A Proangiogenic Signature Is Revealed in FGF-Mediated Bevacizumab-Resistant Head and Neck Squamous Cell Carcinoma

Rekha Gyanchandani1,2, Marcus V. Ortega Alves3, Jeffrey N. Myers3, and Seungwon Kim1,2

Abstract

Resistance to antiangiogenic therapies is a critical problem that has limited the utility of antiangiogenic agents in clinical settings. However, the molecular mechanisms underlying this resistance have yet to be fully elucidated. In this study, we established a novel xenograft model of acquired resistance to bevacizumab. To identify molecular changes initiated by the tumor cells, we performed human-specific microarray analysis on bevacizumab-sensitive and -resistant tumors. Efficiency analysis identified 150 genes upregulated and 31 genes downregulated in the resistant tumors. Among angiogenesis-related genes, we found upregulation of fibroblast growth factor-2 (FGF2) and fibroblast growth factor receptor-3 (FGFR3) in the resistant tumors. Inhibition of the FGFR in the resistant tumors led to the restoration of sensitivity to bevacizumab. Furthermore, increased FGF2 production in the resistant cells was found to be mediated by overexpression of upstream genes phospholipase C (PLCg2), frizzled receptor-4 (FZD4), chemokine [C-X3-C motif] (CX3CL1), and chemokine [C-C motif] ligand 5 (CCL5) via extracellular signal-regulated kinase (ERK). In summary, our work has identified an upregulation of a proangiogenic signature in bevacizumab-refractory HNSCC tumors that converges on ERK signaling to upregulate FGF2, which then mediates evasion of anti-VEGF therapy. These findings provide a new strategy on how to enhance the therapeutic efficacy of antiangiogenic therapy.

Implications: Novel xenograft model leads to the discovery of FGF as a promising therapeutic target in overcoming the resistance of antiangiogenic therapy in HNSCC. Mol Cancer Res; 11(12); 1585–96. ©2013 AACR.

Introduction

Resistance to antiangiogenic therapies limits the clinical benefit of these agents in cancer patients. The single-agent response rate to antiangiogenic drugs such as bevacizumab (a monoclonal antibody to VEGF-A) is less than 10%, and even in patients who do respond, the duration of response is typically less than 3 months (1–3). Similar responses are seen in HNSCC (4–6), where bevacizumab is being evaluated in phase III clinical trials (NCT00588770). Available evidence suggests that tumors can adapt to the effects of VEGF blockade by acquiring alternative signaling pathways that sustain growth and survival. Studies using relevant preclinical models that identify these escape mechanisms will help develop reliable biomarkers of resistance and allow the development of cotargeting approaches to overcome resistance.

In recent years, there have been an increasing number of reports that described potential mechanisms of resistance to antiangiogenic therapy (7–11). In a xenograft model of Wilms tumor, vascular remodeling was seen in tumors that relapsed due to prolonged anti-VEGF therapy. In addition, remodeled vessels were associated with increased expression of ephrinB2 and PDGF-β (12). Angiogenic revascularization was observed in pancreatic-islet tumors, after a transient decrease in microvessel density (MVD) with anti-VEGF antibody. This revascularization was accompanied by increased expression of members of the FGF family (13). In murine tumor models, infiltrating myeloid cells produced proangiogenic factors including Bv8 and tumor cells secreted HGF, which were shown to mediate intrinsic resistance to anti-VEGF antibody and sunitinib, respectively (14, 15). Increased plasma interleukin IL-8 expression was reported in renal cell carcinoma xenografts that acquired resistance to sunitinib (16). Our previous work has also demonstrated IL-8 as a contributor of innate resistance to bevacizumab in HNSCC xenograft models (17). Upregulated stromal EGFR and FGFR have been implicated in bevacizumab-resistant non–small cell lung cancer (NSCLC) xenografts (18). High expression of proinflammatory factors has been associated with increased aggressiveness of bevacizumab-resistant pancreatic tumors (19).
Above studies indicate that tumors can rely on multiple mechanisms of resistance using a variety of angiogenic proteins secreted by both tumor cells and stromal cells. Hence, selection of one critical mediator of resistance for cotargeting with VEGF remains a daunting task. To address this issue, preclinical models that test combinatorial therapies along with VEGF inhibitors are needed to assist selection of suitable cotargets. Also, studies that elucidate the mechanism of upregulation of these resistance-contributing proteins will enable identification of functional networks that integrate additional upstream genes as potential contributors of resistance.

