Human and Mouse VEGFA-Amplified Hepatocellular Carcinomas Are Highly Sensitive to Sorafenib Treatment

Elad Horwitz1, Ilan Stein1,3, Mariacarla Andreozzi5, Julia Nemeth6, Avit Shoham1, Orit Pappo1, Nora Schweitzer12, Luigi Tornillo3, Naama Kanarek1, Luca Quagliata5, Farid Zreik3, Rinnat M. Porat3, Rutie Finkelstein5, Henrik Reuter7, Ronald Koschny11, Tom Ganten11, Carolin Mogler9, Nora Schweitzer12, Luigi Terracciano5, Peter Angel6, Yinon Ben-Neriah1, and Eli Pikarsky1,3

ABSTRACT
Death rates from hepatocellular carcinoma (HCC) are steadily increasing, yet therapeutic options for advanced HCC are limited. We identify a subset of mouse and human HCCs harboring VEGFA genomic amplification, displaying distinct biologic characteristics. Unlike common tumor amplifications, this one seems to work via heterotypic paracrine interactions: stromal VEGF receptors (VEGFR), responding to tumor VEGF-A, produce hepatocyte growth factor (HGF) that reciprocally affects tumor cells. VEGF-A inhibition results in HGF downregulation and reduced proliferation, specifically in amplicon-positive mouse HCCs. Sorafenib—the first-line drug in advanced HCC—targets multiple kinases, including VEGFRs, but has only an overall mild beneficial effect. We found that VEGFA amplification specifies mouse and human HCCs that are distinctly sensitive to sorafenib. FISH analysis of a retrospective patient cohort showed markedly improved survival of sorafenib-treated patients with VEGFA-amplified HCCs, suggesting that VEGFA amplification is a potential biomarker for HCC response to VEGF-A–blocking drugs.

SIGNIFICANCE: Using a mouse model of inflammation-driven cancer, we identified a subclass of HCC carrying VEGFA amplification, which is particularly sensitive to VEGF-A inhibition. We found that a similar amplification in human HCC identifies patients who favorably responded to sorafenib—the first-line treatment of advanced HCC—which has an overall moderate therapeutic efficacy. Cancer Discov; 4(6); 1–14. © 2014 AACR.

Authors’ Affiliations: 1The Lautenberg Center for Immunology; 2Department of Developmental Biology and Cancer Research, IMRIC, Hadassah Medical School, Hebrew University; 3Department of Pathology, Hadassah-Hebrew University Medical Center, Jerusalem; 4Liver Unit, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel; 5Institute of Pathology, University Hospital Basel, Basel, Switzerland; 6Division of Signal Transduction and Growth Control (A100); 7Division of Molecular Genetics (B060); 8Junior Group Molecular Mechanisms of Head and Neck Tumors (A102), German Cancer Research Center (DKFZ), DKFZ-ZMBH Alliance; 9Institute of Pathology, University Hospital Heidelberg; Departments of 10Otolaryngology, Head and Neck Surgery and 11Internal Medicine, University Hospital Heidelberg, Heidelberg; and 12Department of Gastroenterology, Hepatology and Endocrinology, Hannover Medical School, Hannover, Germany

Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

Corresponding Authors: Eli Pikarsky, The Lautenberg Center for Immunology, IMRIC, Hadassah Medical School, Hebrew University, Kiryat Hadassah, P.O.B. 12272, Jerusalem 91120, Israel. Phone: 972-2-675-8202; Fax: 972-2-642-6268; E-mail: peli@hadassah.org.il; and Yinon Ben-Neriah, The Lautenberg Center for Immunology, IMRIC, Hadassah Medical School, Hebrew University, Kiryat Hadassah, P.O.B. 12272, Jerusalem 91120, Israel. Phone: 972-2-675-8718; E-mail: yinonb@ekmd.huji.ac.il
DOI: 10.1158/2159-8290.CD-13-0782
©2014 American Association for Cancer Research.
INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide, with the highest increase rate in North America (1, 2). Sorafenib is the mainstay of therapy for advanced HCC and the only systemic drug that has shown any survival advantage in HCC so far (3, 4). However, patients’ response is modest, and sorafenib treatment is associated with side effects (3–8). Thus, several studies looked for predictive markers for sorafenib response (9–11), yet no such biomarkers have entered the clinical setting. Predictive biomarkers, identifying patient subsets to guide treatment choices, are usually based on distinct pathogenetic mechanisms, and are the cornerstone of personalized medicine (12, 13). Prominent examples include ERBB2 amplification and KRAS mutations that serve as key determinants of treatment with trastuzumab or cetuximab, respectively (14, 15).

