Acquired resistance to anti-VEGF therapy in glioblastoma is associated with a mesenchymal transition

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Conflicts of Interest

The authors report no conflicts of interest.
Translational Relevance

Acquired resistance remains a central barrier to the long-term efficacy of antiangiogenic therapy in patients with glioblastoma. To ascertain the underlying mechanisms of resistance, we developed glioma cell lines with acquired resistance to bevacizumab. Tumor tissue transcriptome analysis revealed that chronic in vivo administration of antiangiogenic therapy promotes a proneural to mesenchymal transition (PMT). Several genes were strongly correlated with the mesenchymal phenotype and might provide surrogate markers for early identification of tumor cell transition to a more aggressive and resistant phenotype. Multiple genes related to a pro-inflammatory environment were also overexpressed. Myeloid chemokines and proangiogenic factors were identified that might represent new targets for therapy to overcome or delay the development of resistance. In the future, interim analyses of therapy-induced changes in tumor biology may aid in the identification of rescue agents and the optimization of antiangiogenic therapy administration.
Abstract

Purpose: Antiangiogenic therapy reduces vascular permeability and delays progression but may ultimately promote an aggressive treatment-resistant phenotype. The aim of the present study was to identify mechanisms responsible for glioblastoma resistance to antiangiogenic therapy.

Experimental Design: Glioma stem cell (GSC) NSC11 and U87 cell lines with acquired resistance to bevacizumab were developed from orthotopic xenografts in nude mice treated with bevacizumab. Genome wide analyses were used to identify changes in tumor subtype and specific factors associated with resistance.

Results: Mice with established parental NSC11 and U87 cells responded to bevacizumab, whereas glioma cell lines derived at the time of acquired resistance to anti-VEGF therapy were resistant to bevacizumab and did not have prolongation of survival compared to untreated controls. Gene expression profiling comparing anti-VEGF therapy-resistant cell lines to untreated controls demonstrated an increase in genes associated with a mesenchymal origin, cellular migration/invasion, and inflammation. Gene Set Enrichment Analysis (GSEA) demonstrated that bevacizumab-treated tumors showed a highly significant correlation to published mesenchymal gene signatures. Mice bearing resistant tumors showed significantly greater infiltration of myeloid cells in NSC11 and U87 resistant tumors. Invasion-related genes were also upregulated in both NSC11 and U87 resistant cells which had higher invasion rates in vitro compared with their respective parental cell lines.

Conclusions: Our studies identify multiple pro-inflammatory factors associated with resistance and identify a proneural to mesenchymal transition (PMT) in tumors resistant to antiangiogenic therapy.
Introduction

Glioblastoma is the most common malignant brain tumor and is characterized by rapid angiogenesis-dependent (re)growth, cell heterogeneity, and extensive local tissue infiltration. Transcriptional profiling of human gliomas has allowed for the creation of classification schemes for high-grade glioma that assign tumors to subtypes based on similarity to defined expression signatures (1, 2). These classification schemes, although a continuum, identified molecular subtypes of glioma based on their resemblance to a distinct set of tissues that are enriched for markers of different aspects of tissue growth. The proneural (PN) class is characterized by the expression of genes associated with normal brain and the process of neurogenesis and is distinguished by better long-term prognosis. The worst prognostic class, termed mesenchymal (MES), is associated with a uniformly poor prognosis (2). Analysis of paired tumor specimens from the time of diagnosis and recurrence has indicated that there is a shift from a proneural to mesenchymal phenotype at the time of tumor recurrence (2) which may result from the effects of radiation therapy (3). However, it is not known if this shift represents the accumulation of genetic changes inherent in the progression of the tumor or if treatment itself can accelerate this transition. The latter scenario highlights the importance of understanding the impact of a treatment on the biologic response and selective pressure within the tumor and its subsequent behavior.

The antiangiogenic agent bevacizumab (Avastin; Roche/Genentech, South San Francisco, CA), a humanized monoclonal antibody that sequesters vascular endothelial growth factor (VEGF), is FDA approved for multiple solid tumors including recurrent glioblastoma. Anti-VEGF therapies including bevacizumab have been shown to rapidly decrease vascular permeability which manifests as a decrease in contrast enhancement (4, 5). Clinical trials of bevacizumab have demonstrated impressive radiographic response rates leading to prolongation of progression free survival but the impact of this therapy on overall survival are not known (6, 7). It is typical for patients to have an initial benefit lasting three to four months but
Unfortunately these tumors continue to progress (6). Clinical observations and retrospective studies indicate that glioblastoma become more aggressive and treatment resistant at the time of bevacizumab failure (8-10). A significant fraction of patient’s failing bevacizumab therapy progress with a diffusely non-enhancing tumor without imaging evidence of vascular permeability (11). Histologic examination of both human tumor specimens and tumors from animal xenograft studies demonstrates vessel co-option and diffuse neuropil infiltration suggesting these tumors are highly invasive (11). In the clinic, very few responses to salvage chemotherapy have been reported (8, 10), suggesting the emergence of a highly treatment-resistant phenotype.

