Gene Expression Profile Identifies Tyrosine Kinase c-Met as a Targetable Mediator of Antiangiogenic Therapy Resistance

Arman Jahangiri1,5, Michael De Lay1,5, Liane M. Miller1,5, W. Shawn Carbonell1,5, Yu-Long Hu1,5, Kan Lu1,5, Maxwell W. Tom1,5, Jesse Paquette5,6, Taku A. Tokuyasu5,6, Sean Tsao1,5, Roxanne Marshall2,5, Arie Perry2,5, Kirsten M. Bjorgan3, Myriam M. Chaumeil4,5, Sabrina M. Ronen4,5, Gabriele Bergers1,5, and Manish K. Aghi1,5

Abstract

Purpose: To identify mediators of glioblastoma antiangiogenic therapy resistance and target these mediators in xenografts.

Experimental Design: We conducted microarray analysis comparing bevacizumab-resistant glioblastomas (BRG) with pretreatment tumors from the same patients. We established novel xenograft models of antiangiogenic therapy resistance to target candidate resistance mediator(s).

Results: BRG microarray analysis revealed upregulation versus pretreatment of receptor tyrosine kinase c-Met, which underwent further investigation because of its prior biologic plausibility as a bevacizumab resistance mediator. BRGs exhibited increased hypoxia versus pretreatment in a manner correlating with their c-Met upregulation, increased c-Met phosphorylation, and increased phosphorylation of c-Met–activated focal adhesion kinase and STAT3. We developed 2 novel xenograft models of antiangiogenic therapy resistance. In the first model, serial bevacizumab treatment of an initially responsive xenograft generated a xenograft with acquired bevacizumab resistance, which exhibited upregulated c-Met expression versus pretreatment. In the second model, a BRG-derived xenograft maintained refractoriness to the MRI tumor vasculature alterations and survival-promoting effects of bevacizumab. Growth of this BRG-derived xenograft was inhibited by a c-Met inhibitor. Transducing these xenograft cells with c-Met short hairpin RNA inhibited their invasion and survival in hypoxia, disrupted their mesenchymal morphology, and converted them from bevacizumab-resistant to bevacizumab-responsive. Engineering bevacizumab-responsive cells to express constitutively active c-Met caused these cells to form bevacizumab-resistant xenografts.

Conclusion: These findings support the role of c-Met in survival in hypoxia and invasion, features associated with antiangiogenic therapy resistance, and growth and therapeutic resistance of xenografts resistant to antiangiogenic therapy. Therapeutically targeting c-Met could prevent or overcome antiangiogenic therapy resistance. Clin Cancer Res; 19(7); 1773–83. ©2012 AACR.

Introduction

VEGF contributes to the abnormal vascularity of glioblastomas (GBM), which promotes their growth and recurrence following standard treatment. Encouraging results from clinical trials of humanized mouse antihuman VEGF antibody bevacizumab in patients with GBM (1, 2) led to the 2009 U.S. Food and Drug Administration approval of bevacizumab for recurrent GBM treatment.

However, as with other cancers (3), the response to antiangiogenic therapy in GBM is often transient, with 40% to 60% rates of radiographic progression after initial tumor regression (1, 2). GBMs developing antiangiogenic therapy resistance respond poorly to available treatments, with median overall survival less than 6 months after acquired bevacizumab resistance (4).

Preclinical studies suggest that tumor cells become resistant to antiangiogenic therapy through transcriptional upregulation of compensatory pathways increasing production of alternative proangiogenic stimuli, recruitment of marrow-derived cells, or invasion, allowing tumor cells to survive and potentially proliferate, whereas the antiangiogenic target remains inhibited (5). This paradigm represents a departure from resistance to traditional DNA-damaging chemotherapy, which typically involves gene mutations. Because transcriptional changes occur more readily than mutations (5), these changes may occur to some extent in all...
Translational Relevance