Sorafenib is a multikinase inhibitor, the targets of which include BRAF, CRAF, PDGFR2, c-KIT, and the VEGF receptors (VEGFR; refs. 10, 16). Although VEGFRs were postulated as mediators of sorafenib response in HCC, testing for VEGF-A serum levels was not found to be predictive of sorafenib treatment success (10). Moreover, bevacizumab, an antibody against VEGF-A, only shows minimal responses in HCC (17–20). A possible explanation for this is that the mechanism of action of sorafenib is predominantly independent of VEGF-A inhibition. Alternatively, a small subset of patients who can respond to VEGF-A blockers may exist that was underrepresented in bevacizumab studies.

VEGF-A is a master regulator of angiogenesis whose role in tumor vessel recruitment is well established (21). However, additional roles for VEGF-A in tumorigenesis are emerging. VEGF-A was shown to promote tumor cell growth in an autocrine manner, in skin and lung cancer cells expressing VEGFRs (22, 23). Moreover, VEGF-A also has nonangiogenic functions in normal physiology. In liver tissue, VEGF-A elicits hepatocyte proliferation by elevating expression of hepatocyte mitogens in liver sinusoidal endothelial cells (24, 25).

HCC is most commonly the outcome of chronic injury and inflammation, resulting in hepatocyte regeneration and dysregulated growth factor signaling (1, 26). It has become clear that inflammatory signaling pathways can support survival, growth, and progression of cancer. Accordingly, secreted cytokines and effector molecules, which are abundant in the tumor microenvironment, could constitute suitable targets for treatment and primary prevention of HCC (26). Here, we used Mdr2-deficient mice (Mdr2−/−), which develop chronic liver inflammation, eventually leading to inflammation-induced liver tumors similar to human HCC (27, 28), to look for candidate treatment targets modulating the tumor microenvironment that are relevant to human HCC.

RESULTS

Array CGH Reveals Recurrent Gains in the VEGFA Locus Identifying a Molecularly Distinct Tumor Subpopulation

In search of microenvironment-affecting factors whose amplification or deletion plays a role in inflammation-induced HCC development, we applied array-based comparative genomic hybridization (aCGH) to 10 HCCs obtained from 16-month-old Mdr2−/− mice (Supplementary Fig. S1A). We detected several amplifications and deletions, including an amplification in the qB3 band of murine chromosome 17 (Chr17qB3; Supplementary Table S1), encoding among others the gene for VEGF-A. Genomic amplification of Vegfa was of interest as it is a cytokine gene that can modulate several components of the tumor microenvironment and induce liver cell growth (24, 25). To determine the frequency of this amplification, we tested a larger cohort of Mdr2−/− tumors by quantitative PCR (qPCR) of tumor DNA (Supplementary Fig. S1B) and chromogenic in situ hybridization (CISH; Fig. 1A); 13 of 93 (~14%) HCCs harbored this amplicon. To map the minimal amplified region of this amplicon, we used DNA qPCR directed at several loci along murine chromosome 17 in a cohort of tumors bearing this amplification (Fig. 1B). We found that the common proximal border lies between 43.3 and 48.5 mega base pairs (Mb) from the chromosome 17 start. This minimal amplified region spanned 53 genes (Supplementary Table S2).