Although multiple mechanisms are likely to be involved, the underlying drivers of this resistant phenotype need to be identified. By pruning tumor vasculature, chronic administration of anti-VEGF therapies may be reducing the blood supply within glioblastoma tumors leading to a more hypoxic microenvironment (12, 13) which in turn promotes the attraction of bone marrow derived myeloid cells to hypoxic glioblastoma tumors (13). BMDCs especially CD11b+/Gr1+ myeloid cells have been associated with refractoriness to anti-VEGF therapy (14). Both hypoxia (15, 16) and antiangiogenic therapies (17, 18) are known to promote epithelial to mesenchymal transformation (EMT) in several epithelial tumor types. Therefore, we hypothesized that antiangiogenic therapy may be inducing a highly inflammatory environment and creating resistance by promoting a proneural to mesenchymal phenotypic shift.

Materials and Methods

Cell line, reagents, and treatment

Glioma stem cell line NSC11 was derived from a recurrent glioblastoma specimen as previously described who did not receive bevacizumab (19). Human glioblastoma cell line U87 was obtained from American Type Culture Collection (ATCC). NSC11 cells were cultured in DMEM-F12 (1:1) media with B27 (Invitrogen, Carlsbad, CA), bFGF (Sigma, St. Louis, MO) and
EGF (Sigma, St. Louis, MO) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, and U87 cells were maintained in DMEM containing 10% fetal bovine serum. All cell lines were grown in antibiotic-free medium and were free of *Mycoplasma* contamination.

Establishment of two glioblastoma cell lines (NSC11R and U87R) with acquired resistance to bevacizumab. Intracranial tumor tissue was collected from bevacizumab-treated moribund mice. Mouse brain tumor tissues were immediately put in DMEM/F12 serum free media and then prepared using MACS Neural Tissue Dissociation Kit (Miltenyi Biotec GmbH, Teterow, Germany) according to the manufacturer’s instruction. Briefly, the tissues were cut into small pieces, and incubated in the pre-warmed enzyme mix with agitation at 37°C. The tissue was further mechanically dissociated, and the suspension was applied to a 30 µm cell strainer. The collected cells were immediately cultured in DMEM-F12 (1:1) media containing B27 (Invitrogen, Carlsbad, CA), bFGF (Sigma, St. Louis, MO) and EGF (Sigma, St. Louis, MO.).

**Animal xenografts**

For the *in vivo* experiments, we used 4- to 6-week-old female nude mice strictly inbred at The University of Texas - MD Anderson Cancer Center (MD Anderson) and maintained in the MD Anderson Isolation Facility in accordance with Laboratory Animal Resources Commission standards and treated according to an approved protocol. NSC11 (3 x 10⁵) or U87 glioblastoma cells (5 x 10⁵) and were implanted intracranially into nude mice as previously described (20). Beginning 4 days after implantation, bevacizumab (10mg/kg) or vehicle was administered by i.p. injection twice a week (21). When the mice developed signs and symptoms of advanced tumor growth, they were compassionately euthanized with CO₂ in accordance with animal welfare guidelines. At the time of euthanasia, brains were removed and processed for analysis. The institutional animal care and use committee of The University of Texas MD Anderson Cancer Center approved all of the experiments on our study.
**Gene expression microarrays, pathway analysis and gene set enrichment analysis**

Fresh frozen tumor tissue was processed and total RNA extracted using standard methods (Qiagen, Hilden, Germany) for transcriptome analysis using GeneChip HG-U133Plus 2.0 microarrays (Affymetrix, Santa Clara, CA, USA). Raw gene expression files (CEL) were normalized using a kernel smoothing technique and median centering. Hierarchical clustering was performed using the Ward method for cluster joining. Data normalization and clustering were performed using JMP Genomics 5.0 (SAS Institute, Cary, NC, USA). Differentially expressed transcripts were tested for network and functional interrelatedness using the IPA software program (Ingenuity Systems, Redwood City, CA). Gene set enrichment analysis was performed using class gene lists describing the three molecular subtypes of high-grade gliomas which correlate with survival and were labeled based on putative function of overexpression of genes in each subclass as proneural or mesenchymal (2). Correlations were performed based on either calculating a centroid value for each gene set and normalizing to the highest value among the data or by conducting Gene Set Enrichment Analysis (GSEA v2.07, Broad Institute, Massachusetts Institute of Technology) (22). Gene expression data deposited in Gene Expression Omnibus (GEO); accession number GSE45161.