The anticancer efficacy of angiogenesis inhibitors like VEGF-neutralizing antibody bevacizumab has been hindered by lack of enduring responses. We conducted microarray analysis of glioblastomas (GBM) that acquired resistance to bevacizumab, and chose c-Met, an upregulated gene expressing a receptor tyrosine kinase, for further investigation because of its role in invasion and VEGF-independent angiogenesis. Elevated hypoxia occurred after bevacizumab resistance in a manner correlating with increased c-Met expression. Growth of a novel xenograft model of antiangiogenic therapy resistance was inhibited by a c-Met inhibitor. Transducing these xenografts with c-Met short hairpin RNA inhibited their invasion and survival in hypoxia and converted them from bevacizumab-resistant to responsive. Transducing bevacizumab-responsive GBM cells to express constitutively active c-Met rendered them bevacizumab-resistant. These findings suggest that c-Met upregulation can mediate antiangiogenic therapy resistance, and that c-Met disruption warrants investigation as a means of overcoming antiangiogenic therapy resistance, allowing these treatments to fulfill their promise.

Immunohistochemistry

Immunohistochemistry and FISH details are described in Supplementary Methods. c-Met immunostaining was subjectively scored 1 to 5 by 2 independent observers blinded to treatment group, and was quantified using ImageJ (NIH; Bethesda, MD). CA9, c-Met, and phosphorylated focal adhesion kinase (FAK), STAT3, and c-Met immunostaining were quantified in ImageJ using the measure RGB feature (6) or the immunoRatio macro to measure tissue percentage staining positive.

Cells

Human umbilical vein endothelial cells and U87 were obtained from and authenticated by American Type Culture Collection and passed in less than 6 months. Human microvascular endothelial cells were provided by the lab of Gabriele Bergers (UCSF) and were not authenticated. Cell culture, transductions, scratch and brain slice assays, Western blotting, and flow cytometry are described in Supplementary Methods.

Animal work

Animal protocols are listed in Supplementary Methods. VEGF blockade used 5 mg/kg B20-4.1.1, an antibody targeting mouse and human VEGF (Genentech; ref. 7) or 10 mg/kg bevacizumab intraperitoneally twice weekly, with multiple xenografts showing identical response to B20-4.1.1 or bevacizumab at these doses.

MRI

MRI protocol is listed in Supplementary Methods. Tumor permeability (PS) and fractional blood volume (BV) histograms were averaged for each treatment group.

Statistics

Kaplan–Meier analysis compared treatment group survivals. Wilcoxon rank-sum test analyzed nonparametric comparisons. Fisher test measured associations between discrete variables. Spearman rank correlation determined correlation between continuous variables. Box and whisker plot outliers were outside 1.5 times the interquartile range. P < 0.05 was statistically significant.

Results

Patient characteristics

Of 234 bevacizumab-treated patients with GBM from 2006 to 2010 at our institution, 22 were identified who developed radiographic progression after initial response requiring surgery with pretreatment tissue available (Supplementary Table S1). BRG resection occurred an average of 32 days after last bevacizumab dose (range, 14–49 days).

