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Molecular Cancer Therapeutics

Dual HER/VEGF Receptor Targeting Inhibits *In Vivo* Ovarian Cancer Tumor Growth

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Abstract

Ovarian cancer mortality ranks highest among all gynecologic cancers with growth factor pathways playing an integral role in tumorigenesis, metastatic dissemination, and therapeutic resistance. The HER and VEGF receptor (VEGFR) are both overexpressed and/or aberrantly activated in subsets of ovarian tumors. While agents targeting either the HER or VEGF pathways alone have been investigated, the impact of these agents have not led to overall survival benefit in ovarian cancer. We tested the hypothesis that cotargeting HER and VEGFR would maximize antitumor efficacy at tolerable doses. To this end, ovarian cancer xenografts grown intraperitoneally in athymic nude mice were tested in response to AC480 (pan-HER inhibitor, "HERi"), cediranib (pan-VEGFR inhibitor "VEGFRi"), or BMS-690514 (combined HER/VEGFR inhibitor "EVRi"). EVRi was superior to both HERi and VEGFRi in terms of tumor growth, final tumor weight, and progression-free survival. Correlative tumor studies employing phosphoproteomic antibody arrays revealed distinct agent-specific alterations, with EVRi inducing the greatest overall effect on growth factor signaling. These data suggest that simultaneous inhibition of HER and VEGFR may benefit select subsets of ovarian cancer tumors. To this end, we derived a novel HER/VEGF signature that correlated with poor overall survival in high-grade, late stage, serous ovarian cancer patient tumors. *Mol Cancer Ther; 12(12); 2909–16. ©2013 AACR.*

Introduction

Ovarian cancer symptoms are difficult to identify and as a result, patients commonly present with advanced disease at the time of diagnosis (1). While age, performance status, tumor histology, optimal cytoreduction, and chemotherapy (e.g., platinum-based regimens) are key prognostic indices and despite improvements in disease management, the overall survival rate for patients with high-grade ovarian cancer remains poor (2, 3).

Despite marked interpatient tumor heterogeneity, conserved oncogenic mutations (e.g. *TP53*, *BRCA1/2*, etc.) leading to aberrant receptor tyrosine kinase (RTK)-mediated signaling support the use of targeted therapies in ovarian cancer (4–6). The VEGF pathway functions via RTK-mediated activation and plays a key role in ovarian cancer growth, angiogenesis, and dissemination (7). As a result, anti-VEGF agents have garnered attention as potential therapeutic adjuncts for ovarian cancer (8–10). For example, combined use of adjuvant bevacizumab (anti-VEGF) with standard chemotherapy improved overall response rates in patients with advanced ovarian cancer (11). In addition, the palliative use of bevacizumab has been explored in the recurrent setting (12, 13). For example, addition of bevacizumab to platinum-based chemotherapy significantly improved progression-free survival (PFS) in heavily pretreated and recurrent epithelial ovarian cancer patients (14). However, recent meta-analysis from four independent studies (>4,000 patients) found that combined chemotherapy plus bevacizumab improved PFS but not overall survival (8, 15–17). Furthermore, multiple VEGF family members are simultaneously expressed in ovarian tumors and may account for bevacizumab resistance (18). Therefore, alternative anti-VEGF strategies are warranted.

Aberrant EGF receptor (HER) activity has been reported in ovarian cancer (19). AC480, a highly selective and potent inhibitor of all erbB/HER receptor tyrosine kinases (HER1, HER2, and HER4), abrogated ovarian tumor cell proliferation (20). Unfortunately, the utility of anti-HER targeting in the patient setting remains unclear, as numerous clinical trials examining the efficacy of anti-HER agents in ovarian cancer have been negative (21). A recent phase II clinical trial evaluating the concomitant use of topotecan with lapatinib, a small-molecule pan-HER inhibitor, demonstrated no benefit in patients with chemoresistant ovarian cancer (22).