Amplification of human Chr6p21 (the region syntenic for murine Chr17qB3) and whole chromosome gains were previously reported in several whole-genome analyses of human HCC with a frequency ranging between 7% and 40% (29–33). Accordingly, through FISH, we found VEGFA amplifications and chromosome 6 polysomies in 11% of human HCCs we tested (21 of 187; Fig. 1C; Supplementary Table S3). To elucidate the tumor relevance of these chromosomal gains, we analyzed the expression of mouse Chr17qB3 amplicon genes in amplicon-positive (herein Amppos) and amplicon-negative (Ampneg) tumors. Matched increases in mRNA and DNA levels were found for Vegfa, Tjp1, and Xpo5 (Fig. 1D and Supplementary Fig. S2A). We further found a correlation between Chr17qB3 amplification and VEGF-A protein levels in both tumor extracts and serum (Fig. 1E and Supplementary Fig. S2B). Furthermore, double immunostaining for VEGF-A and E-cadherin in Amppos tumors showed that the tumor cells are indeed the main origin of VEGF-A in these tumors (Fig. 1F). Thus, amplification of murine Chr17qB3 is a recurrent event in HCC associated with an elevated expression of several resident genes, including Vegfa.

Amppos Tumors Display Distinct Histologic Features

Tumor cell proliferation is a strong and consistent marker of poor prognosis in human HCC (34). Bromodeoxyuridine (BrdUrd) immunostaining revealed that Amppos mouse HCCs displayed a 2-fold higher proliferation index compared with Ampneg tumors (Fig. 2A and B, top). No differences were noted in apoptosis (Supplementary Fig. S2C) or in the presence of neutrophils or fibroblasts (data not shown). Other histologic features, differing between Amppos and Ampneg mouse HCCs, include a 6-fold higher vessel density (Fig. 2A and B, middle) and 4-fold higher macrophage content (Fig. 2A and B, bottom). Moreover, several signature genes of tumor-associated macrophages (TAM), including those encoding Arginase 1, TGFβ, and YM1 (35, 36), were elevated in the Amppos group (Fig. 2C), whereas markers of classically...
Figure 1. A recurrent gain in the VEGFA locus identifying a molecularly distinct tumor subpopulation. A, representative photomicrographs of CISH using probes specific for the murine Chr17qB3. B, DNA qPCR analysis using primers specific for different loci on the qB3 arm of chromosome 17. Each vertical line represents a single Amp\(^{\text{pos}}\) tumor. The thin line represents nonamplified regions, and the thick line represents amplified regions. The list includes several of the residing genes (a full list is available as Supplementary Data). C, representative photomicrographs of FISH of human HCC using Chr6p12 probe (red) and chromosome 6 centromere probe (green). D, qPCR analysis of the mRNA levels of murine genes encoded on the amplified region. Each dot represents a different tumor. The cross line signifies geometric mean (n.s., not significant; **, \(P < 0.01\); ***, \(P < 0.0001\)). E, qPCR and ELISA analyses of VEGF-A performed on extracts of wild-type (WT) livers, Amp\(^{\text{neg}}\), and Amp\(^{\text{pos}}\) Mdr2\(^{-/-}\) tumors in matching pairs show a correlation between the increase in mRNA and protein levels. F, confocal microscopy images of an Mdr2\(^{-/-}\) Amp\(^{\text{pos}}\) tumor immunostained for E-cadherin and VEGF-A. Hoechst 33342 marks nuclei. Scale bar, 20 μm.
Figure 2. Amp\textsuperscript{neg} tumors are a distinct tumor subpopulation. A, representative IHC photomicrographs for BrdUrd, vWF, and F4/80. Scale bar, 50 μm (BrdUrd) and 100 μm (vWF and F4/80). B, IHCs were quantified using automated image analysis (n ≥ 7; *, P < 0.01; **, P < 0.05). C, qPCR analysis of tumor-associated (protumorigenic) and classically activated (antitumorigenic) macrophage markers. Each dot represents a different tumor. The cross line signifies geometric mean (n.s., not significant; ***, P < 0.001). D, immunofluorescent stain for MRC1 on Amp\textsuperscript{neg} and Amp\textsuperscript{pos} tumors. Scale bar, 40 μm. E, quantification of MRC1 immunofluorescence. Bars represent geometric mean; **, P < 0.01.
activated macrophages, including TNFα, inducible nitric oxide synthase (iNOS), CXCL10, and IL12p40, were similar in both tumor groups (Fig. 2C and Supplementary Fig. S3A). Along with these, immunofluorescent staining for the protumorigenic macrophage marker MRC1 revealed a higher presence of MRC1-expressing cells in Amppos tumors (Fig. 2D and E). Of note, VEGF-A was shown to act as a chemoattractant for naïve myeloid cells, which facilitate the generation of new blood vessels (37). Together, these data signify Amppos tumors as a distinct subgroup of HCCs characterized by enhanced presence of specific microenvironmental components and increased proliferation rate.

