Raw staining data from which H-scores were drawn of good responders to bevacizumab.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **KEGG Gene Set Enrichment** | | | | |
| *Genset* | *Normalized Enrichment* | *Adjusted P-value* | | *Core Enrichment* | |
| PROTEASOME | -2.4956553 | < 0.001 | PSMD13/PSMB7/PSMC5/PSMB6/PSMC3/PSME4/PSMB10/PSMC2/PSME1/PSMA2/PSMB1/PSMD7/SEM1/PSMD1/PSMA1/POMP/PSMB5/PSMD11/PSMD12/PSMC4/PSMA3/PSMD8/PSMC6/PSMB3/PSMB2/PSMB4/PSMA6/PSMC1/PSMA5/PSMA7/PSME2/PSMD14 | | |
| OXIDATIVE PHOSPHORYLATION | -2.3884212 | < 0.001 | NDUFA5/COX17/NDUFS3/PPA1/NDUFA2/COX11/NDUFC2/ATP6V0B/NDUFA10/ATP5MC3/SDHC/NDUFB2/COX7C/NDUFS4/NDUFS6/NDUFB5/NDUFB8/ATP6V1F/NDUFB3/ATP5MG/NDUFS1/ATP6V1E1/COX5B/NDUFA8/ATP5F1C/NDUFB9/NDUFS8/ATP5ME/NDUFA9/ATP5MF/UQCRC2/ATP6AP1/NDUFA3/NDUFA7/UQCR10/ATP6V1D/ATP5PB/NDUFB4/ATP5F1A/NDUFA4/NDUFB7/UQCRFS1/NDUFA6/NDUFC1/COX7A2/COX4I1/NDUFB1/NDUFAB1/UQCRQ/ATP5PD/COX6C/NDUFV2/UQCRH/UQCR11/ATP6V0E1/NDUFB10/COX6B1/NDUFA1/NDUFB6/COX7B/NDUFS5/COX7A2L/SDHB/ATP5F1E/UQCRHL/PPA2 | | |
| PARKINSONS DISEASE | -2.2591287 | < 0.001 | NDUFC2/NDUFA10/ATP5MC3/UBE2G1/CASP3/SDHC/PPID/NDUFB2/COX7C/NDUFS4/NDUFS6/NDUFB5/NDUFB8/NDUFB3/NDUFS1/COX5B/NDUFA8/ATP5F1C/NDUFB9/VDAC2/NDUFS8/NDUFA9/VDAC1/UQCRC2/NDUFA3/NDUFA7/CYCS/UQCR10/ATP5PB/NDUFB4/UBB/UBE2L3/ATP5F1A/NDUFA4/NDUFB7/UQCRFS1/NDUFA6/NDUFC1/COX7A2/COX4I1/SLC25A5/NDUFB1/NDUFAB1/UQCRQ/ATP5PD/COX6C/NDUFV2/UQCRH/UQCR11/VDAC3/NDUFB10/COX6B1/NDUFA1/NDUFB6/COX7B/NDUFS5/COX7A2L/SDHB/ATP5F1E/UBE2J1/UQCRHL | | |
| RIBOSOME | -2.2267832 | < 0.001 | RPL30/RPS20/RPS15/RPL7/RPS9/RPL27A/RPLP2/RPL10A/RPL31/RPL19/RPS8/RPS11/RPL26L1/RPL22/RPL37A/RPS15A/RPL21/RPS27L/RPL14/RPL5/RPL36A/RPS10/RPS27/RPS5/RPS27A/RPS2/RPL11/RPL18A/RPL38/RPL10/RPLP0/RPS7/RPS21/RPS19/RPS29/RPS16/RPS28/RPS12/RPL34/RPS6/RPL28/RPL13A/RPL12/RPL23A/RPS26/UBA52/RPL35/RPS3A/RPL18/RPL39/MRPL13/RPL4/RPL36/RPS4X/RPL17/RPL41/RPL9/RPL36AL/RPLP1 | | |

Table 2. Positive normalized enrichment indicates upregulation in poor responders to bevacizumab. Genes listed in core enrichment are sorted in decreasing order of differential expression.