In the present study, we established a novel HNSCC xenograft model of acquired resistance to bevacizumab and identified upregulation of FGF signaling in resistant tumors. Angiogenesis-related genes \( \text{PLCg2, FZD4, CX3CL1, and CCL5} \) regulated increased expression of FGF2 via increased ERK signaling. Cotargeting VEGF and FGFR sensitized resistant tumors to bevacizumab by disrupting angiogenesis. Overall, our results indicate that bevacizumab-refractory HNSCC tumors utilize FGF signaling as a path of least resistance using a battery of proangiogenic genes that converge on ERK signaling to upregulate its expression and mediate resistance to anti-VEGF therapy.

Materials and Methods

Cell lines and reagents

Tu138 cells were kindly provided by Dr. Jeffrey N. Myers (The University of Texas MD Anderson Cancer Center, Houston, TX; ref. 20). Tu138 cells and the bevacizumab-resistant isogenic clones were maintained in Dulbecco’s modified Eagle medium (DMEM)/F12 medium supplemented with 10% FBS. Both cell lines were validated by genotyping using short tandem repeat analysis within 12 months of their use. Bevacizumab (Avastin, Genentech Inc.) and small tumor fragments were implanted to generate xenografts \( (n = 8) \) for validation in a separate in vivo study with a short-term treatment regime \( (4 \text{ weeks}) \). Parental Tu138 cells were also inoculated in mice \( (n = 8) \) as a positive control for sensitivity to bevacizumab. Two weeks after tumor cell inoculation, the mice were randomized to receive vehicle or bevacizumab \( (4 \text{ mg/kg}) \).

Combination experiments. For the combination treatment study, small fragments from the resistant tumor were implanted in mice \( (n = 12) \). Mice were randomized into four treatment groups receiving saline, bevacizumab, PD173074, or a combination of bevacizumab and PD173074. Bevacizumab and PD173074 were administered intraperitoneally at 8 mg/kg (biweekly) and 25 mg/kg (daily) respectively. Tumors were measured daily and tumor growth was assessed for 2 weeks.

Immunohistochemistry and immunofluorescence

Immunohistochemical staining for CD31 and immunofluorescence staining for CD31/TUNEL was performed on frozen tumor sections as previously described \( (21) \). Vessels completely stained with anti-CD31 antibodies were counted in 10 random 0.04 mm\(^2\) fields with a 20\( \times \) objective and mean MVD was expressed as number of vessels per square millimeter. Quantification of CD31\(^+\)/TUNEL\(^+\) staining was done as the average percentage of apoptotic endothelial cells in 10 random 0.01 mm\(^2\) fields using a 40\( \times \) objective.

Microarray

Total RNA was extracted from frozen tumors using TRIzol reagent (Invitrogen/Life Technologies) and purified using the RNaseasy Kit (Qiagen). RNA amplification and biotin labeling was done using Illumina Total Prep RNA Amplification Kit (Ambion/Life Technologies). Biotinylated cRNA was hybridized to human HT-12 v4 BeadChips (Illumina Inc.) and scanned using an Illumina BeadChip Array Reader.

Efficiency analysis was used to determine the optimal methods for data normalization, transformation, and feature selection that produced the most internally consistent gene set \( (22) \). Raw data were normalized using a log2 and z-transformation and differentially expressed genes were identified using J5 test. This test computes a J5 score by comparing the mean difference in expression intensity between two groups for any gene to the average mean group difference over the whole array. Gene expression changes were considered to be statistically significant for genes bearing a J5 score higher than the threshold value 8.0. Gene expression pattern grids were generated for differentially expressed genes with the GEDA web application \( (23) \).

A pathway level impact analysis \( (24) \) was performed to provide both statistical and biologic significance in suggesting the potential pathways affected by the observed changes in gene expression. Differentially expressed genes between bevacizumab-sensitive and -resistant tumors were also
subjected to the functional interaction network analysis using ingenuity pathway analysis (IPA) software.