Histologic analysis of human HCCs revealed several characteristic traits of HCCs harboring VEGFA expression. A higher incidence of vascular invasion was found in the Amppos group (9 of 20 Amppos tumors vs. 1 of 20 Ampneg tumors; \(P < 0.01\); Supplementary Table S4). A lower incidence of fibrosis within tumor tissue was found in the Amppos group as well. Few additional traits nearly reaching statistical significance were identified (Supplementary Table S4). We found no differences in several clinical characteristics of HCC including underlying disease, gender, or tumor size (Supplementary Fig. S4A–S4C). Altogether, these results indicate that in both murine and human HCCs, tumors that harbor genomic gains in the VEGFA locus are distinct from those that do not.

**Macrophase–Tumor Cell Cross-talk in Amppos Tumors**

Previous studies have delineated hepatocyte-endothelial cross-talk taking place in non-neoplastic liver, wherein VEGF-A stimulates endothelial cells to secrete several mitogens including hepatocyte growth factor (HGF; refs. 24, 25). We hypothesized that Amppos HCCs exploit this interaction for promoting tumor cell proliferation. Following this notion, we detected a 3-fold elevation of Hgf mRNA levels in Amppos versus Ampneg mouse tumors (Fig. 3A). We did not find significant changes in other angiocrine-produced molecules (24, 25)—Wnt2, IL6, and heparin binding EGF-like growth factor (HB-EGF; data not shown). Immunostaining for endogenous HGF expression only in Amppos tumors, exclusively in the non-neoplastic stromal cells (Fig. 3B). Immunofluorescent staining for von Willebrand Factor (vWF), F4/80, and HGF suggested that macrophages are the major cell type expressing HGF (Fig. 3C).

To understand the VEGF-A–HGF relationship, we isolated hepatocytes and macrophages from Mdr2\(^{-/-}\) livers at the age of 8 months (Fig. 3D and Supplementary Fig. S3B), a time point signified by marked dysplasia, yet no overt HCC formation (27). mRNA profiling of these fractions showed that the genes encoding VEGFRs (FLT1 and KDR) and coreceptors [Neuropilin (Nrp)1 and 2] were more abundant in hepatocytes whereas the HGF receptor (c-MET) was more abundant in hepatocytes (Fig. 3E). This aligns with previous work showing that hepatocytes are innate to direct activation with VEGF-A (24). Immunostaining for KDR in Amppos tumors demonstrated that its expression in these tumors was restricted to non-neoplastic cells (Fig. 3F). This correlated with a modest increase in mRNA levels of both Kdr and Flt1 in total tumor lysates, which was comparable with the increase in mRNA levels of recruited macrophage and endothelial markers Mvr1 and Cd105, respectively (Fig. 3G). Recapitulating this interaction in vitro, we treated peritoneal macrophages with recombinant VEGF-A and detected an increase in Hgf mRNA levels (Fig. 3H). This raises the possibility that VEGF-A in Amppos tumors does not provide autocrine signals to hepatocytes, but rather acts through manipulation of the microenvironment to induce HGF secretion.