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Resistance to anti-VEGF and anti-HER agents in ovarian cancer is significant and new strategies are warranted (23). Early evidence demonstrating EGF-induced VEGF production in tumor cells support crosstalk between the two pathways (24). As a result, numerous preclinical studies have demonstrated benefit via combined use of VEGF and HER agents (25–30). In ovarian cancer, HER ligand overexpression was sufficient to induce VEGF production in ovarian carcinoma cells (31). Therefore, we hypothesized that a dual HER/VEGF targeting strategy would effectively limit ovarian cancer tumor growth as compared to either pathway alone. To this end, the pan HER/VEGFR small-molecule inhibitor BMS-690514 was employed (32). In a comprehensive kinase inhibition screen, BMS-690514 demonstrated the highest selectivity and potency toward both HER (HER1, HER2, and HER4) and VEGF (VEGFR1, VEGFR2, VEGFR3) family members. While the antitumor activity of BMS-690514 has been established in non–small cell lung cancer xenografts, its utility in ovarian cancer is unknown (33). Intraperitoneal ovarian cancer xenografts were tested in response to single agent AC480 (pan-HER inhibitor, "HERi"), single agent cediranib [pan VEGFR (VEGFR1, VEGFR2 and VEGFR3) inhibitor, here in referred to as "VEGFRi"], or dual targeting BMS-690514 (combined HER/VEGFR inhibitor, here in referred to as "EVRi"). EVRi was superior to both HERi and VEGFRi in terms of tumor growth, final tumor weight, and PFS. In addition, we identified a subset of patients with ovarian cancer with increased VEGF/HER activity and may benefit from dual targeting.

Materials and Methods

Cell lines and reagents

SKOV3.ip1 cells were generously provided by Dr. Ellen Vitetta (passaged for less than 1 month before experimentation), cultured in DMEM-F12 (Sigma Chemical Co.) supplemented with 10% FBS (HyClone) and 100 U/mL penicillin G and maintained at 37°C in a humidified atmosphere of 5% CO_2. SKOV3.ip1 cells were authenticated as per our prior work and stably transduced with luciferase-expressing lentivirus (SKOV3.ip1-LUC) as previously described (34, 35). Cediranib (Recentin, AZD2171) was purchased from Selleckchem and Bristol-Myers Squibb generously provided both AC480 (BMS-599626) and BMS-690514. The chemical structures for cediranib, BMS-599626, and BMS-690514 have previously been reported (20, 32, 36).

Xenografts and *in vivo* imaging

Female nu/nu athymic nude mice (Harlan Laboratories) were housed in a pathogen-free environment under controlled conditions of light and humidity receiving food and water *ad libitum*. All animal studies were conducted according to the Mayo Clinic Institutional Animal Care and Use Committee. Eight- to 10-week-old mice were injected intraperitoneally with SKOV3.ip1-LUC cells (1.0×10^6) for subsequent drug treatment and imaging studies. Specifically, daily treatments were initiated 72 hours post intraperitoneal tumor injection for a total of 15 days and tumor growth was measured noninvasively via whole animal bioluminescence (Xenogen) initiated the following day (96 hours postinoculation). Whole body bioluminescence (photons/sec) was carried out using the IVIS 200 Bioluminescence Imaging System (Perkin Elmer). Immmediately before image acquisition, anesthetized animals (ketamine/xylazine) were injected intraperitoneally with 100 μL of D-luciferin (150 mg/kg; Gold Biotechnology, Inc.). Dosing was carried via oral gavage according to the predetermined maximally tolerated dose for each of the following cohorts with $n \geq 10$ mice per cohort: HERi (180 mg/kg), VEGFRi (6 mg/kg), and EVRi (50 mg/kg). Animals were sacrificed 35 days post drug initiation and tumors harvested for subsequent correlative analyses.