**Real-time reverse transcription PCR**

Real-time RT-PCR was performed using TaqMan one-step RT-PCR master mix kit and TaqMan gene expression assay kits (Applied Biosystems/Life Technologies) on a 7900HT Real-Time PCR system (Applied Biosystems/Life Technologies). Samples were prepared in triplicates in a 20 µL reaction volume containing 200 ng input RNA. RT-negative controls were run on each plate to ensure no amplification in the absence of input RNA. Standard cycling conditions were programmed as: 95°C for 12 minutes, 40 cycles of: 95°C for 15 seconds, 60°C for 1 minute. β-Actin

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**Figure 1.** Generation of HNSCC xenograft model of acquired resistance to bevacizumab. A, growth curve of Tu138 xenografts (n = 5 per group) treated with saline or bevacizumab at an initial dose of 4 mg/kg followed by incrementing the dose by 4 mg/kg with every subsequent increase in tumor volume. Three of 5 xenograft tumors (TuR1, TuR2, and TuR3) showed resistance with growth rates comparable with the saline control. B–D, resistant xenografts were excised and small tumor fragments were reimplanted into new mice (n = 2) to propagate the model. Reimplanted tumors were subjected to a second phase of treatment with saline or bevacizumab (increasing concentrations, 8–20 mg/kg). Emergence of resistance was observed in bevacizumab-treated tumor TuR1 and TuR3 while TuR2 remained fairly sensitive. E, validation of acquired resistance in reimplanted TuR3-resistant tumors by treating xenografts (n = 4 per group) with saline or bevacizumab. Parental Tu138 tumors were sensitive to bevacizumab resulting in 88% growth inhibition (*, P = 0.0171). In contrast, the resistant tumors showed no significant reduction in tumor growth.
was used as endogenous control. Following gene-specific TaqMan gene expression assay kits were used; FGF2: Hs00266645_m1, FGFR3: Hs00179829_m1, and PLCg2: Hs00182192_m1.

**Western blot analysis**

Parental Tu138 and bevacizumab-resistant cells were plated in 10 cm dishes. The following day, complete medium was replaced with serum-free medium. After 24 hours, cells were treated with MEK inhibitor U0126 for 6 hours and whole-cell lysates were prepared and resolved on 10% SDS-PAGE gels. Following transfer onto nitrocellulose membranes, antibody staining was done using: pERK (Thr202/Tyr204), ERK, FGF2, and β-Actin (Cell Signaling Technology Inc.). Reactive bands were detected by chemiluminescence using ECL plus western blotting detection kit (Amersham Biosciences). Similarly, immunoblots were performed using untreated cells and siRNA transfected cells using the following antibodies; FGFr1, FGFr3, pPLCg1 (Tyr783), PLCg1, pPLCg2 (Tyr759), PLCg2, pSrc (Tyr416), Src, pAKT (S473), AKT, CCL5 (Cell Signaling Technology Inc.) FGFR2, FGFR4, FZD4, and CX3CL1 (Abcam).

**siRNA transfection**

Parental Tu138 and bevacizumab-resistant cells were transfected with siRNA targeting PLCg2, FZD4, CX3CL1, CCL5, and negative control siRNA (non-targeting) using Opti-MEM media, Lipofectamine-2000 (Invitrogen/Life Technologies). After 4 hours, the media were replaced with complete medium and cells were incubated at 37°C for 1 hour. Following incubation, transfected cells were replated in 6 cm plates for Western blot analysis after 48 hours of transfection. Following gene-specific siRNAs were used; CX3CL1: s12629, FZD4: s15840, PLCg2: s10634, and CCL5: s12575 (Invitrogen/ Life Technologies).

**Results**

**Generation of preclinical model of acquired resistance to bevacizumab**

To establish xenograft model of acquired resistance, we inoculated mice with HNSCC cell line Tu138, which has been previously shown to be highly sensitive to bevacizumab in vivo (17). After generation of subcutaneous tumors, these...
Figure 3. Bevacizumab-resistant tumors upregulate angiogenesis genes in response to chronic anti-VEGF therapy. A, gene expression grid displaying 181 differentially expressed genes between bevacizumab-resistant tumors (n = 4; left column/A) and bevacizumab-sensitive tumors (n = 4; right column/B) indicates that higher number genes are upregulated than downregulated in the resistant tumors. Also, there is relatively less heterogeneity in gene expression (black color) among the replicates within each group. B, functional interaction network analysis involving differentially expressed genes between bevacizumab-resistant tumors and bevacizumab-sensitive tumors using IPA tool.
mice were randomized to receive vehicle or bevacizumab at an initial dose of 4 mg/kg followed by incrementing the dose by 4 mg/kg with every subsequent increase in tumor volume. Drug concentration was increased up to the maximum-tolerated dose in patients (20 mg/kg). Such an escalating dosing regimen eliminated the sensitive tumor cells and sequentially selected for the resistant clones. Initially, 3 of 5 xenograft tumors showed resistance quite early in the treatment cycle with growth rates comparable to the saline control (Fig. 1A). Mean tumor volumes for the resistant xenografts TuR1, TuR2, and TuR3 were 2,351.2, 1,329.4, and 1,194.0 mm³, respectively.