**VEGF-A Increases Cellular Proliferation in HCC**

To prove the functional role of VEGFA in this genomic amplification, we set out to inhibit VEGF-A in Mdr2\(^{-/-}\) tumors. To this end, we injected intravenously adenoviral vectors encoding GFP alone or GFP and the soluble VEGFR1 (sFLT), a potent inhibitor of VEGFA (38), into 56 tumor-harboring Mdr2\(^{-/-}\) mice of ages 14 to 18 months and sacrificed them 10 days following injection (Supplementary Fig. S5A). Vgfα amplification status was determined after sacrifice by both DNA qPCR and Vgfα mRNA expression (Fig. 4C and data not shown). Liver damage, measured through plasma aspartate aminotransferase (AST) activity, was similar in all groups (Supplementary Fig. S5B). Immunostaining for BrdUrd, Ki67, and phosphorylated histone H3 (pHH3) revealed that blocking VEGF-A markedly inhibited tumor cell proliferation in Amppos tumors, but not in Ampneg ones (Fig. 4A and B and Supplementary Fig. SSC and SSD). This decrease in proliferation was accompanied by reduced Hgf mRNA levels (Fig. 4C). Treatment with adenovirus encoding GFP alone did not induce any change in either group. Macrophage infiltration and protumorigenic macrophage expression profile did not decrease following the sFLT treatment (Supplementary Fig. SSC and SSD and data not shown). Macroscopic and histologic analyses revealed multiple foci of coagulative necrosis only in sFLT-treated Amppos tumors (3 of 6 vs. 0 of 10 in Ampneg; \(P < 0.05\); Fig. 4D). In these specific tumors, we also found an elevation of the hypoxia-inducible factor-1α (HIF1α) target genes Glut1 and Pkg1 (Fig. 4C), indicative of tissue hypoxia. Immunostaining for vWF revealed a trend of decrease in vasculature only in VEGF-A–blocked Amppos tumors, particularly in the hypoxic tumors (Supplementary Fig. SSC and SSD). These data denote Amppos tumors as hypersensitive to direct inhibition of VEGF-A.

**Overexpression of VEGF-A in HCC Cells Increases Proliferation Only In Vivo**

To further substantiate the tumor–stroma relationship with respect to VEGF amplification, in particular hepatocyte VEGF-A eliciting a macrophage HGF–heterotypic circuit in tumor growth, we injected human Hep3B HCC cells transduced with a lentivirus overexpressing human VEGF-A into immunodefi cient mice (Supplementary Fig. S6A). The in vivo growth rate of VEGF-A–overexpressing cells was higher than control vector–transduced cells (Fig. 5A and Supplementary Fig. S6B). This correlated with higher proliferation rate and increased HGF expression (Fig. 5B–D and Supplementary Fig. S6C and S6D), phenocopying the Amppos Mdr2\(^{-/-}\) HCCs. Supporting the non–cell-autonomous role, VEGF-A overexpression did not affect the in vitro growth rate of Hep3B cells (Fig. 5E). We also found no difference in expression of the hypoxia markers prolyl hydroxylase domain 3 (PHD3) and lactate dehydrogenase A (LDHA) in these xenografts, lending further support to a nonangiogenic role for VEGF-A in HCC (Supplementary Fig. S6E). Immunofluorescence confirmed VEGF-A expression in human HCC cells transduced with a lentivirus overexpressing human VEGF-A.
transduced tumor cells and revealed HGF expression in macrophages (Supplementary Fig. S7A).

Next, we generated single-cell suspensions from VEGF-A-overexpressing or control Hep3B ZsGreen-labeled xenografts and isolated macrophages (ZsGreen-CD45^+ F4/80^+) and endothelial cells (ZsGreen-CD45^− Meca32^+) using FACS sorting. qPCR analysis of the macrophage-specific and endothelium-specific genes, 

\[ 	ext{Msr1} \quad \text{Cd105} \]

respectively, confirmed successful separation of cell populations (Supplementary Fig. S7A). Although VEGF-A and control tumor groups did not show significant differences in the TAM genes Tgfβ, Ym1, Tnfα, and Nos2 (Supplementary Fig. S7C), we found a 2-fold increase in Hgf expression in macrophages, but not endothelial cells, isolated from VEGF-A-overexpressing tumors (Fig. 5F). Thus, VEGF-A overexpression by HCC cells is sufficient to induce upregulation of HGF, mostly in...
Figure 4. VEGF-A inhibition impedes proliferation in Amppos tumors. Mdr2+/− mice were treated with adenovectors expressing either GFP alone or GFP and sFLT for 10 days. A, representative photomicrographs of IHC for BrdUrd. Tumor-infiltrating cells remain proliferative. Scale bar, 100 μm. B, BrdUrd immunostaining was quantified using automated image analysis (n ≥ 6; *P < 0.05). C, mRNA qPCR analysis of the indicated genes in Amppos and Amppos tumors treated with the indicated adenovectors. Each dot represents a different tumor. The cross line signifies geometric mean (**P < 0.0001). D, left, histologic section stained with H&E depicting necrosis, representing three out of the six sFLT-treated Amppos tumors. Scale bar, 500 μm. Right, a macroscopic picture of a tumor with hemorrhagic necrosis. Scale bar, 0.5 cm.