Microarray analysis of BRGs versus pretreatment-paired specimens

Of more than 24,000 transcripts analyzed by microarrays in the 15 BRGs and their pretreatment GBMs with sufficient RNA, there were 25 upregulated and 14 downregulated genes in BRGs versus their paired pretreatment specimens.
with raw $P < 0.005$ (Fig. 1A and Supplementary Tables S2 and S3). When comparing published microarray data (8) of 16 bevacizumab-naïve GBMs and their recurrences with similar age at recurrence as our BRGs ($P = 0.1$), of the top 1,000 genes whose transcription was altered in bevacizumab-naïve recurrent GBMs (raw $P < 0.04$; Supplementary Table S4), only 33 were also altered with raw $P < 0.05$ in BRGs, suggesting that most BRG transcriptional alterations could be unique to bevacizumab resistance. Receptor tyrosine kinase c-Met, the fifth most upregulated BRG gene (Fig. 1B and Supplementary Table S3), was further investigated because its roles in invasion (9) and VEGF-independent angiogenesis (10) offered prior biologic plausibility as a mediator of antiangiogenic therapy resistance. While c-Met intensity in microarray data increased nearly 40% in the 15 BRG samples versus pretreatment, c-Met intensity did not change in the 16 bevacizumab-naïve recurrent GBMs versus their earlier GBMs (Fig. 1B). Real-time PCR (RT-PCR) verified c-Met upregulation in BRGs versus pretreatment and verified microarray data showing unaltered BRG transcription of other factors with prior plausibility as mediators of antiangiogenic therapy resistance: VEGF, VEGF receptor-2 (VEGFR2), and basic fibroblast growth factor (Supplementary Table S4). C-Met ligand hepatocyte growth factor (HGF) expression was unaltered in BRG microarray analysis (Supplementary Table S2). FISH revealed unchanged degree of c-Met gene amplification or polysomy 7 in paired pretreatment GBMs and BRGs (Supplementary Fig. S2 and Supplementary Table S5) and no c-Met gene amplification in cultured BRG cells (Supplementary Table S5), confirming that c-Met upregulation occurred at the transcription level. To determine whether upregulated c-Met expression in BRGs was part of a transition toward expressing genes like c-Met associated with a mesenchymal phenotype, we measured changes in the Pearson correlation coefficients of tumor gene signatures relative to the centroids of 3 GBM subtypes (mesenchymal, proneural, and proliferative; ref. 8). While published microarray data (8) from 16 paired
bevacizumab-naïve GBMs and their recurrences analyzed above showed that these GBMs gained similarity to the mesenchymal centroid, our 15 BRGs analyzed by microarray did not gain similarity to any centroid (Supplementary Fig. S3). When dividing BRGs into 2 types we previously described (11), enhancing BRGs gained more similarity to the mesenchymal centroid than bevacizumab-naïve recurrent GBMs, although this difference was insignificant ($P > 0.05$), whereas nonenhancing BRGs gained more similarity to the proneural centroid.

**c-Met protein expression, downstream effectors, and regulators in BRGs**

Having confirmed 6.4-fold transcriptional upregulation of c-Met by RT-PCR in our 22 BRG samples (Supplementary Fig. S1), we analyzed c-Met protein expression in BRGs (Fig. 2A). Compared with pretreatment, BRGs exhibited 35% increased subjective c-Met staining score ($P = 0.006$; Fig. 2B) and 48% increased automated quantification of c-Met staining ($P = 0.008$; Fig. 2B), with 16 of 22 BRGs exhibiting increased C-Met staining by both scoring methods (Supplementary Table S1). Western blot analysis revealed 3.4-fold increased c-Met expression in a BRG versus pretreatment (Fig. 2C). Of 2 BRG types we recently described (11), c-Met expression increased comparably in nonenhancing versus enhancing BRGs ($P = 0.8$, data not shown).

We then verified that the increased c-Met expression in BRGs versus pretreatment caused increased c-Met activity. Phosphorylated c-Met immunostaining nearly doubled in BRGs versus pretreatment ($P < 0.05$; Supplementary Fig. S4). Expression of phosphorylated c-Met downstream effectors, FAK (12) and STAT3 (13), increased by more than 40% and 100%, respectively, in BRGs versus pretreatment ($P = 0.01–0.03$; Supplementary Figs. S5 and S6).

We investigated hypoxia, a c-Met transcription regulator (14), after bevacizumab resistance by immunostaining for hypoxia marker CA9. CA9 expression increased 43% after bevacizumab resistance than before ($P < 0.05$; Supplementary Fig. S7), with the magnitude of CA9 increase correlating with c-Met changes (Spearman $r = 0.6; P < 0.05$). Unlike BRGs, a control group of 22 bevacizumab-naïve recurrent GBMs and paired initial tumors from our institution with similar average age at recurrence to our BRGs ($P = 0.3$) exhibited unchanged CA9 ($P = 0.9$), c-Met ($P = 0.2–0.4$), or phosphorylated STAT3 ($P = 0.9$) staining upon recurrence (Fig. 2A and B, Supplementary Table S6, and Supplementary Figs. S6 and S7), suggesting that the increased hypoxia, c-Met upregulation, and phosphorylation of downstream effectors of c-Met in BRGs could be specific to bevacizumab resistance.