The resistant xenografts were excised and small tumor fragments were reimplanted into new mice to propagate the model (Fig. 1B–D). These reimplanted tumors were subjected to a second phase of treatment where the resistant tumor-bearing mice were exposed to saline or bevacizumab (increasing concentrations, 8–20 mg/kg) for a period of 2 months. We observed a slow emergence of resistance in bevacizumab-treated tumor TuR1 with mean tumor volumes equivalent to the respective saline control (Fig. 1B). Bevacizumab-treated tumor TuR2 remained moderately sensitive throughout the treatment suggesting that it failed to retain the initial resistance under incremental drug-selective pressure (Fig. 1C). However, we observed a steep increase in tumor growth in the bevacizumab-treated tumor TuR3 beyond day 36 (Fig. 1D) indicating emergence of resistance. Mean tumor volumes at the end of the treatment were 2,571.1 mm³ as compared to 1,326.7 mm³ in tumor TuR1. Hence, we selected tumor TuR3 to validate the acquisition of resistance.

In the validation study, we inoculated parental Tu138 cells as a positive control for sensitivity to bevacizumab and implanted small fragments from the bevacizumab-treated tumor TuR3 to generate xenografts (Fig. 1E). The parental tumors were sensitive to bevacizumab, as expected, resulting in 88% growth inhibition. In contrast, TuR3 tumors showed complete resistance to bevacizumab as the growth rate of bevacizumab-treated tumors was almost identical to the saline-treated tumors.

**Table 1. Pathway level impact analysis using genes differentially expressed in resistant/bevacizumab tumors compared with parental/bevacizumab tumors**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Pathway name</th>
<th>Genes upregulated</th>
<th>Impact factor</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Phosphatidylinositol signaling system</td>
<td>PLCg2, ARHGEF16, FGF2, FGR3, PLCG2, FZD4, IGFBP6, IRS1, GRB10, CCNB2, CCND3, CDC42EP4, TP63, CAPN1</td>
<td>25.009</td>
<td>3.58E-10</td>
</tr>
<tr>
<td>2</td>
<td>Pathways in cancer</td>
<td>CCR1, CCR2, CCR5, CCND1, CDC2474, TP63, TP63RA, CCND3, CDC42EP4, TP63, CAPN1</td>
<td>8.601</td>
<td>1.77E-4</td>
</tr>
<tr>
<td>3</td>
<td>Cytokine–cytokine receptor interaction</td>
<td>CXCR7, CX3CL1, CCL5, IL20RB</td>
<td>5.551</td>
<td>2.5E-2</td>
</tr>
<tr>
<td>4</td>
<td>Cell cycle</td>
<td>CCNB2, CCND3, CDC42EP4</td>
<td>5.163</td>
<td>3.5E-2</td>
</tr>
<tr>
<td>5</td>
<td>Apoptosis</td>
<td>IRF1, TNFSF10, PARP9, CRABP2, CASP1</td>
<td>4.866</td>
<td>4.52E-2</td>
</tr>
</tbody>
</table>