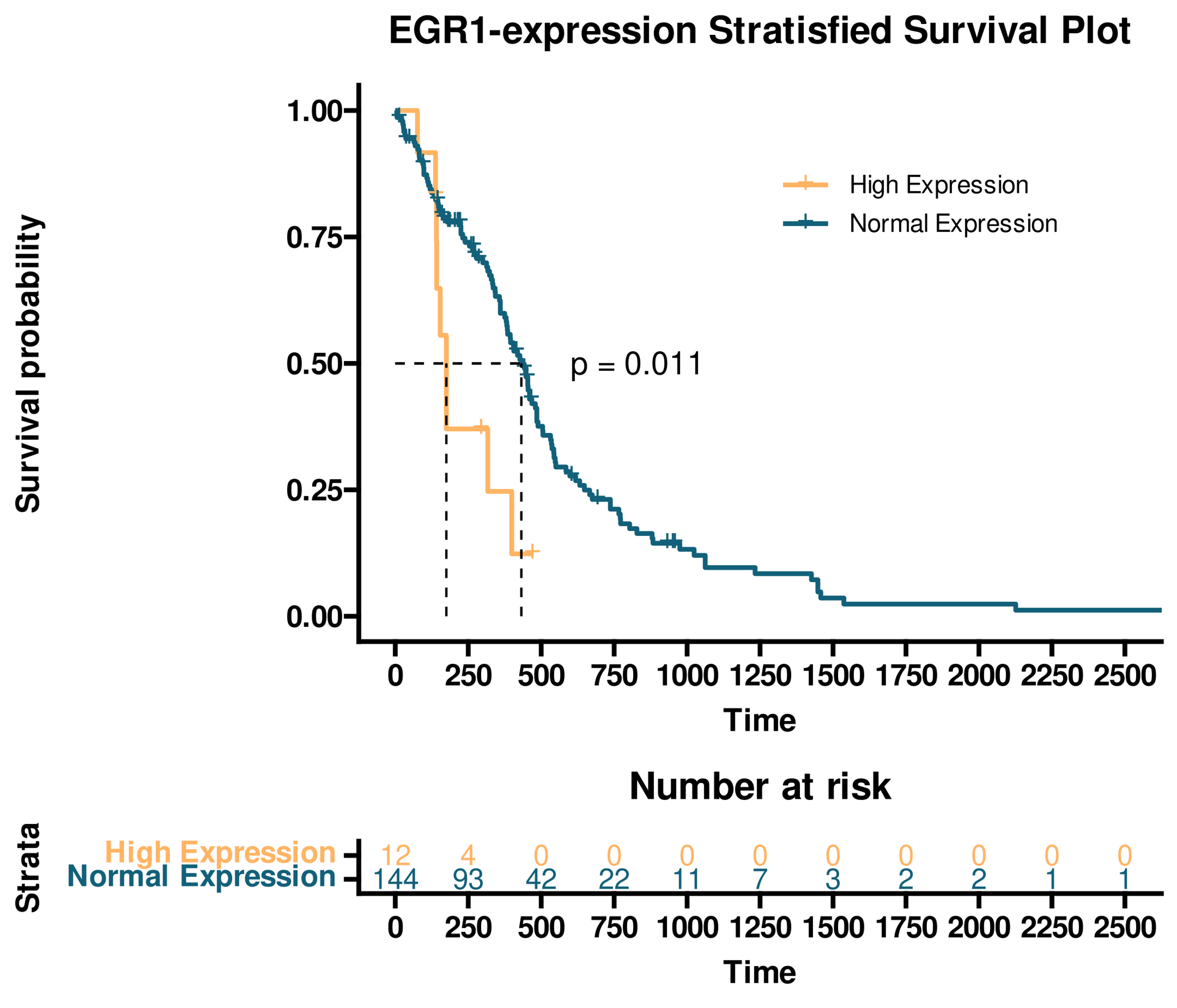


Figure 4. Kaplan Meier survival curve of GBM patients with elevated levels of *EGR1* mRNA. Log-rank survival analysis shows a significant difference in median overall survival.