**Immunohistochemistry and immunofluorescence**

Tissues were fixed in 4% paraformaldehyde for 24h, embedded in paraffin, sectioned serially (4µm), and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich). For immunohistochemical stains, slides were deparaffinized and subjected to graded rehydration. After blocking in 5% serum and antigen retrieval (citrate buffer, pH 6.0), we incubated the slides with the primary antibodies overnight at 4°C. After the slides were washed in PBS with Tween 20, the primary antibody reactions were detected using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) with the respective secondary antibody. Immunohistochemical analysis of the slides was assessed for microvascular density (von Willebrand factor/ Factor
VIII, A0082, diluted 1:500; DAKO, Carpinteria, CA), the cell proliferation marker Ki-67 (M7240, diluted 1:80; DAKO, Carpinteria, CA) and the mesenchymal markers phospho-STAT3(Tyr705) (9145,diluted 1:50, Boston, MA) and c-Met (SP44, diluted 1:1, Ventana, Tucson, AZ). For immunofluorescence studies, the tissue sections were incubated with the macrophage marker F4/80 (RB6-8C5, 108401, diluted 1:50, Biolegend, San Diego, CA), the hypoxia marker HIF-1 alpha (NB100-449, diluted 1:50, Novus, Littleton, CO), and the mesenchymal marker TGFβ (ab66043, diluted 1:50, Abcam, Cambridge, MA). For double immunofluorescence studies, tissue sections were blocked with 10% FBS serum following heat-induced antigen retrieval and then incubated with anti-CXCR3 (2Ar1, AB64714, diluted 1:25, Abcam, Cambridge, MA), or CD74 (LN2, AB9514, diluted 1:25, Abcam, Cambridge, MA) and anti-Nestin (AB5922, diluted 1:1000, Millipore, Billerica, MA). Texas red fluorescein isothiocyanate-conjugated secondary antibodies and green fluorescein isothiocyanate-conjugated antibodies (Invitrogen, Grand Island, NY), respectively, were used for 1 hour at room temperature.

The number of positively stained cells in the area of highest tumor cell density in nine non-overlapping high-power microscopic fields (at 200× magnification) from at least three different tumor-bearing brains from each group was counted. The expression of each marker was quantified by calculating the number of positively stained cells or the area of antibody staining per unit area of tumor using Photoshop CS4 software (Adobe). Microvessel density (MVD) was determined by calculating the area of Factor VIII, using the Image-Pro Plus system version 7.0 (Media Cybernetics) in × 20 fields of at least three tumor samples per group and three to fourth different sections per tumor sample.

**Invasion assay**

Matrigel basement membrane matrix (BD Labware, Franklin Lakes, NJ) was used to perform the *in vitro* cell invasion assays. Transwell inserts for 24-well plates were coated with diluted Matrigel, and cells were added to the transwell in triplicate. Serum-free medium or
macrophage (CRL-2470, ATCC) conditioned media as a positive control was added to the bottom of the plate. Cells were allowed to invade for 24h at 37°C. The filters were then fixed and stained with 0.1% crystal violet in 20% methanol. The invasive cells were visualized using bright-field microscopy. Transwell membranes were incubated with 2% deoxycholic acid for 20min and the absorbance at 595 nm was recorded.

**Real–time polymerase chain reaction (PCR)**

Total RNA was extracted from tumor bearing mouse brain tissue using RNeasy Mini Kit coupled with DNase treatment (QIAGEN) and reverse transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Each cDNA was analyzed in triplicate using real-time TaqMan probes (Applied Biosystems). Quantitative PCR analysis was performed using a chromo 4 sequence-detection system (Bio-Rad, Hercules, CA, USA). Relative quantification of mRNA levels was performed using the comparative CT method with GADPH as the reference gene and the formula $2^{-\Delta\Delta C_t}$.

**Statistical analysis**

All statistical analyses were performed with GraphPad (InStat) software for Windows (Graphpad Software, La Jolla, CA). Survival analysis was performed using the Kaplan-Meier method, and cohorts were compared using the log-rank test. All other data were compared using an unpaired two-tailed Student's test. Summary statistics for continuous data are expressed as the mean ± standard error of the mean. P values less than 0.05 were considered statistically significant.

**Results**

*Isolation and characterization of two glioblastoma cell lines with acquired resistance to bevacizumab*
To develop a model of acquired resistance to anti-VEGF therapy, we treated mice bearing glioblastoma xenografts with bevacizumab until they became moribund at which time tumor tissue was collected. As expected and consistent with previous studies (21), bevacizumab treatment significant prolonged survival compared to controls. In NSC11 bearing mice (n=8/group), bevacizumab increased median survival from 27 days to 39 days (P<0.0001, Fig. 1A). Likewise, bevacizumab improved median survival from 25 to 51 days in the U87 bearing mice (P=0.002, Fig. 1C).