**Table 2. Angiogenesis-related genes upregulated in resistant/bevacizumab tumors compared with parental/bevacizumab tumors**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Entrez gene name</th>
<th>J5 score</th>
<th>Accession #</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Insulin-like growth factor binding protein 6 (IGFBP6)</td>
<td>27.040</td>
<td>NM_002178.2</td>
</tr>
<tr>
<td>2</td>
<td>Frizzled homolog 4 (Drosophila; FZD4)</td>
<td>18.239</td>
<td>NM_012193.2</td>
</tr>
<tr>
<td>3</td>
<td>Chemokine (C-X3-C motif) ligand 1 (CX3CL1)</td>
<td>13.329</td>
<td>NM_002996.3</td>
</tr>
<tr>
<td>4</td>
<td>Chemokine (C-X-C motif) receptor 7 (CXCR7)</td>
<td>13.142</td>
<td>NM_002661.1</td>
</tr>
<tr>
<td>5</td>
<td>Phospholipase C, gamma 2 (phosphatidylinositol-specific; PLCG2)</td>
<td>12.871</td>
<td>NM_002661.1</td>
</tr>
<tr>
<td>6</td>
<td>Biglycan (BGN)</td>
<td>12.165</td>
<td>NM_001711.3</td>
</tr>
<tr>
<td>7</td>
<td>Growth factor receptor-bound protein 10 (GRB10)</td>
<td>11.518</td>
<td>NM_005311.3</td>
</tr>
<tr>
<td>8</td>
<td>Chemokine (C-C motif) ligand 5 (CCL5)</td>
<td>10.718</td>
<td>NM_002985.2</td>
</tr>
<tr>
<td>9</td>
<td>Fibroblast growth factor 2 (basic; FGFR2)</td>
<td>9.741</td>
<td>NM_002006.3</td>
</tr>
<tr>
<td>10</td>
<td>Caspase 1, apoptosis-related cysteine peptidase (CASP1)</td>
<td>9.477</td>
<td>NM_033292.2</td>
</tr>
<tr>
<td>11</td>
<td>Insulin receptor substrate 1 (IRS1)</td>
<td>8.817</td>
<td>NM_005544.1</td>
</tr>
<tr>
<td>12</td>
<td>Fibroblast growth factor receptor 3 (FGFR3)</td>
<td>8.424</td>
<td>NM_000142.2</td>
</tr>
</tbody>
</table>
validation study; Fig. 2A). The parental tumors showed significant decrease in MVD and increase in endothelial apoptosis in response to bevacizumab treatment. However, treatment of resistant tumors with bevacizumab did not result in statistically significant changes in MVD or endothelial apoptosis (Fig. 2B and C). These results suggest that the bevacizumab-resistant tumors were able to maintain tumor angiogenesis and prevent endothelial apoptosis despite the sequestration of VEGF by bevacizumab.

**Bevacizumab-resistant tumors upregulate angiogenesis genes in response to chronic anti-VEGF therapy**

To identify molecular changes initiated by the tumor cells to mediate bevacizumab resistance, we performed whole-genome microarray analysis. Bevacizumab-sensitive and -resistant isogenic tumor models were compared for differences in gene expression using HumanHT-12 v4 BeadChips. Efficiency analysis was used to determine the best statistical method and the differentially expressed genes were identified using J5 test.

We found 150 genes upregulated and 31 genes downregulated in the resistant tumors (Fig. 3A; Supplementary Table S1). Using these differentially expressed genes, we carried out a pathway level impact analysis and found several cancer-related pathways to be affected by the observed changes in gene expression (Table 1). Among the angiogenesis-related genes, we found upregulation of FGF2, FGFR3, PLCG2, FZD4, CX3CL1, and CCL5 in
Among the four FGFR receptors, FGFR1, 2, and 3 were downregulated whereas FGFR4 was upregulated in the resistant cells. Among the downstream proteins, higher levels of phospho PLCγ1, total PLCγ1, phospho PLCγ2, total PLCγ2, phospho AKT, and phospho ERK were seen in the resistant cells.

Increased ERK activation upregulates FGF2 expression in resistant cells

Studies have shown that activated ERK can positively regulate FGF levels (25). We next examined if increased expression of FGF2 was due to increased ERK activation in the bevacizumab-resistant cells. Parental and resistant cells were treated with the MEK inhibitor U0126 to block ERK activation and FGF2 expression was examined by Western blotting (A) and ELISA (B). Complete abrogation of ERK activation was observed within 6 hours of inhibitor treatment and a significant decrease in FGF2 expression (\( P = 0.0003 \)).

Upregulated angiogenesis genes induce increased expression of FGF2 in resistant cells by activating ERK

On the basis of the above finding, we investigated other upregulated genes in the microarray that were known to activate ERK. Using the IPA tool, we generated a functional gene interaction network, and found high J5 score-bearing differentially expressed genes PLCγ2, FZD4, CX3CL1, CCL5, and FGFR3 as known activators of ERK (Fig. 6A). We hypothesized that in the resistant cells these upregulated genes lead to increased activation of ERK and subsequent increase in FGF2 expression. To test this, we first validated overexpression of these upstream genes in the resistant cells (Fig. 6B and C). We then used siRNA approach to downregulate PLCγ2, FZD4, CX3CL1, and CCL5 in resistant cells and examined the effect on pERK and FGF2 expression (Fig. 6D–G). We observed that downregulation of these genes resulted in a significant decrease in ERK activation and a corresponding decrease in FGF2 expression.