Human phospho-RTK array

Pan-RTK phosphorylation was assessed in xenograft tumors via the Proteome Profiler 96 (R&D Systems) as previously described (37). Briefly, tumor lysates were subjected to the provided two-site sandwich ELISA plate and subsequent procedures carried out as per the manufacturer's guidelines. Signals were detected using a luminescent image analyzer (LAS-4000; FUJIFILM) and spot intensities quantified via the Quantity One software package (Bio-Rad).

Statistical analysis

Statistical significance between two groups was tested using ANOVA with Bonferroni post hoc test for multiple comparison analysis using Prism 5.0 (GraphPad Software). Pearson coefficient correlations were calculated for each treatment sample, hierarchical clustering performed (Cluster 3.0) and visualized (Java TreeView; refs. 38, 39). A strong correlation between a pair of samples is revealed by close proximity on the heatmap. Univariate survival analyses were performed using the Kaplan–Meier method and corresponding log-rank test for intergroup differences. Time to event was employed as a surrogate marker of PFS was calculated as time for whole body bioluminescence of each animal to reach five times the initial whole body bioluminescence. All analyses were conducted using JMP 9.0 software.

Results

EVRi inhibits tumor growth and progression of ovarian cancer xenografts

To determine whether cotargeted inhibition of these pathways via the single agent BMS-690514 (EVRi) effectively limits ovarian cancer tumor growth, SKOV3.ip1-LUC xenografts were established and drug response determined (Fig. 1). Tumor growth was significantly abrogated in response to both EVRi and VEGFRi (although to a lesser extent) while no significant difference between the HERi and control group was observed (Fig. 1A). Representative bioluminescent images were acquired at the time of study cessation (35 days after tumor inoculation) and included for depiction of the *in vivo* antitumor effect of

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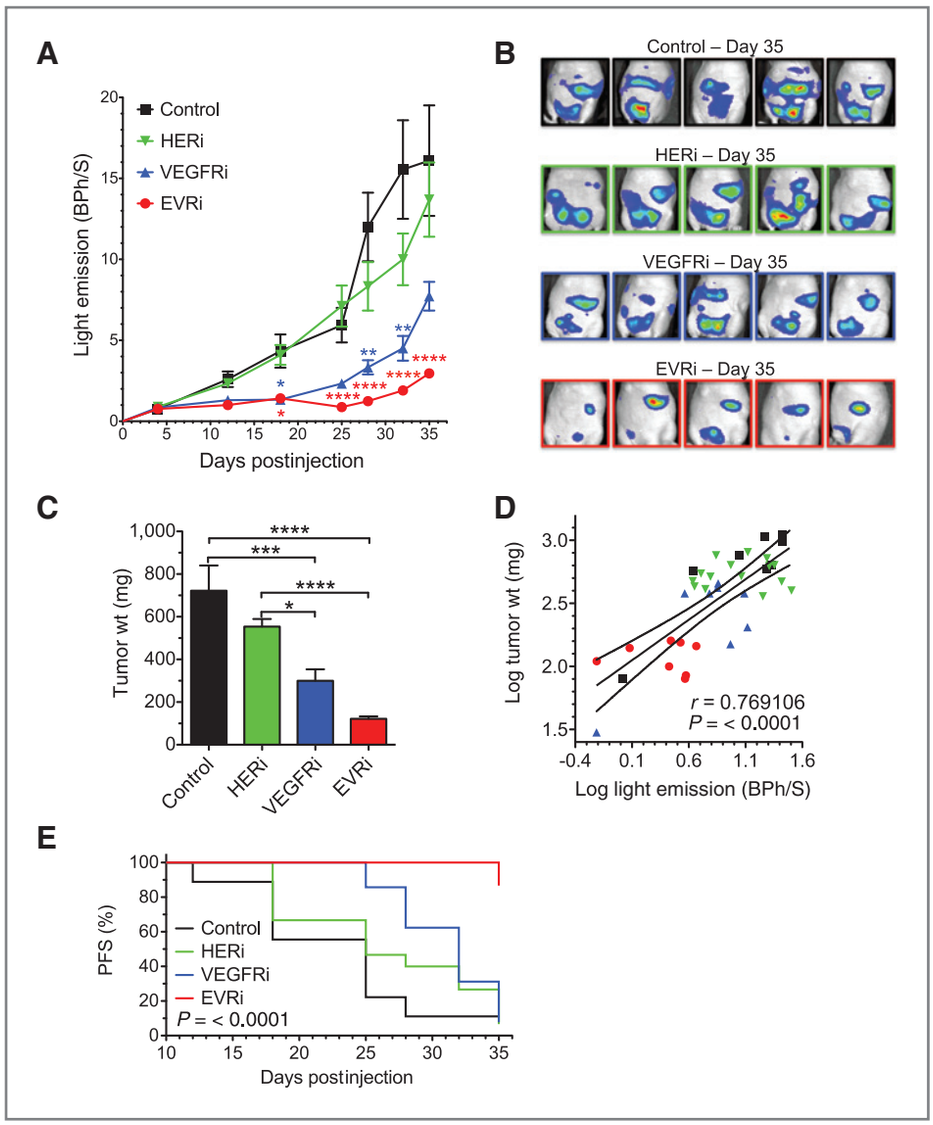