Tumor tissue collected from bevacizumab-treated mice extracted from moribund animals was immediately cultured in vitro as described in the Methods section. NSC11R and U87R (cell lines resistant to bevacizumab) were expanded and then injected orthotopically into nude mice (n=5/group). Mice were randomized to treatment twice a week with either bevacizumab or vehicle. As expected, the tumors formed by parental U87 and NSC11 cells were sensitive to bevacizumab (data similar to Fig. 1A, C, not shown). Tumors formed by the injection of NSC11R and U87R cells were resistance and no survival benefit was observed in animals treated with bevacizumab (Fig. 1B, D). Median survival for NSC11R controls and NSC11R animals treated with bevacizumab was 16 and 14 days, respectively (P=0.82, Fig. 1B). Similarly, U87R controls survived for a median of 14.5 days compared to 19 days in bevacizumab treated animals (P=0.34, Fig. 1D) confirming a resistant phenotype in these cell lines. Serial passage in vitro did not diminish in vivo resistance to bevacizumab (data not shown).

**Bevacizumab resistant tumors are primed for enhanced angiogenesis**

One important mechanism of escape from anti-VEGF inhibitors is the development of VEGF-independent angiogenesis via secretion of a variety of pro-angiogenic factors (21, 23). We evaluated the relative changes in tumor vascularity in parental and bevacizumab-resistant cell lines with and without bevacizumab treatment. At the time of tumor progression, we found a significant increase in tumor vascularity in bevacizumab treated tumors compared with
untreated controls in both NSC11 and U87 tumors. Fractional area of Factor VIII staining in parental NSC11 tumors significantly increased from 4.6 ± 0.4% to 6.9 ± 0.4% in bevacizumab treated animals (Fig. 2A, B P<0.05). When NSC11R resistant cells were re-injected into nude mice and allowed to grow without treatment until mice became moribund, tumor vascularity was 6.1 ± 0.2%, not statistically significant compared to NSC11 parental cells treated with bevacizumab. However, treatment of NSC11R with bevacizumab resulted in a greater increase in tumor vascularity to 8.9 ± 0.7% (Fig. 2B, P<0.05 compared to NSC11R control). There was also a further increase in vascular staining in NSC11R upon treatment with bevacizumab compared to NSC11 (Fig. 2B).

A similar trend was observed for the U87 parental and resistant cell lines. Fractional area of Factor VIII staining in U87 parental cells increased from 11.8 ± 0.5 to 20.2 ± 1.1% following bevacizumab treatment. U87R tumors re-injected into mice in the absence of treatment continued to have greater tumor vascularity than the untreated parental cell line at 16.3 ± 0.7% (Fig. 2C, D, P<0.05). As in NSC11, treatment of U87R with bevacizumab further increased tumor vascularity to 26.3 ± 2.4% (Fig. 2D, P<0.05 compared to U87R untreated controls), a greater than 2-fold increase compared to the original parental cell line.

To investigate whether hypoxia is involved in antiangiogenic therapy-induced vascular resistance in glioblastoma, we evaluated the hypoxia marker HIF-1α expression in parental and bevacizumab-resistant cell lines with and without bevacizumab treatment. We found that HIF-1α expression was enhanced by bevacizumab treatment in a similar manner to the changes in Factor VIII staining in NSC11 and U87 tumors (Supplemental 1A,B).

**Anti-VEGF treatment induces a mesenchymal shift in glioblastoma cells**

To gain insight into the molecular mechanisms underlying the aggressive phenotype observed in bevacizumab resistant tumors, we analyzed data from the gene expression profiles compared to published mesenchymal gene signatures (1, 2). Hierarchical clustering showed
significant similarity between gene expression changes in bevacizumab treated tumors compared to known mesenchymal signatures (Supplemental Fig. 2A). Using Gene Set Enrichment Analysis, we show that tumors treated with bevacizumab demonstrated a highly significant correlation to published mesenchymal gene signatures. Plots of running enrichment scores in NSC11 tumors showed highly significant enrichment of proneural signatures in untreated tumors (Supplemental Fig. 2B) and mesenchymal signatures following bevacizumab treatment (Supplemental Fig. 2C). Untreated resistant tumors maintained some degree of mesenchymal gene expression (Supplemental Fig. 2D) but retreatment with bevacizumab further increased mesenchymal enrichment (Supplemental Fig. 2E). Data are summarized using a composite mesenchymal score (Fig. 3A). The parental glioma cell line NSC11 demonstrated a more proneural phenotype whereas the parental U87 cell line had a signature more consistent with a mesenchymal (MES) phenotype (Fig 3B). Of note, U87 has a higher baseline mesenchymal score and following in vivo anti-VEGF treatment this expression signature further increased.