Cotargeting VEGF and FGFR sensitize HNSCC tumors to bevacizumab

To test the contribution of FGF signaling in bevacizumab-resistant cells, we inhibited FGFRs in the resistant xenografts using PD173074 small-molecule inhibitor in combination with bevacizumab (Fig. 7A). Treatment with FGFR inhibitor alone resulted in a modest but statistically significant decrease in tumor growth. However, cotargeting VEGF and FGFR completely abrogated tumor growth in these resistant xenografts. Furthermore, CD31 staining in the tumor sections revealed a significant decrease in MVD in the combination group compared to bevacizumab or PD173074 alone (Fig. 7B and C). These data suggest that cotargeting VEGF and FGFR sensitize resistant tumors to bevacizumab by disrupting angiogenesis.
Discussion

HNSCC is the eighth leading cancer by incidence worldwide. Although there have been significant advances in surgery and chemoradiotherapy for HNSCC in the past 50 years, the overall survival has remained unchanged. Hence, there is a pressing need to develop new therapeutic strategies in HNSCC. Preclinical studies have shown antiangiogenic therapy to be a promising therapeutic strategy. However, clinical use of antiangiogenic therapy has been hampered by the phenomenon of resistance. Furthermore, there are no reliable predictive biomarkers to identify those patients who are likely to benefit or show resistance to this therapeutic approach. Studies using relevant preclinical models that identify mechanisms of resistance to antiangiogenic agents will help meet these challenges.

There is growing evidence that suggests that the FGF/FGFR axis influences the sensitivity of tumors to antiangiogenic therapy (13, 18, 19, 26). Recent studies have shown that dual inhibition of VEGFR and FGFR in preclinical models can overcome anti-VEGF therapy resistance (27, 28). However, there are no reports that describe the underlying mechanisms that drive the overexpression of the FGF pathway in response to treatment with antiangiogenic agents. Knowledge of such regulatory mechanisms is necessary to discover functional networks that can integrate additional upstream genes as potential contributors of resistance.

In the present study, we established a novel HNSCC xenograft model of acquired resistance to bevacizumab and identified upregulation of FGF signaling in resistant tumors. Increased expression of FGF2 was regulated by upstream genes including PLCγ2, FZD4, CX3CL1, and CCL5 via increased ERK signaling. We also showed that modulation of FGF signaling in the resistant xenografts regulated the sensitivity to bevacizumab.

FGFs are known to play an important role in a variety of cellular processes including differentiation, cell proliferation,
apoptosis, angiogenesis, and inflammation (29–31). These FGF signaling-mediated functions can greatly contribute to the process of tumorigenesis. Accumulating evidence highlights the deregulation of FGF/FGFRs in cancer through different mechanisms, including aberrant expression, mutations, and gene amplifications (32). There are 4 known FGFRs, FGFR1 through FGFR4. These receptors differ in the distribution patterns of specific isoforms on tumor cells and stromal cells, collectively mediating autocrine and paracrine signaling in tumors. Studies have shown that FGF2/FGFRs autocrine signaling contributes to EGFR-TKI resistance in NSCLC lines (33, 34).

In our HNSCC xenograft model of acquired resistance to bevacizumab, we have shown upregulation of FGF signaling specifically in the tumors cells. Several members of the FGF pathway were found to be overexpressed including FGF2, FGFR1-3, downstream proteins phospho PLCγ1, PLCγ1, phospho PLCγ2, PLCγ2, phospho AKT, and phospho ERK. Finally, we showed that the cotargeting of VEGF and FGF pathways resulted in restoration of sensitivity to anti-VEGF therapy in the resistant tumors. Taken together, our data suggest that the upregulation of FGF/FGFR autocrine signaling may be one of the ways by which bevacizumab-resistant tumors cells circumvents VEGF inhibition.

We next addressed the molecular basis for overexpression of FGF2 in the resistant tumors. We observed that the resistant cells had higher levels of pERK and that the expression of FGF2 was dependent at least in part to ERK activity. Using the IPA tool, we then found several high J5 score bearing differentially expressed genes including PLCγ2, FZD4, CX3CL1, and CCL5, which are well-known activators of ERK. Downregulation of these upstream genes in resistant cells resulted in a significant decrease in activation of ERK and a corresponding decrease in FGF2 levels. Although FGF has been implicated, as a mediator of resistance to antiangiogenic therapy in a handful of other reports, our study is the first to demonstrate a mechanism by which the overexpression of FGF occurs in resistant tumors (13, 27, 28). This mechanism involved ERK as a central regulator of FGF expression, but there may be other potential contributors such as HOXB7, CTNNB1, and PKC, which merit further study (Fig. 6A; refs. 35, 36).