# 4 Discussion

*4.1. Key findings*

Our data suggests that GBM tumors with a poor-response to bevacizumab have a distinct molecular phenotype. In tumor cells, there is upregulation of two transcription factors, *EGR1* and *SOX10*, which regulate angiogenesis and tumor cell migration. Furthermore, there is upregulation of an angiogenesis gene set and of collagen genes.

*4.2. EGR1 regulates angiogenesis genes in relation to your findings.*

Our data indicate that *EGR1* mRNA and protein is significantly upregulated in the poor-responder tumors and *EGR1* protein is largely localized to the nucleus in the poor-responder tumors, consistent with its role as a transcription factor. *EGR1* is known to regulate multiple genes involved in angiogenesis (Refs), and it has been inferred to transcriptionally regulate additional angiogenesis genes based on ChipSeq data (Refs). We evaluated the homogeneity of *EGR1* nuclear expression in the poor- versus the good-responder xenograft tumors using a Chi Square analysis and did not find a significant difference (data not shown).

(Roshan, did you indicate in the angiogenesis gene set table which genes EGR1 regulates or is inferred to regulate.)

*4.3. α7-nAChR promotes cancer cell migration and proliferation and is regulated by EGR1.*

The nicotinic acetylcholine receptors (nAChRs) regulate complex stimulatory and inhibitory networks, as well as regulate the release of angiogenic factors in cancer cells51,52. In addition to nicotine, other ligands for α7-nAChR have been reported; thus, the absence of identification of the ligand for α7-nAChR in cancer cells does not negate the important role of this receptor in promoting cancer cell migration and proliferation53.

Our co-localization data of EGR1 and α7-nAChR in xenograft tumor cells by multiplex staining suggests that EGR1 may positively regulate expression of α7-nAChR in the poor-responder tumors and thereby increase α7-nAChR signaling that can promote cancer cell migration and proliferation. Although EGR1 can transcriptionally regulate α7-nAChR, there can also be bi-directional crosstalk between EGR1 and α7-nAChR in specific cells or microenvironments. For example, in the retina, choroidal neovascularization and upregulation of α7-nAChR expression are induced with laser treatment25 and nicotine treatment of this model induced signaling of α7-nAChR that resulted in increased EGR1 transcription of FGF-2, promoting angiogenesis25.

A common co-morbidity occurring in GBM patients is aging54. There is a peak in the incidence of GBM in the sixth and seventh decade and aging is associated with an increased deposition in the brain of amyloid-β-A4 protein (APP)54. The APP protein is a ligand for α7-nAChR55. This suggests that older adults with GBM that demonstrate increased tumor cell expression of α7-nAChR may be at risk for increased activation of tumor cell α7-nAChR by the ligand APP, followed by increased α7-nAChR signaling that can promote tumor cell migration and/or proliferation.

*4.4. Poor responders to bevacizumab demonstrated upregulation of a collagen gene set.*

## 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 glioblastoma56,57. Existing biomarkers are largely MRI biomarkers of the tumor microenvironment58. 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*, *SOX10*, 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 a protein level as potential contributors to a poor-response to bevacizumab therapy in GBM tumors, the relatively small number of GBM PDX-tumors and, broadly, 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 tumor59. Overall, computational analysis of glioblastoma prevents causative associations as it is difficult to account for genetic confounders. However, the genes and pathways identified in our analysis describe a distinct molecular phenotype of bevacizumab with demonstrated clinical effect.