Figure 1. EVRi inhibits tumor growth and progression of ovarian cancer xenografts. A, tumor growth curves for mice bearing intraperitoneal SKOV3.ip1-LUC xenografts treated with HERi, VEGFRi, EVRi, or saline control. Representative bioluminescent images for each of the treatment groups at 35 days post tumor inoculation. In addition, final tumor weight (C), correlation between bioluminescence and actual tumor weight (D), and progression-free survival (PFS; E) are depicted. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

EVRi compared to HERi and VEGFRi (Fig. 1B). In addition, animals were sacrificed and tumors harvested immediately following day 35 bioluminescent imaging (Fig. 1C) to correlate bioluminescence with actual tumor weight (Fig. 1D). These data demonstrate that whole body *in vivo* imaging was an accurate surrogate of ovarian cancer xenograft tumor burden. Importantly, non-specific cytotoxicity was not a factor in any of the treatment groups as bodyweight was maintained throughout the course of the experiment (Supplementary Fig. S1).

Finally, in an effort to translate the potential benefit of EVRi towards tumor suppression, PFS was assessed in ovarian cancer xenografts (Fig. 1E). For each animal, an event was defined as ≥five-fold increase from baseline bioluminescent tumor signal. In conjunction with both tumor growth and final weight, EVRi treatment resulted in the greatest antitumor effect as is demonstrated by significant improvement in PFS versus control (P = <0.0001; Table 1). No change in PFS was observed in the HERi or VEGFRi versus control.

EVRi abrogates oncogenic growth factor signaling

To investigate the mechanism of the antitumor effects of EVRi compared with HERi and VEGFRi at the molecular

Table 1. HRs and 95% CIs of HERi, VEGFRi, and EVRi versus control related to PFS

Group	HR (95% CI)	*P*
Control	—	—
HERi	0.6339 (0.268–1.603)	0.3210
VEGFRi	0.4275 (0.174–1.109)	0.0789
EVRi	0.0558 (0.014–0.194)	<0.0001