Identification of specific mesenchymal genes was performed to identify novel markers of anti-VEGF therapy associated with the mesenchymal transformation. Analysis of Affymetrix gene expression profiling data identified that NSC11 tumors treated with bevacizumab had an increase in mesenchymal genes (gene name, fold-increase treated/untreated): YKL-40 7.5, NNMT 4.5, and CEBPD 2.3 (2, 24, 25). We validated the higher expression of these genes in bevacizumab treated tumors by real time PCR (Fig 3C). The gene expression changes for NNMT and CEBPD were similar to the aggregate mesenchymal gene score changes whereas YKL-40 expression did not remain elevated in the absence of treatment suggesting YKL-40 may not be an optimal marker of persistent treatment-induced mesenchymal shift.

The tyrosine kinase c-Met, a known mediator of the glioma mesenchymal phenotype after treated with antiangiogenic therapy was increased in glioma tumors treated with Bevacizumab. The fractional area of c-Met staining was significantly increased from 6.7 ± 1.7% to 17.2 ± 2.9%
following bevacizumab treatment in NSC11 tumors. Similarly, c-Met was markedly increased in resistance cell lines treated with bevacizumab in the NSC11 xenograft mice model (Fig 3D, P<0.05). The fractional area of c-Met staining was increased from 28.1±4.5% in untreated NSC11R tumors compared to 45.3±6.3% following bevacizumab treatment (Fig 3D, P<0.05). After bevacizumab treatment, c-Met staining was also increased in NSC11R tumor compared to NSC11 tumors (Fig 3D).

Similar changes were observed in the U87 xenograft model. The fractional area of c-Met staining in U87 parental cells was increased from 8.3±2.4% to 17.2±2.9% after bevacizumab treatment (Figure 3E, P<0.05). The fractional area of c-Met staining was increased from 28.1±4.5% to 45.3±5.3% after bevacizumab treatment (Figure 3E, P<0.05).

**Anti-VEGF therapy-induced mesenchymal shift is associated with an invasive phenotype**

Anti-VEGF therapy induced an increase in invasive characteristics in both NSC11 and U87 cell lines despite increasing median overall survival. However, to our surprise, NSC11R and U87R cell lines that are resistant to bevacizumab were more invasive even in the absence of bevacizumab treatment (Fig. 4A, B, white arrows) suggesting these induced invasive characteristics are persistent. With additional bevacizumab treatment, the NSC11R and U87R cells appeared more aggressive on histologic examination as evidenced by changes in tissue architecture such as the development of spindle-shaped and elongated tumor cells as they infiltrated into the surrounding tissue.

To determine if the invasive behaviors persist outside of the tumor microenvironment, we next investigated the invasive capacity of acquired resistant cell lines in vitro using a Matrigel transwell migration assay. We found that the NSC11R and U87R cell lines showed significantly higher invasive capacity compared with their respective parental control in the absence of drug treatment. Representative photomicrographs demonstrate an increase in cellular migration in the NSC11R (Supplemental Fig. 3A) and U87R (Supplemental Fig. 3B) cell lines. Quantification
revealed a significant 35 ± 9% increase in invasion in NSC11R cells compared to control (P<0.05, Fig. 4C) and a 21 ± 8% increase in invasion in U87R cells compared to control (P<0.05, Fig. 4D). Proliferation analysis, as measured by Ki-67, did not reveal that resistant cell lines were more proliferative, but rather there was a slight decrease in proliferation in resistant cell lines compared to untreated controls (Supplemental Fig. 4A, B). Genes over expressed in tumors with acquired resistance to antiangiogenic therapy and related to the mesenchymal and invasive phenotype are shown in Supplemental Table 1.

**Identification of relevant genes and biological processes using global transcript profiling**

To obtain insight into some of the molecular mechanisms underlying the resistant phenotype in glioblastoma, we compared gene expression profiles from tumors in mice not treated with bevacizumab compared to those with acquired resistance using microarray analysis. Genes that were significantly up-regulated at least 2.5-fold in the untreated controls versus acquired resistant tumors were analyzed using the IPA software program. Antiangiogenic therapy increased the expression of genes involved in multiple biologic processes including mesenchymal-related pathways, cellular migration/invasion and multiple aspects of inflammation including chemokine secretion, myeloid cell chemotaxis and multiple markers reflective of a pro-inflammatory environment (Fig. 5 A, B). For example, we found that numerous pro-inflammatory factors, including CXCL10, CXCL11, NFKBIA and BMPRIA where overexpressed in resistant NSC11 tumors compared to controls whereas CXCR4, CCL24 and TRIM were elevated in U87 cells compared to controls. Inflammatory and proangiogenic gene expression changes observed in tumors with acquired resistance to antiangiogenic therapy are shown in Supplemental Table 1.