**Acknowledgements**:

# 5 Appendix

## 5.1 Supplemental Figures

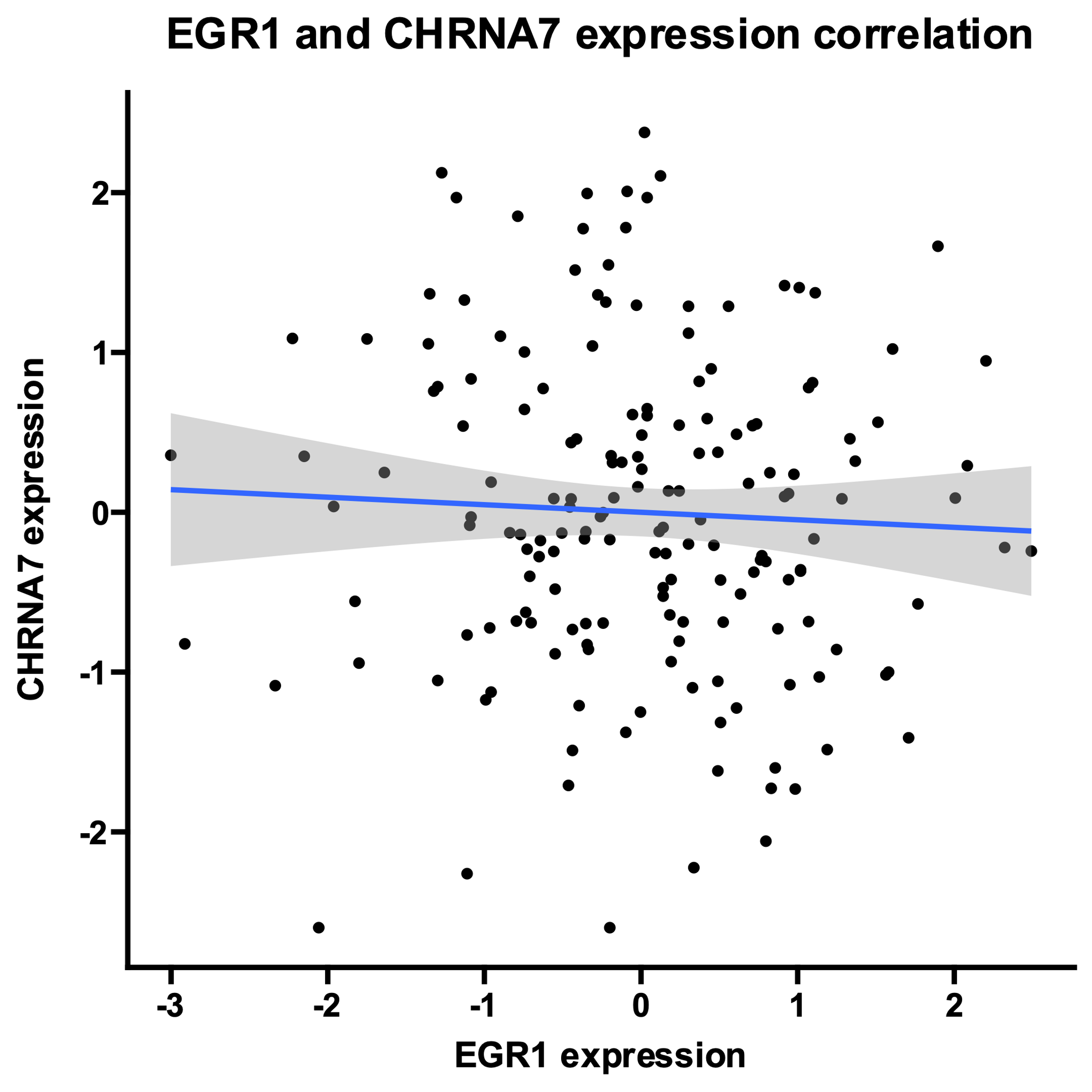
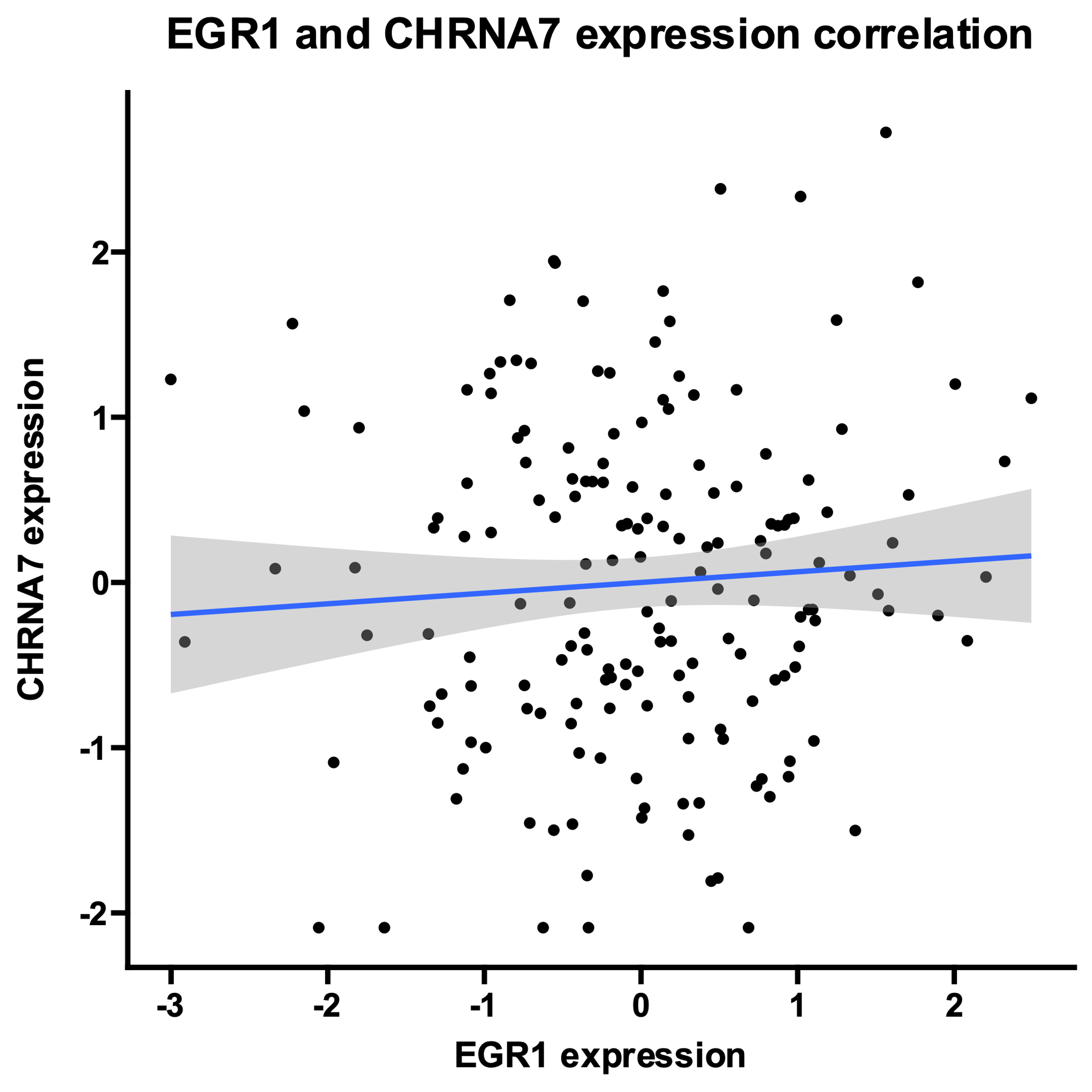