Figure 5. VEGF-A overexpression enhances tumor cell proliferation. Immunodeficient mice were subcutaneously injected with Hep3B cells transduced with control vector or with a human VEGF-A vector. A, growth curve of xenografts transduced with control vector (black line) or with VEGF-A lentivector (gray line). Tumor volumes were measured topically with a caliper (*P < 0.05; **P < 0.01). B, representative photomicrographs of IHC for BrdUrd in control or VEGF-A lentivirus-transduced xenografts. Scale bar, 100 μm. C, BrdUrd immunostaining was quantified using automated image analysis (n ≥ 3; *P < 0.05; a representative experiment of two performed is shown). D, mRNA qPCR analysis in control or VEGF-A lentivirus-transduced xenografts. Bars represent geometric mean (n ≥ 4; *P < 0.05; **P < 0.01). E, XTT in vitro proliferation assay of the indicated lentivirus-transduced cultured cells. F, expression of Hgf in macrophage and endothelial fractions from tumors overexpressing VEGF-A and controls determined by qPCR. Bars represent geometric mean; n ≥ 3; *P < 0.05; ND, not detected—amplification did not occur in any of the sample’s wells.
macrophages, and leads to increased proliferation of tumor cells.

**Profiling Proangiogenic-Factor Expression in Mdr2−/− HCC**

As Ampneg tumors did not respond to sFLT treatment, we profiled other proangiogenic factors that can support tumor vascularization. mRNA qPCR analysis of the genes encoding angiopoietin 1 and 2, angiopoietin-like 2, FGF 1 and 2, platelet-derived growth factor (PDGF)-A, -B, and -C, placent al growth factor (PLGF), and VEGF-B revealed that several of these factors were overexpressed in Mdr2−/− HCCs compared with normal livers, irrespective of the amplicon status. Notably, Pdgfc levels were significantly higher (2.4-fold) in Ampneg compared with Amppos tumors (Fig. 6). This is in line with a previous report showing that PDGF-C can promote angiogenesis in a VEGF-A-independent manner (39) and could provide a plausible explanation for the lack of effect on vessel density in Ampneg tumors in response to sFLT.

**Murine Amppos Tumors Are Uniquely Sensitive to Sorafenib**

Sorafenib is the only systemic drug showing a clinical advantage in patients with advanced HCC who are not eligible for local therapies and is a first-line treatment for these patients (3). As sorafenib inhibits VEGFRs and BRAF—a downstream effector of both VEGFRs and the HGF receptor c-MET (21, 40, 41)—we tested whether sorafenib may have a selective advantage in Amppos tumors. We treated 58 Mdr2−/− mice ages 14 to 18 months with sorafenib or vehicle alone for 3 days, after which they were sacrificed and tumor tissue was analyzed (experimental design depicted in Supplementary Fig. S5A). Amplification status was assessed after sacrifice by DNA qPCR and verified by Vegfa mRNA expression (Fig. 7C and data not shown). Immunostaining for BrdUrd and pH3 demonstrated decreased proliferation in sorafenib-treated mice in Amppos but not Ampneg tumors (Fig. 7A and Supplementary Fig. S8A and S8B). Similar to VEGF-A inhibition, Hgf levels were decreased only in sorafenib-treated Amppos tumors (Fig. 7C). Although no effects were observed on tumor macrophage density (Supplementary Fig. S8A and S8B), expression of the TAM marker TGFβ was decreased in sorafenib-treated Amppos tumors (Supplementary Fig. S8C). Blood vessel density changes or signs of hypoxia were absent (Supplementary Fig. S8A–S8C), possibly due to the short treatment duration, implying that the early inhibitory effect of sorafenib in VEGF-A-amplified tumors may be independent of angiogenesis.