We then investigated whether increased c-Met expression in BRGs originated from endothelial cells. Hypoxia and bevacizumab lowered endothelial c-Met expression in 2 endothelial cell lines and in endothelial cells isolated from a BRG (Supplementary Fig. S8A). Immunostaining BRGs revealed no endothelial and c-Met costaining before or after bevacizumab resistance (Supplementary Fig. S8B). These findings support GBM cells, and not endothelial cells, as the source of c-Met upregulation in BRGs.

**Figure 2.** c-Met upregulation in BRGs versus pretreatment. A, representative c-Met immunostainings from areas of dense tumor nuclei or less dense tumor nuclei in normal brain (> 630; scale bar, 10 μm). B, both subjective and automated ImageJ scoring revealed increased c-Met staining after bevacizumab resistance ($P = 0.0003–0.002$) with unchanged c-Met staining after bevacizumab-naïve recurrence ($P = 0.2–0.4$). C, Western blot analysis revealed 3.4-fold increased c-Met expression in a BRG versus the pretreatment GBM from the same patient. Numbers represent band densities normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the same sample.
Generating a xenograft model of acquired bevacizumab resistance

We established a xenograft model of acquired antiangiogenic therapy resistance by treating subcutaneous U87 glioma cell line-derived xenografts with bevacizumab. The least responsive xenograft was reimplanted and treated with bevacizumab, and the process was repeated 3 times, producing a U87-BevR xenograft. A control U87-IgG xenograft was generated in parallel with immunoglobulin G (IgG) treatment. U87-BevR exhibited no response to bevacizumab versus IgG treatment ($P = 0.3$; Fig. 3A), and subcutaneous U87-IgG tumors regressed with bevacizumab, whereas U87-BevR grew exponentially during bevacizumab treatment ($P = 0.02$; Fig. 3A). Intracranial U87-BevR xenografts were unresponsive to bevacizumab ($P = 0.1$), whereas intracranial U87-IgG xenografts responded to bevacizumab ($P = 0.006$; Fig. 3B). U87-BevR cells exhibited nearly 4-fold more c-Met protein expression as U87-IgG cells (Fig. 3C). Hypoxia increased HGF expression in U87-IgG and U87-BevR cells 3.5- and 4.5-fold, respectively (Supplementary Fig. S9). Intracranial U87-BevR xenografts exhibited greater discontinuous invasion, percentage of invading tumor cells 10 μmol/L from a vessel, and invasive islands than U87-IgG ($P < 0.05$; Fig. 3D).

Generating a xenograft model of intrinsic bevacizumab resistance

We implanted fresh patient GBM tissue subcutaneously into immunodeficient mice, establishing xenografts from 2 GBMs, which were resected because they acquired...
bevacizumab resistance after initial responsiveness (SF7796 and SF8106) and one GBM, which proved after tissue acquisition to be intrinsically bevacizumab-resistant without radiographic response (SF8244). These xenografts were serially passed subcutaneously in vivo and formed tumors intracranially. Histologically, SF8106 and SF7796 xenografts exhibited greater distance of white matter invasion ($P = 0.04$) than xenografts from bevacizumab-naïve GBMs (Supplementary Figs. S10–S13). While the percentage of invasive cells 10 μm from vessels, a marker of perivascular invasion, and islands of 3 or more cells clustered together invading away from the primary mass were higher in BRG-derived xenografts than in most xenografts from bevacizumab-naïve GBMs, these tendencies were insignificant ($P = 0.1$). SF8244, derived from a GBM with intrinsic bevacizumab resistance, exhibited discontinuous and perivascular invasion, albeit less than SF7796 and SF8106.