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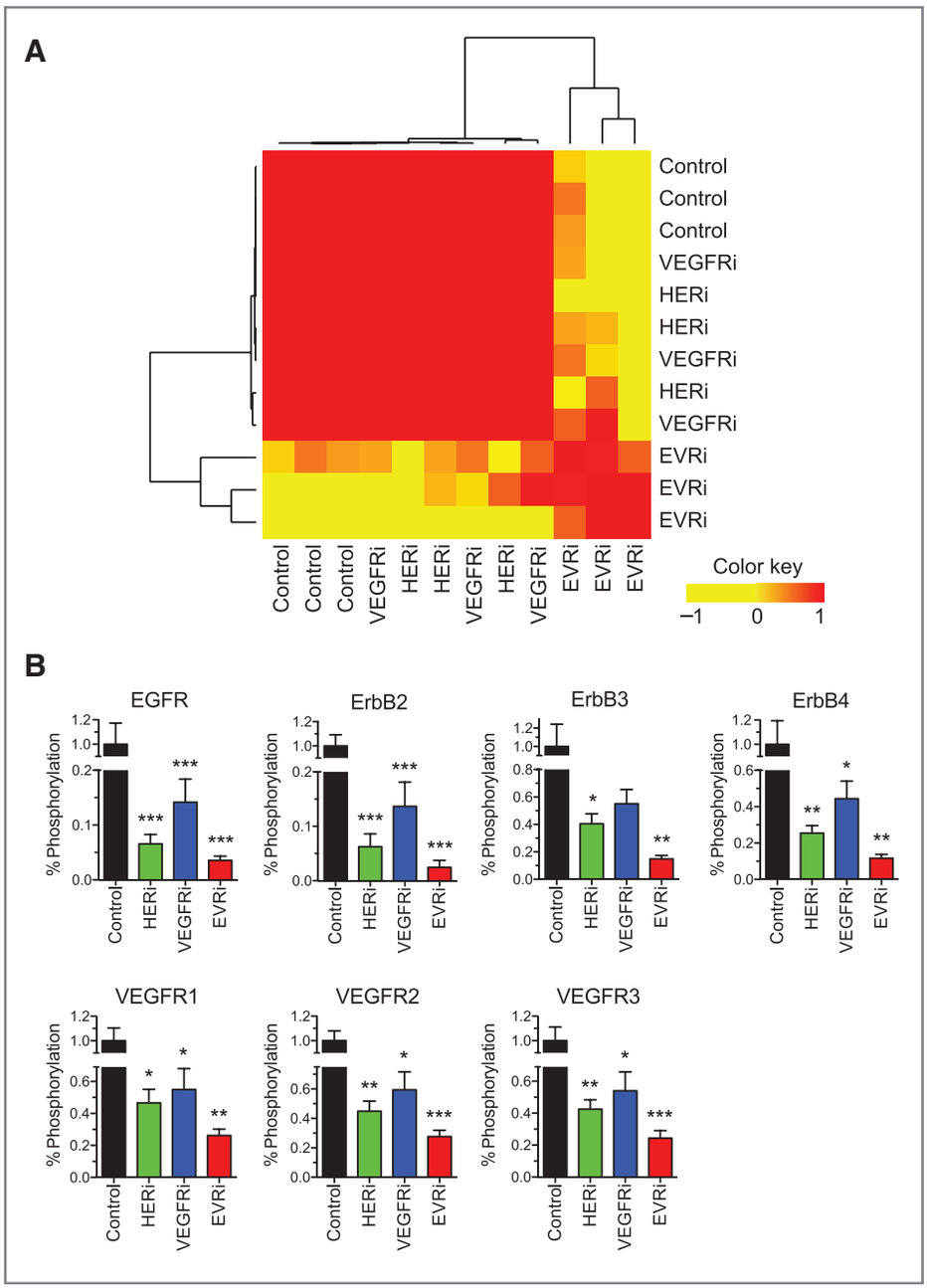