The mechanism by which FGF allows evasive resistance to VEGF inhibition remains to be elucidated further. However, our observation that the resistant tumors were able to maintain tumor vasculature and resist endothelial cell apoptosis despite bevacizumab treatment suggests that the primary effects of FGF may be on tumor endothelium. Although we have not analyzed the levels of FGFRs on the endothelial cells, a recent report by Cascone and colleagues described upregulation of stromal FGFR in a NSCLC xenograft model of acquired resistance to bevacizumab (18). It is well known that FGF is a potent angiogenic cytokine (37–41). Studies have shown that FGF pathway may interact with the VEGF pathway in regulating tumor angiogenesis. Initial studies using in vitro models showed that addition of both FGF and VEGF resulted in greater tube formation by endothelial cells compared with either cytokines alone (41). Furthermore, in vivo studies have shown that tumor overexpressing both FGF and VEGF have higher growth rate and MVD compared with tumors that were engineered to overexpress either cytokine alone (42). Finally, it has been shown that VEGFR2 antagonists can inhibit both VEGF- and FGF-induced angiogenesis suggesting interplay between the two pathways (43).

Our study puts forward an important concept in the phenomenon of acquired resistance. In response to anti-VEGF therapy, our HNSCC tumors showed an increased expression of a number of angiogenesis-related genes. Despite changes in the expression of many different signaling proteins, FGF signaling continued to play a critical role in maintaining tumor growth and resistance to antiangiogenic therapy.
proteins, the cumulative effect of these changes appears to feed through a common protein or pathway to exert its effect. In our acquired model, the overexpression of a proangiogenic signature comprising PLG2, FZD4, CX3CL1, and CCL5 has the common effect of ERK activation, which eventually leads to the overexpression of FGF2.

On the basis of this observation, we can put forth a hypothesis that although treatment with antiangiogenic agents lead to pleiotrophic changes to the tumor, therapeutic attempts to reverse the resistance may not require the inhibition of all these differentially expressed proteins but rather one or two integral pathways that these changes mainly feed through. In our model, that common pathway appeared to be ERK and eventually FGF2. These data also provide an added rationale for the use of ERK inhibitors in conjunction with bevacizumab as a means to overcome resistance to anti-VEGF therapy. A multicenter phase I trial of the ERK Inhibitor BAY 86-9766 in patients with advanced cancer indicated some evidence of clinical benefit across a range of tumor types (44), an encouraging trend for future clinical trials.

In our model of acquired resistance, we observed increased expression of FGF2 and not FGFl in the resistant cells. In our previous study, which focused on the mechanisms of bevacizumab resistance in the intrinsic models we observed overexpression of FGFl in the resistant cells (17). Also, IL-8 was the primary mechanism of bevacizumab resistance in these intrinsic models. In contrast, IL-8 was downregulated in the acquired resistant tumors, suggesting that the tumors excluded this mechanism to maintain angiogenesis and sustain tumor growth in presence of VEGF blockade (Fig. 3B; Supplementary Table S1). These findings indicate that there might be an inherent difference in the underlying mechanisms of intrinsic and acquired resistance to antiangiogenic therapies. These distinct mechanisms could correspond to different patient subpopulations. Identification of these subsets can help improve pretreatment patient selection for personalized medicine and enhance the therapeutic efficacy of antiangiogenic therapies.

In summary, our work has identified a proangiogenic signature including PLG2, FZD4, CX3CL1, and CCL5 genes that converge on ERK signaling to upregulate FGF2 expression, which mediates resistance to anti-VEGF therapy. Knowledge of these regulatory networks provides a stronger mechanistic rationale for targeting VEGF and FGF in future clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: R. Gyanchandani, S. Kim

Development of methodology: R. Gyanchandani, M.V. Ortega Alves, S. Kim

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Gyanchandani, M.V. Ortega Alves, J.N. Myers

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Gyanchandani, M.V. Ortega Alves, J.N. Myers, S. Kim

Writing, review, and/or revision of the manuscript: R. Gyanchandani, S. Kim

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Gyanchandani

Study supervision: J.N. Myers

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