To determine whether these xenografts maintained the resistance or response to antiangiogenic therapy found in their patient tumors, we treated xenografts with B20-4.1.1 murine monoclonal antibody or PBS treatment ($n = 10$). SF8244, derived from a GBM with intrinsic bevacizumab resistance, exhibited discontinuous and perivascular invasion, albeit less than SF7796 and SF8106.

Western blot analysis revealed that combined c-Met and human HGF expression distinguished xenografts derived from GBMs with intrinsic (SF8244) or acquired (SF8106 and SF7796) resistance versus those derived from bevacizumab-naïve GBMs (Fig. 4B). We then investigated whether growth of bevacizumab-resistant xenograft SF7796 could be attenuated by XL184 (Exelixis), a tyrosine kinase inhibitor targeting c-Met. Treating SF7796 subcutaneous tumors with XL184 for 28 days reduced tumor volume nearly 3-fold ($P = 0.01$; Fig. 4C). XL184 prolonged the median survivals of mice with subcutaneous SF7796 tumors from 28 to 72 days ($P < 0.0001$; Fig. 4C) and intracranial SF7796 tumors from after B20-4.1.1 treatment ($P = 0.4–0.9$; Fig. 4A).

Figure 4. Xenograft model of intrinsic antiangiogenic therapy resistance exhibits growth suppression with pharmacologic c-Met blockade. A, whereas immunodeficient mice with intracranial U87, SF8557, SF7300, or SF7227 xenografts exhibited prolonged survival with B20-4.1.1 or bevacizumab versus ragweed control antibody or PBS treatment ($n = 10$); U87 and SF8557, $P = 0.0007$; SF7300, $P = 0.0009$; and SF7227, $P = 0.002$. SF7300, $P = 0.0007$ U87, $P = 0.0009$ SF8557, $P = 0.002$ SF7300, $P = 0.0003$ SF7227). B20-4.1.1 did not affect survival of mice with intracranial xenografts derived from a GBM with intrinsic (SF8244) or acquired (SF7796) bevacizumab resistance ($n = 10$). Arrows indicate time of treatment initiation. B, Western blot analysis showing c-Met, human HGF, and mouse HGF expression of 9 xenografts from bevacizumab-naïve GBMs (SF7227, SF7300, and GBM14) and from GBMs with intrinsic (SF8244) or acquired (SF7796 and SF8106) bevacizumab resistance. Numbers represent band densities normalized to GAPDH from the same sample. C, c-Met inhibitor XL184 prevented volumetric growth of subcutaneous SF7796 xenografts after 4 treatment weeks ($P = 0.01$). D, XL184 prolonged survival of mice with intracranial SF7796 xenografts ($n = 5$). Water phospho-Met and XL184 phospho-Met. P < 0.05.
27 to 47 days ($P = 0.01$; Fig. 4D), with nearly 4-fold less phosphorylated c-Met staining after XL184 treatment of intracranial SF7796 ($P = 0.03$; Fig. 4D).

**Transducing a bevacizumab-resistant xenograft with c-Met shRNA reduces survival in hypoxia and invasiveness and causes bevacizumab responsiveness**

We engineered SF7796 cells to express 3 short hairpin RNAs (shRNA) targeting c-Met, each diminishing c-Met protein expression by more than 65% versus control shRNAs, with the shRNA diminishing c-Met protein expression the most causing 91% and 55% loss of c-Met mRNA and protein, respectively, relative to control shRNA (Supplementary Fig. S15). We also engineered U87-BevR cells to express 2 shRNAs targeting c-Met, each diminishing c-Met protein expression versus cells transduced with control shRNA and parental U87-BevR cells (Supplementary Fig. S16A).

To determine whether c-Met upregulation in BRGs promotes survival in the increased hypoxia we identified in BRGs, we investigated the impact of c-Met knockdown on the survival of cells derived from a bevacizumab-resistant xenograft in hypoxia. SF7796/shCmet1 cells grew slightly faster in culture and exhibited 28% less cells after 48 hours in hypoxia versus normoxia ($P < 0.001$), whereas cultured SF7796/shControl cells exhibited unaltered cell numbers after 48 hours in hypoxia ($P = 0.9$; Fig. 5A).