Figure 2. EVRi abrogates oncogenic growth factor signaling. Phosphoproteomic RTK antibody arrays were performed on xenografts harvested at the end of study. A, heatmap depicting sample-to-sample correlation by unsupervised hierarchical clustering of Pearson coefficient values. B, HER (EGFR, ErbB2, ErbB3, ErbB4) and VEGF (VEGFR1, VEGFR2, VEGFR3) receptor phosphorylation. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

level, phosphoproteomic analysis of multiple growth factor receptor tyrosine kinases (RTK) known to play a role in cancer was performed via antibody microarray. Briefly, SKOV3.ip1-LUC xenograft tumors were established, randomized to receive a single dose of HERi, VEGFRi or EVRi and tumors harvested 72 hours posttreatment. Tumor protein lysates ($n = 3$/cohort) were prepared and subjected to antibody microarray analysis for quantification of the following cancer-related RTKs: EGFR, ErbB2, ErbB3, ErbB4, HGFR, IGF-1R, INSR, M-CSF, MSPR, PDGFRa, PDGFRb, SCFR, Tie-2, VEGFR1, VEGFR2, and VEGFR3. While strong similarities existed between HERi, VEGFRi, and untreated control samples, EVRi treatment samples clustered both according to treatment and independently of HERi, VEGFRi and untreated control samples (Fig. 2A). EVRi resulted in the most significant downregulation of HER and VEGF RTK phosphorylation (Fig. 2B). It is noteworthy to mention that a combined single agent strategy (HERi plus VEGFRi) did not inhibit pan-RTK phosphorylation to that of EVRi (data not shown). These data demonstrate that EVRi inhibits ovarian cancer tumors via pan-RTK suppression and subsequent disruption of functional proteomic dynamics.

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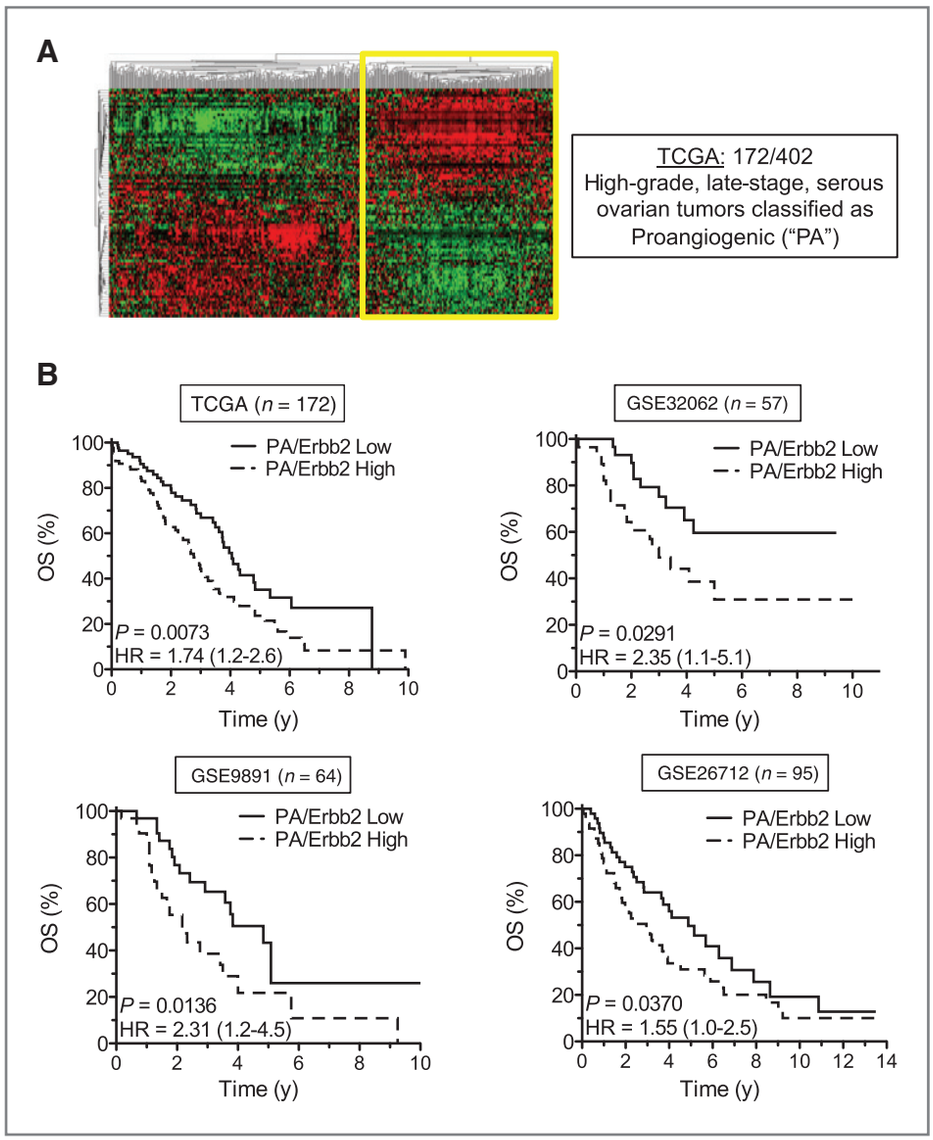