**Acquired bevacizumab resistance leads to persistent attraction of myeloid cells which promote tumor invasion**
To evaluate the role of chemokines and myeloid cells in the resistant phenotype, we confirmed the infiltration of myeloid cells expressing two receptors identified in our gene expression screen. Representative changes in myeloid cell infiltration are demonstrated using immunofluorescence imaging (Supplemental Fig. 5A, B). CD74, a marker for macrophages and microglia and the receptor for macrophage migratory inhibitor factor (MIF), significantly increased from a mean of 42 ± 4.9 positive cells/200X HPF to 70 ± 2.9 cells/HPF following bevacizumab treatment in NSC11 tumors (Fig. 6A, P<0.05). The number of CD74 cells increased from 96.7 ± 10.2 cells/HPF in untreated NSC11R tumors to 123.7 ± 17 cells/HPF following bevacizumab treatment (P<0.05). No difference was observed between NSC11 bevacizumab-treated and NSC11R untreated cells. Qualitatively, CXCR3 the receptor for CXCL10 was increased in a manner similar to CD74 (Supplemental Fig. 5C). In U87 cell lines, F4/80 (a pan-macrophage marker) increased from 582 cells/20X HPF to 866 cell/HPF following bevacizumab treatment (Fig. 6B, P<0.05). Untreated U87R tumors attracted 1272 cells/HPF which did not increase further following bevacizumab treatment (P>0.05). These data suggest that one mechanism for the persistent phenotype of bevacizumab resistance in this model may be genes associated with inflammation and myeloid chemotaxis.

Factors secreted by myeloid cells may contribute to the mesenchymal phenotype. TGF-beta, a factor known to promote the glioma mesenchymal phenotype (3, 28), was significantly increased in resistant cell lines compared to untreated controls (Supplemental Fig. 6A, B). Likewise, the mesenchymal marker STAT3 (25) was found to be elevated in glioma cell lines following the development of antiangiogenic therapy resistance (Supplemental Fig. 7A, B).

Finally, to determine the potential contribution of myeloid cell infiltration on the invasive phenotype, we performed a transwell migration assay with NSC11 and U87 cells cultured with macrophage conditioned media (representative photomicrographs, Fig. 6C). Glioma cell transwell migration increased by 89 ± 0.48% and 51 ± 0.1% in the presence of macrophage media compared to controls in NSC11 and U87 cells, respectively (Fig. 6D, P<0.05), suggesting
that the infiltration of macrophages may be partially responsible for the invasive phenotype of bevacizumab-resistant tumors.

**Discussion**

The goal of this study was to identify mechanisms by which glioblastoma develop resistance to anti-VEGF therapy which may lead to the identification of potential therapeutic targets of resistance. We developed two preclinical models of acquired resistance to anti-VEGF therapy both verifying the acquisition of a mesenchymal phenotype (or greater mesenchymal phenotype). From gene expression and tumor tissue analyses, the predominant biologic process occurring during this mesenchymal transition are markers of inflammation including secreted chemokines and cytokines and the influx of immune cells. These inflammatory mediators may act in an autocrine or paracrine fashion to facilitate a mesenchymal shift and to promote tumor cell invasion. These data are also consistent with preclinical studies implicating a putative role for the mesenchymal marker c-met in the development of aggressive gliosarcoma tumors (26) and clinical experience demonstrating extreme resistance to salvage therapy following tumor progression on bevacizumab and other antiangiogenic therapies (10).

As expected, treatment of tumors with anti-VEGF therapy resulted in an increase in tumor vascularity at the time of tumor progression. In the absence of continued treatment with antiangiogenic therapy, there was not a significant increase in vascularity in tumors formed from resistant cells. However, there appears to be a priming of these resistant cells to become more angiogenic in the setting of re-exposure to antiangiogenic therapy. These data suggest once a tumor becomes resistant to antiangiogenic therapy, removal of anti-VEGF therapy could reduce the selective pressure that leads to enhanced release of angiogenic factors. However, continued treatment or re-exposure may trigger an enhanced release of factors that leads to further increases in angiogenesis. Consistent with this premise is data demonstrating that exposure of glioblastoma tumors to bevacizumab therapy is ineffective in the setting of tumor
progression after prior VEGFR-targeted therapy (9). Although the potential mechanisms for this priming effect are unknown, one contribution could be the persistent influx of macrophages which may be more likely to release angiogenic factors in the setting of VEGF inhibition.

Prognostic classification schemes have been created for high-grade gliomas that assign tumors to subtypes based on similarity to defined expression signatures (1, 2). These classification schemes identified molecular subtypes of glioma based on their resemblance to a distinct set of tissues and are enriched for markers of different aspects of tissue growth. The proneural (PN) class is characterized by the expression of genes associated with normal brain and the process of neurogenesis and is distinguished by markedly better long term prognosis (median survival 3.3 years). The worst prognostic class, mesenchymal (MES), is associated with a uniformly poor prognosis (median survival 1.2 years) (2). Although glioma subtype was initially thought to represent a stable phenotype with a predictable prognosis, our data suggest that treatment-induced conditions in the microenvironment promote a shift in subtype leading to treatment resistance. Comparing the MES gene signature from Phillips et al(2) with the gene changes in our experiments using GSEA, we show that glioma stem cell line NSC11 treated with bevacizumab showed a highly significant correlation to this published MES gene signature. These results were confirmed with qRT-PCR data and provide confidence that the changes in our preclinical models are likely representative of the human disease. Not surprisingly, the more mesenchymal cell line U87 had a significant increase in the MES gene score following bevacizumab treatment, although this cell line started with a higher baseline MES score.