Because antiangiogenic therapy resistance can be associated with increased tumor cell invasiveness (15), the impact of c-Met knockdown on the morphology and invasiveness of cells derived from a bevacizumab-resistant xenograft was investigated. SF7796/shCmet1 cells exhibited 40% reduced inverse shape factor, a unitless parameter measuring a cell’s dendricity (16, 17), versus SF7796/shControl cells ($P < 0.05$; Supplementary Fig. S17). SF7796/shCmet1 cells were less migratory than SF7796/shControl cells, as scratch assays revealed 91% versus 21% scratch reduction at 14 hours with SF7796/shControl and SF7796/shCmet1 cells, respectively ($P = 0.002$; Fig. 5B). In brain slice invasion assays, SF7796/shControl exhibited more invasive multiple cell islands ($P = 0.03$) and single cells ($P = 0.04$) after 3 days than SF7796/shCmet1 (Supplementary Fig. S18). Similarly, the mean fluorescence intensity outside of the core collection of cells after 96 hours was higher after injecting U87-BevR/shControl cells into brain slices than after injecting either U87-BevR/shCmet2 or U87-BevR/shCmet3.
cells into brain slices (P < 0.01; Supplementary Fig. S16B). Thus, c-Met knockdown altered 2 features, survival in hypoxia and invasiveness, associated with antiangiogenic therapy resistance.

We then established xenografts from SF7796/shCmet1 and SF7796/shControl cells. Intracranial SF7796/shCmet1 xenografts exhibited sharper borders with shorter discontinuous invasion from continuous tumor edge; fewer percentage of invasive cells 10 μm from a vessel; and fewer invasive islands (P < 0.05) than intracranial SF7796/shControl xenografts (Fig. 5C and Supplementary Fig. S19). Serial volumetric analysis of subcutaneous SF7796/shCmet1 and SF7796/shControl xenografts revealed that the latter maintained the B20-4.1.1 resistance of parental SF7796 cells (P = 0.2), whereas SF7796/shCmet1 xenografts exhibited B20-4.1.1 responsiveness (P = 0.002; Fig. 6A), suggesting c-Met necessity for xenograft resistance to VEGF blockade. There were similar vessel densities in B20- versus PBS-treated SF7796/shControl xenografts (P = 0.8; Fig. 6B). c-Met staining was reduced in PBS-treated SF7796/shCmet1 xenografts versus PBS-treated SF7796/shControl xenografts (P = 0.03), whereas B20-treated SF7796/shCmet1 xenografts regressed and could not be analyzed (Fig. 6B).

**Transduction of a bevacizumab-responsive xenograft with constitutively active c-Met causes bevacizumab resistance**

We engineered U87 cells to express Tpr-met, a cytoplasmic fusion protein escaping lysosomal degradation, and causing constitutive c-Met activation (18). Transduction generated U87/pBABE-Puro and U87/pBABE-Puro-Tpr-met cells, with only the former exhibiting phosphorylated Tpr-Met (Supplementary Fig. S20). Treating U87/pBABE-Puro-Tpr-met subcutaneous xenografts with bevacizumab caused no response versus PBS treatment (P = 0.7), consistent with resistance, whereas bevacizumab caused U87/pBABE-Puro subcutaneous xenograft regression (P < 0.001; Fig. 6C). Immunohistochemistry revealed fewer but larger vessels in U87/pBABE-Puro-Tpr-met xenografts versus U87/pBABE-Puro xenografts (P < 0.05), with bevacizumab not altering the U87/pBABE-Puro-Tpr-met xenografts vascular pattern (Fig. 6D). Western blot analysis revealed Tpr-met and phosphorylated Tpr-met in U87/pBABE-Puro-Tpr-met xenografts, but not in U87/pBABE-Puro xenografts (Fig. 6D). Bevacizumab increased Tpr-Met and phosphorylated Tpr-Met levels in U87/pBABE-Puro-Tpr-met xenografts (Fig. 6D).