Figure 3. A novel HER/VEGF signature correlates with poor overall survival in high-grade serous ovarian cancer patient tumors. A, heatmap depicting unsupervised hierarchical clustering of high-grade, late stage, serous ovarian tumors from the TCGA using a proangiogenic (PA) gene signature. B, Kaplan–Meier analysis of PA tumors stratified by ErbB2 expression into PA/ErbB2 High (dotted line) versus PA/ErbB2 Low (solid line) and confirmed in four independent cohorts (identified by TCGA or GEO accession number).

A novel HER/VEGF signature correlates with poor overall survival in high-grade serous ovarian cancer patient tumors

One of the greatest challenges towards the clinical advancement of novel therapeutics as a means to improve patient outcome is the ability to accurately identify targetable tumor subsets. In the context of HER and VEGF signaling, both tumor angiogenesis and ErbB2 amplification have been independently examined as poor outcome markers in ovarian cancer (40–42). Moreover, ErbB2 expression directly correlates to EVRi sensitivity (32). It is therefore plausible that combining these two variables (tumor angiogenesis and ErbB2 expression) may provide a predictive surrogate for anti-HER/VEGF agents.

In an effort to first identify VEGF pathway-dependent tumors, a recently defined angiogenic ovarian-specific gene set was applied to high-grade, late stage, serous ovarian tumors from The Cancer Genome Atlas (TCGA; refs. 43). Unsupervised hierarchical clustering stratified patients into two groups, one of which is defined as proangiogenic ("PA"; Fig. 3A). Amplification of *ErbB2* mRNA correlates with increased HER activity and may predict anti-HER–targeted therapeutic response (44, 45). As a result, PA tumors were ranked according to *ErbB2* expression and split evenly into two cohorts, where the top 50% was defined as "PA/ErbB2 High" and the bottom 50% defined as "PA/ErbB2 Low". Univariate analysis revealed that overall survival was significantly poorer for the PA/ErbB2 High cohort ($P = 0.0073$; Fig. 3B). The prognostic value of this novel HER/VEGF signature was validated independently within three additional public data sets comprised exclusively of high-grade, late state, serous ovarian tumors (GEO accession numbers GSE32062, GSE9891, and GSE26712) (46–48). In combined

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Table 2. ORs of the combined cohorts depicting OS at 5 and 10 years in the PA/ErbB2 High versus PA/ErbB2 Low groups

OS, y	No. of patients	PA/ErbB2 High (%)	PA/ErbB2 Low (%)	OR (95% CI)	*P*
<5	192	60	40	2.68 (1.68–4.28)	<0.0001
>5	123	36	64		
<10	213	60	40	2.76 (1.80–4.25)	<0.0001
>10	153	35	65		

analyses, the 5- and 10-year estimates of overall survival are presented (Table 2). At 5 and 10 years, patients with PA/ErbB2 High tumors had shorter survival (60%) rates than patients with PA/ErbB2 Low tumors (40%).