MES related genes as evidenced by the composite MES score and NNMT and CEBPD remained elevated in the absence of continued anti-VEGF treatment. Expression of YKL-40, a secreted protein involved in tumor invasion (27), returned to baseline levels in the absence of anti-VEGF treatment and may not be an adequate marker of a mesenchymal shift when evaluating therapy-induced mesenchymal behavior. These data suggest that once transformed into a more mesenchymal phenotype, removal of antiangiogenic therapy does not lead to
complete reversal of the mesenchymal shift. This observation is borne out with clinical data suggesting that patients failing bevacizumab do not respond to salvage chemotherapy and changes in antiangiogenic therapy do not result in radiographic responses (9, 10). Although there may be multiple mechanisms leading to resistance, stable changes in tumor genetics could be responsible. For example, resistant tumors may increase expression of CXCL10, CXCL11 and other myeloid chemokines that attract tumor promoting bone marrow derived cells. In turn, these cells may promote invasion and secrete factors such as TGF-beta that promote the mesenchymal shift (28). A recent report identified a role for TGF-beta in promoting radiation-induced mesenchymal transformation in glioblastoma which was inhibited using the TGF-beta inhibitor LY2109761 (3). In addition to STAT3, c-MET expression was also increased in the setting of bevacizumab failure and could be a potential target for therapy (29-31). The specific mediators of anti-VEGF therapy-induced mesenchymal shift are currently under investigation.

Previously we and others have shown that chronic antiangiogenic therapy leads to excessive pruning of tumor vessels promoting hypoxia. Both hypoxia (15, 16) and antiangiogenic therapy (17, 18) is known to promote the epithelial to mesenchymal transformation (EMT) in several epithelial tumor types. Hypoxia likely contributes to both the inflammatory microenvironment and directly or indirectly promotes mesenchymal changes. Gene expression profiling identified persistent changes in key signaling nodes that regulates the mesenchymal phenotype in glioblastoma such as CEBPB/D (25). Since several of these genetic changes were not reversible following withdrawal of therapy, monitoring changes in MES gene expression may provide a robust mechanism to monitor the impact of treatment on the development of resistance in preclinical models. We are currently evaluating alternative dosing paradigms in preclinical studies in an effort to minimize the development of resistance (32).

Consistent with a mesenchymal phenotype, tumor cells with acquired resistance to bevacizumab retained an increase in invasion both in vivo and in vitro. We have previously evaluated invasion related gene expression changes in U87 following treatment with
antiangiogenic therapy (21). As in this previous study, multiple mechanisms may explain this phenotype including the expression of multiple genes that enhance tumor invasion. Changes in the tumor microenvironment such as the persistent increase in macrophages may be partly responsible for the increase in tumor invasion as was demonstrated in this study. Inhibition of infiltrated myeloid cells will be essential to overcome the resistant phenotype.

We did not observe significant changes in the PI3K pathway or metabolism as previously described (12). Multiple factors may be responsible for these differences including cell line selection and differences in the microarray analyses. The two cell lines evaluated in our experiments exhibit widely different patterns of growth (U87 does not invade whereas the glioma stem cell line is diffusely invasive at baseline). Also different are their level of baseline angiogenesis. U87 is more angiogenic than the glioma stem cell line. Although both cell lines in our studies exhibited a mesenchymal shift following treatment with anti-VEGF therapy, there was little overlap in specific gene expression changes between the two cell lines. This makes generalization of these results to other cell lines difficult and may indicate that each tumor develops a unique gene changes following exposure to antiangiogenic therapy. Tumor cell heterogeneity and their disparate genetic backgrounds may dictate a wide range of genetic responses that culminate in a mesenchymal phenotype. It is important to recognize that there are multiple overlapping pathways that may contribute to resistance. Further investigation will determine if there are common or overlapping signaling nodes that can be blocked to prevent resistance in most tumors.

In summary, we demonstrate a proneural to mesenchymal shift (PMT) in glioma cells following chronic exposure to antiangiogenic therapy. Several markers have been identified that may provide a robust measure of mesenchymal change. The role of antiangiogenic therapy in driving proneural and mesenchymal tumors towards a (more) mesenchymal phenotype has enormous clinical implications. If confirmed in additional studies, patients may have a worse outcome if exposed to inappropriate dosing of antiangiogenic therapy. Furthermore, the current
inability to salvage patients failing antiangiogenic therapy may be due to a persistent and irreversible mesenchymal shift. Antiangiogenic therapy has a very important role in the treatment of glioblastoma. However, optimal translation of these agents into the clinic is dependent upon a better understanding of the effect of the treatment on tumor biology and the optimal ways of delivering the therapy to our patients.