---

**Figure 6.** Angiogenic factors elevated in murine Mdr2−/− HCC. mRNA qPCR analysis of Mdr2−/− tumors for the indicated angiogenesis regulators. Values shown are fold over normal livers’ average. Each dot represents a different tumor. The cross line signifies geometric mean, significance marks immediately above each group refer to comparison with normal livers. Topmost significance marks represent the comparison between Ampneg and Amppos tumors (n = 4 normal liver; n ≥ 6 in tumor groups; n.s., not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001). PDGF, platelet-derived growth factor.
Figure 7. Amp<sup>pos</sup> tumors are uniquely sensitive to sorafenib. A, representative BrdUrd immunostains. Scale bar, 100 μm. B, BrdUrd immunostaining was quantified using automated image analysis (n ≥ 6; *, P < 0.05). C, qPCR analysis of Amp<sup>neg</sup> and Amp<sup>pos</sup> tumors treated as indicated. Each dot represents a different tumor. The cross line signifies geometric mean (n.s., not significant; *, P < 0.01). D, growth curves of xenografts transduced with control (dashed lines) or VEGF-A (solid lines) lentivectors, treated daily with sorafenib (gray lines) or nontreated (NT, black lines). Tumor volumes were measured with a caliper (n ≥ 6; *, P < 0.05). E, Kaplan-Meier curves showing survival of resected HCC patients negative (n = 96) or positive (n = 14) for VEGFA gain (p log-rank > 0.05). F, Kaplan-Meier curves showing survival of patients with HCC treated with sorafenib, negative (n = 47, 10 months median) or positive for VEGFA gain (n = 7, median undefined; p log-rank = 0.029). G and H, genomic gains in VEGFA promote tumorigenesis through the microenvironment. G, an increase in VEGFA gene copy number in liver tumor cells leads to elevated VEGF-A secretion. VEGF-A modulates the tumor microenvironment in favor of tumor cell growth through several modes: (i) recruitment of TAMs expressing the mitogen HGF and (ii) activating the liver endothelium to secrete angiocrine factors and enhance the tumor blood supply. H, inhibition of VEGF-A through soluble receptor or sorafenib results in decreased HGF signaling and blood supply, impeding tumor growth.
VEGFA Amplification in HCC

Amp<sup>+</sup> tumors did not show any measurable response to sorafenib. In addition, we treated mice bearing Hep3B xenografts, with or without VEGF overexpression, with sorafenib for 10 days. Similar to the Mdr<sup>−/−</sup> HCCs, this treatment markedly reduced growth, proliferation, and HGF expression in VEGF-A-overexpressing HCCs but did not affect control HCCs (Fig. 7D and Supplementary Fig. S9A–S9D). Despite the longer duration of treatment, we still could not detect changes in macrophage or blood vessel densities (Supplementary Fig. S10A–S10C). Of note, a differential response to sorafenib was not evident in vitro, emphasizing the importance of microenvironmental factors (Supplementary Fig. S10D).

**Beneficial Effect of Sorafenib Treatment in Human Patients with HCCs Bearing VEGFA Gains**

Noting the predictive potential of Vegfa gains in the mouse model, we analyzed samples from patients with HCC who underwent tumor resection. This retrospective cohort was collected from three different centers. To assess the correlation between VEGFA gain and survival, we analyzed human tumor samples by FISH (Fig. 1C). Survival of patients with HCC who did not receive sorafenib was independent of VEGFA status (Fig. 7E). However, markedly improved survival was seen in the VEGFA-gain group compared with the non-gain group in sorafenib-treated patients (indefinable median survival from sorafenib treatment start and 11 months, respectively; p log-rank = 0.029; Fig. 7F). Taken together, our mouse data and retrospective analysis of a human cohort imply that VEGFA gains correlate with a particularly beneficial response to sorafenib, and possibly other VEGF-A inhibitors, in HCC.

**DISCUSSION**

Using a mouse model of inflammation-induced HCC, we identified and characterized a unique group of HCC in humans and mice. These tumors are defined by genomic gains of a region encompassing VEGFA, and are distinct in histologic appearance, rate of proliferation, and microenvironmental content. We delineated cytokine-based heterotypic cross-talk between malignant Amp<sup>+</sup> hepatocytes and tumor stromal cells. Importantly, we showed that mouse Amp<sup>+</sup> tumors are uniquely sensitive to VEGF-A inhibition and to sorafenib. A retrospective analysis of human HCCs indicates that genomic gains of VEGFA can predict response to sorafenib.