Supplemental Figure 1. Analysis of GBM samples before filtering. A. The first two principal components of each mouse are plotted with color indicating the response group. Samples GBM44\_poor and GBM5\_good do not cluster in their respective group. B. The same data is shown as a dendrogram.

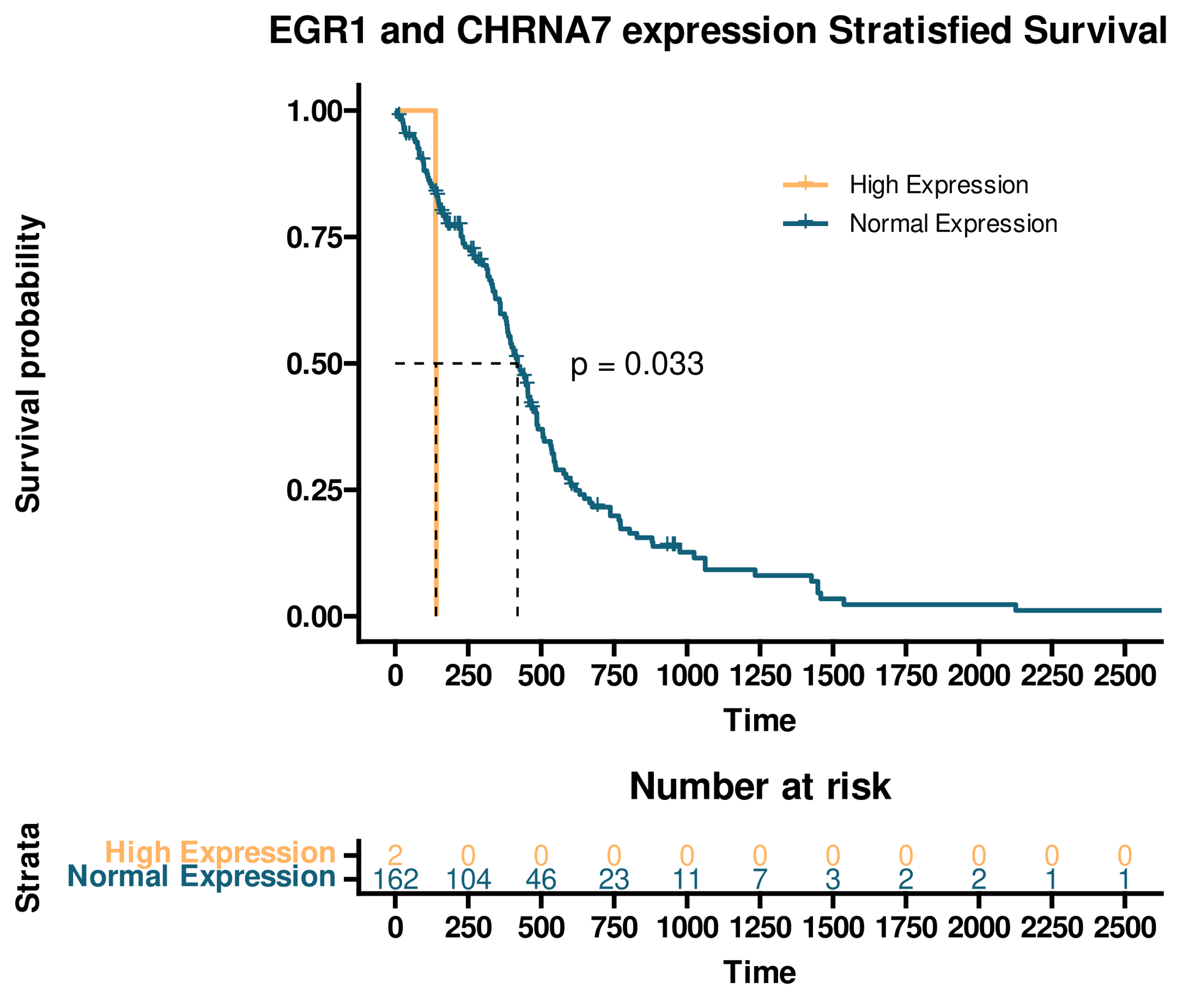
Chart, scatter chart

Description automatically generated

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.



Supplemental Figure 3. Correlation between EGR1 expression and CHRNA7 (A) and RAMP3 (B) expression in TCGA GBM samples.



Supplemental Figure 4. Survival curves of EGR1 and CHRNA7 or RAMP3 upregulated patients.

## 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.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **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.

|  |  |  |  |
| --- | --- | --- | --- |
| **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>

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