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Figure Legends

**Fig. 1.** *In vivo* selection of two glioblastoma cell lines (NSC11R and U87R) with acquired resistance to bevacizumab. Graphs showing Kaplan-Meyer estimates of survival for control and bevacizumab treated mice: (A) parental NSC11 glioma stem cell line, (B) NSC11 with acquired resistance to bevacizumab (NSC11R), (C) parental U87 and (D) U87 with acquired resistance to bevacizumab.

**Fig. 2.** Tumors with acquired resistance to bevacizumab are primed for increases in tumor vascularity. Representative photomicrographs and bar graphs demonstrating the fractional area of tumor vascularity (Factor VIII staining) quantified by measuring the density of stained vessels per HPF (at ×200 magnification) for (A, B) NSC11 and NSC11R and (C, D) U87 and U87R control and bevacizumab-treated tumors.
**Fig. 3.** Bevacizumab induces a mesenchymal shift in glioblastoma tumors. Correlations between MES signature and either (A) GSC11 or (B) U87 following bevacizumab selection and/or treatment. Values are normalized to maximum correlation. (C) Validation of priority MES genes YKL-40, NNMT and CEBPD. Quantitative real time PCR data are expressed as the fold change in RNA expression between the gene of interest and GAPDH. Means and 95% confidence intervals from three independent experiments performed in quadruplicate are shown. Bevacizumab increases c-Met expression in GSC11 (D) and U87 (E) tumors. Representative photomicrographs and bar graphs represent the fractional area of c-Met for each treatment condition under ×200 magnification.

**Fig. 4.** NSC11R and U87R cell lines are more invasive *in vitro* and *in vivo*. Representative photomicrographs of glioma xenograft tumors from (A) NSC11 and NSC11R, and (B) U87 and U87R from control and bevacizumab-treated mice (at ×200 magnification). White arrows point to areas of enhanced tumor invasion. Matrigel invasion assay for (C) NSC11 and NSC11R and (D) U87 and U87R cell lines. Photomicrographs are representative examples from three independent experiments. Bar graphs demonstrate measured numbers of migrated cells as described in the Methods section. *, *P* < 0.05.

**Fig. 5.** Relevant genes and biologic processes during acquired bevacizumab resistance. Microarray analysis was conducted based on biological functions enriched among genes differentially expressed in bevacizumab-resistant cell lines compared to untreated controls. (A, B) Ingenuity Pathway Analysis (IPA) used for the intracellular signaling interaction network...
identifies multiple biologic processes and genes involved in bevacizumab resistance. X-axis represents -log (10) P-Value for enrichment with threshold at $P=0.05$.

**Fig. 6.** The most prominent processes in resistance involve inflammation including genes regulating chemokine secretion and myeloid cell chemotaxis. Infiltrated myeloid cells in NSC11 and U87 were validated using immunofluorescence for CD74 and F4/80, respectively. Bar graph represents the number of tumor infiltrated (A) CD74 or (B) F4/80 positive cells per HPF. (C) Representative photomicrographs and (D) bar graphs demonstrating a significant increase in tumor cell migration when cultured with macrophage medium. Conditioned medium from macrophages significantly increased NSC11 and U87 *in vitro* cell invasion ($P<0.05$).

**References**


Figure 1
Figure 2

A

NSC11 Control  NSC11 Bev
NSC11R Control  NSC11R + Bev

B

Fractional Area Factor VIII (%)

NSC11 Control  NSC11 Bev  NSC11R Control  NSC11R + Bev

* * *
Figure 2

C

D

Fractional Area Factor VIII (%)
Figure 3

A

NSC11

B

U87

C

Resistant clone

Bevacizumab

Cancer Research

on April 14, 2017. © 2013 American Association for Cancer Research.
Figure 3

D
Figure 3

E

U87 Control  U87 Bev

U87R Control  U87R+Bev

Area (%) of c-Met staining

U87 Control  U87 Bev  U87R Control  U87R Bev

*
Figure 4

A

NSC11 Control

NSC11 Bev

NSC11R Control

NSC11R+Bev
Figure 4

B

U87 Control
U87 Bev
U87R Control
U87R+Bev
Figure 4

C

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* indicates statistical significance.
Figure 5

A

B
Figure 6

D

Relative Invasion

NSC11
NSC11 + M-media
U87
U87 + M-media

*
Acquired resistance to anti-VEGF therapy in glioblastoma is associated with a mesenchymal transition

Yuji Piao, Ji Liang, Lindsay S. Holmes, et al.

Clin Cancer Res Published OnlineFirst June 26, 2013.

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