Discussion

The complex and dynamic molecular heterogeneity often associated with aggressive ovarian tumors (e.g., high-grade, metastatic, platinum-refractory disease) has challenged the notion of targeted therapeutics as effective tools to improve overall survival. It is likely that *de novo* and acquired resistance to platinum-based chemotherapy regimens relies on multiple growth factor pathways. Targeted inhibition of oncogenic pathways normally overexpressed/activated (e.g., VEGF pathway) could promote off target compensation by growth factor pathways (e.g., HER pathway). Therefore, an optimal strategy to overcome the limitations of single pathway targeting agents may involve simultaneously inhibition of multiple growth factor pathways. These data suggest that simultaneous inhibition of HER and VEGF pathways via the dual targeting agent EVRi may benefit select subsets of ovarian cancer tumors.

Angiogenesis involves multiple RTK-related pathways and can act as a rate limiting step in tumorigenesis and malignant progression (49). Single agent antiangiogenic (e.g., bevacizumab) resistance and subsequent therapeutic escape is clinically problematic in ovarian cancer and is likely related to the plethora of ligands/cognate receptors involved in ovarian tumor neovascularization [e.g., platelet-derived growth factor (PDGF/R), EGF (EGFR/R), placenta growth factor (PlGF/R), KIT, fibroblast growth factor (FGF/R), hepatocyte growth factor (HGF/R); ref. 50). While EVRi simultaneously targets VEGF and EGFR, it was demonstrated herein to be cross-specific and limit the phosphorylation/activation of additional signaling substrates involved in angiogenesis. It is therefore plausible that the pan-RTK phosphorylation inhibition via EVRi is not due to non-specificity. Instead, we postulate that simultaneous inhibition of two key tumor-dependent growth factor pathways (VEGF and HER) results in global RTK disruption and subsequent tumor growth inhibition. As off-target compensation may provide therapeutic escape from chemotherapy and effectively favor the rapid propagation of more aggressive ovarian tumor cells, the ability of EVRi to inhibit multiple RTK pathways in addition to VEGF and HER is favorable. It is important to note that no other commercially available agents targeting both HER and VEGF currently exist. As result, EVRi is uniquely positioned as a novel dual HER/VEGF inhibitor in ovarian cancer.

Similar to previous reports, targeting either VEGF or HER receptor signaling alone demonstrates evidence of crosstalk (Fig. 2B) (51). It is likely that blockade of either VEGF or HER receptor pathways confer resistance via up regulation of the unblocked pathway, thus resulting in better tumor control when both pathways are blocked simultaneously. Alternatively, it is conceivable that a portion of the activity of EVRI may stem from undefined off-target effects. Nonetheless, given the antitumor activity of EVRI *in vivo*, clinical investigations with a combined HER/VEGFR inhibitor may be an attractive area of development in the treatment of ovarian cancer.

Disclosure of Potential Conflicts of Interest

P. Haluska has commercial research support from and is a consultant/advisory board member for Bristol Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: M.A. Becker, S.C. Harrington, X. Hou, P. Haluska
Development of methodology: M.A. Becker, T. Farzan, S.C. Harrington, P. Haluska
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M.A. Becker, T. Farzan, S.C. Harrington, J. Krempski, S.J. Weroha, X. Hou, K.R. Kalli, T.W. Wong, P. Haluska
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.A. Becker, T. Farzan, S.C. Harrington, S.J. Weroha, X. Hou, T.W. Wong, P. Haluska
Writing, review, and/or revision of the manuscript: M.A. Becker, T. Farzan, S.C. Harrington, S.J. Weroha, X. Hou, K.R. Kalli, P. Haluska
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M.A. Becker, S.C. Harrington, X. Hou, T.W. Wong, P. Haluska
Study supervision: S.C. Harrington, P. Haluska

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Molecular Cancer Therapeutics

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