**Discussion**

While much heralded, the arrival of angiogenesis inhibitors has been associated with mostly transient responses followed by renewed tumor growth. We used microarray analysis and novel murine models of GBM antiangiogenic therapy resistance to provide evidence for the role of increased c-Met expression in acquired antiangiogenic therapy resistance.

While the Bonferroni adjustment to reduce microarray false positives caused significant raw P values to no longer be below 0.05 (Supplementary Table S2), the Bonferroni correction is not crucial for studies like this using microarray data to launch further studies into specific genes with significant raw P values and prior plausibility as candidates (19, 20). C-Met fulfilled these criteria as the fifth most upregulated gene of the 24,000 analyzed and because of its roles in invasion (9) and VEGF-independent angiogenesis (10), features associated with angiogenesis inhibitor resistance (5).

Our finding of upregulated c-Met in BRGs versus their paired pretreatment specimens seems unique to bevacizumab resistance, as c-Met was not upregulated in bevacizumab-naïve recurrent GBMs. Discrepancies between our findings and a study, which noted increased c-Met expression in all recurrent GBMs (21), may reflect that study analyzing c-Met expression as a dichotomic covariate rather than the dual use of subjective and automated scoring in our study.

To functionally examine this observed c-Met upregulation, we established the first 2 GBM xenograft models of antiangiogenic therapy resistance. Our first xenograft model—acquired antiangiogenic therapy resistance and was established by serially treating cell line-derived xenografts with bevacizumab until they became resistant, generating a stably resistant xenograft line. Like the 22 BRGs we analyzed, this resistant xenograft line exhibited increased c-Met expression compared with its parental sensitive xenograft. Our second xenograft modeled intrinsic antiangiogenic therapy resistance and was established by implanting BRG tissue into mice, a technique recapitulating GBM biology (22–25). Resulting xenografts maintained the refractoriness to VEGF blockade found in the BRG and exhibited more invasiveness than xenografts from bevacizumab-naïve GBMs. Maintenance of antiangiogenic therapy resistance in BRG-derived xenografts could reflect persistent resistance-mediating factors from the BRG or invasiveness of the BRG-derived xenograft allowing tumors to grow by...

![Figure 6. Effects of genetic c-Met alteration on xenograft responsiveness to VEGF blockade.](clincancerres.aacrjournals.org)
vessel cooption whereby neovascularization is unnecessary (22). While our U87-derived model allows valuable comparisons between paired bevacizumab-resistant and bevacizumab-responsive cells derived from the same cell line, the origin of these cells from a several decade old cell line that likely carries alterations from passage in culture is a disadvantage versus our second model, which was derived directly from fresh patient specimens.

Further work will need to clarify how c-Met upregulation compensates for VEGF blockade. One possibility is c-Met decreasing apoptosis during VEGF blockade, as shown after epidermal growth factor receptor (EGFR) inhibition (26). Our observation of reduced survival in hypoxia of cells expressing c-Met shRNA supports this mechanism. A second possibility is c-Met signaling to VEGFR independent of VEGF, as shown with c-Met and EGFR (27). A third possibility is that c-Met–induced invasion allows escape from hypoxic areas, particularly if the invasion is perivascular (28) and brings tumor cells closer to vessels remaining after antiangiogenic therapy, as identified in our BRGs and a VEGF-knockout glioma (29). Our finding of bevacizumab resistance in cells expressing cytoplasmic constitutively active c-Met suggests that c-Met upregulation compensates for VEGF blockade even with cytoplasmic c-Met signaling.

The role for c-Met in invasiveness shown in other studies (9), and ours, could reflect 3 c-Met effects. First, invasion could reflect chemotaxis to HGF (9). While c-Met–mediated invasion occurs in response to paracrine or autocrine HGF (30), human HGF expression by bevacizumab-resistant xenografts and the inability of mouse HGF to bind human c-Met suggests that autocrine c-Met signaling, which comprises most GBM c-Met signaling (31), contributes to antiangiogenic therapy resistance. Second, c-Met–induced FAK phosphorylation, which we confirmed in BRGs versus pretreatment, could promote invasion (32). Third, c-Met–induced altered cell morphology, suggested by our finding that c-Met knockdown reduced inverse shape factor, a marker of nonspherical morphology, could promote invasiveness. c-Met–induced morphologic changes could be caused by STAT3 phosphorylation (33), which we confirmed to be increased in BRGs versus pretreatment.

While further work will need to identify regulators of the upregulated c-Met we identified in BRGs, our finding that hypoxia and c-Met expression in BRGs both increased versus pretreatment in correlated fashion suggests that hypoxia caused by antiangiogenic therapy (34) plays a role. Hypoxia-driven c-Met transcription could be mediated by hypoxia-inducible factor-1α (14) or hypoxia-induced NF-κB (35), which promotes c-Met transcription (36).

While bevacizumab-naïve GBMs gained similarity to the mesenchymal centroid more than other GBM subtypes, BRGs did not gain similarity to any centroid. While c-Met is more commonly expressed by mesenchymal GBMs than other subtypes, c-Met is not one of the genes defining subtype centroids (8). Given the nearly ubiquitous nature of c-Met upregulation in our BRGs, a mesenchymal transition may occur more frequently after bevacizumab resistance than after bevacizumab-naïve recurrences, as others have suggested (37), but this transition may not be captured by genes defining GBM subtypes and may involve another combination of genes including c-Met, a hypothesis requiring further study.

While we investigated BRG transcriptional changes, antiangiogenic therapy resistance could also reflect altered translation or posttranslational modifications, as suggested by the demonstration that VEGF suppresses c-Met signaling through a VEGF2/c-Met complex (38), which could mean that bevacizumab-induced VEGF depletion could eliminate basal inhibition of c-Met signaling, which would supplement transcriptional c-Met upregulation we observed in BRGs.

Our findings suggest that c-Met targeting using XL184 or other c-Met inhibitors (39) may be effective as monotherapy for tumors resistant to antiangiogenic therapy, as we investigated. Alternatively, because genetic c-Met knockout restored sensitivity to VEGF blockade, c-Met could be targeted alongside VEGF blockade to reduce antiangiogenic therapy resistance, similar to a report combining a VEGF inhibitor with a selective c-Met inhibitor (40). Our findings validate c-Met as a therapeutic target in tumors resistant to antiangiogenic therapy and provide insight into the mechanisms of antiangiogenic therapy resistance, a problem that has unfortunately limited efficacy of these therapies.

Disclosure of Potential Conflicts of Interest
W.S. Carbonell is employed by OncoSynergy, Inc. and is a founder and CEO of OncoSynergy. M.K. Aghi is a consultant/advisory board member of OncoSynergy. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: A. Jahangiri, M. De Lay, W.S. Carbonell, S.M. Ronen, M.K. Aghi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Jahangiri, M. De Lay, L.M. Miller, W.S. Carbonell, M.W. Tom, S. Tsao, R. Marshall, A. Perry, M.M. Chaumeil, S.M. Ronen, M.K. Aghi
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Jahangiri, M.De Lay, L.M. Miller, M.W. Tom, T.A. Tokuyasu, G. Berkers, M.K. Aghi
Study supervision: A. Jahangiri, M. De Lay, M.K. Aghi

Acknowledgments
The authors thank Jonathan Levy and Alexander Jackson in Roger Nicoll’s lab for assisting in brain slice assays.

Grant Support
This work was supported by funding to M.K. Aghi’s laboratory from the American Brain Tumor Association, the James S. McDonnell Foundation, American Cancer Society, the NIH (SK02NS64167-2), and the UCSF Brain Tumor SPORE CA097257.

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Received April 19, 2012; revised December 21, 2012; accepted December 26, 2012; published OnlineFirst January 10, 2013.
c-Met Mediates Antiangiogenic Therapy Resistance

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Clin Cancer Res; 19(7) April 1, 2013 1783

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