The Mdr<sup>−/−</sup> model of inflammation-induced HCC yields primary tumors each holding specific genetic changes. Therefore, we denote it as a sound platform to test the protumorigenic effects of recurring changes in an unbiased manner. Moreover, the inflammatory background in this model is particularly relevant for studying tumor-microenvironment interactions in clinically relevant settings. Previous work showed that systemic elevation of VEGF-A induces proliferation of normal hepatocytes through factors secreted from endothelial cells (24). Although hepatocytes are inert to VEGF-A, liver sinusoidal cells respond to VEGF-A with counter-secretion of HGF (24). Furthermore, in regenerating livers, hepatocyte proliferation was shown to depend on VEGF-A-induced expression of HGF from endothelial cells (25). Here, we show that recurring genomic gains in VEGFA, detected in a subset of HCCs, can promote tumor growth through a similar cell–cell interaction module.

TAMs are key players in tumor progression and are known to modulate invasion, angiogenesis, immune response, and metastasis (36). TAMs are characterized by a specific phenotype, distinguished by a unique expression signature. Previous studies have shown that VEGF-A recruits myeloid cells that play an active part in vessel growth processes (37, 42). In agreement, we observed that Amp<sup>+</sup> tumors harbor higher numbers of TAMs compared with Amp<sup>−/−</sup> tumors and detected features of protumorigenic macrophages. Our findings therefore raise the interesting possibility that the VEGFA amplicon contributes to the inflammatory microenvironment, which supports developing tumors.

Our data suggest that VEGFA genomic gains facilitate tumor development by several different modes: (i) providing a microenvironment rich in TAMs; (ii) promoting proliferation via stroma-derived HGF secretion (and possibly other cytokines as well); and (iii) enhancing tumor angiogenesis. Interestingly, all these modes entail heterotypic cellular interactions, distinguishing this particular genomic gain from other studied amplicons (43). We maintain that the unique sensitivity of tumors with VEGFA gains to VEGF-A blockade stems from these multiple protumorigenic functions of VEGFA-A (Fig. 7G and H).

An amplicon spanning VEGFA was noted in several different human cancers (29–31, 33, 43–51). A linear correlation was found between mRNA levels of VEGFA and extent of amplification in human HCC (29). Amplifications in the VEGFA locus and juxtaposed regions were associated with advanced-stage HCC (30). In colorectal carcinoma and breast cancer, this amplification was found in correlation with vascular invasion and shorter survival (46, 51). Cumulative analysis of these reported human studies shows that gains and amplifications of VEGFA are found in 7% to 30% of human HCCs (29–33). Our interventional studies in the Mdr<sup>−/−</sup> model indicate that Vegfa is a major driver of this amplicon, which minimally harbors 53 genes in Mdr<sup>−/−</sup> murine tumors and 11 genes in humans (42). Xenograft experiments reveal that overexpression of VEGF-A in a human HCC cell line is sufficient to upregulate HGF expression and increase tumor cell proliferation in vitro and that the tumor growth advantage gained through this overexpression can be negated by sorafenib. Nevertheless, we cannot completely exclude the contribution of other amplicon genes to tumorigenesis.

The first line of treatment for advanced HCC is the multi-kinase inhibitor sorafenib, which prolongs median survival by 10 to 12 weeks (3, 4). The response to sorafenib seems to be variable, and treatment is associated with significant side effects (3–8). Importantly, there are no clinically applied biomarkers for predicting sorafenib response in HCC (10). Among the multiple targets of sorafenib (16) are VEGFRs and BRAF—a downstream effector of both VEGFRs and the HGF receptor c-MET (21, 40). Indeed, in line with our mouse results, sorafenib treatment in patients led to a decrease in serum HGF (10). Unlike most tumors, advanced HCC is usually diagnosed and treated without obtaining tumor tissue, making it difficult to establish tissue-based predictive biomarkers. On the basis of our small-scale retrospective study, we show that VEGFA gains may predict response to
sorafenib in HCC, thus enabling the tailoring of treatment to only those patients who may benefit from this side effect-prone therapy. Notably, the same amplification was also found in lung, colorectal, and breast cancers (44–48); thus, it is plausible that similar considerations could be applied to other tumors harboring VEGFA gains.

METHODS

Human Tissue Samples and Tissue Microarray

Human HCC tissues were obtained from resected patients from the institutes of Basel University Hospital (Basel, Switzerland), Han- nover Medical School Hospital (Hannover, Germany), and Heidel- berg University Hospital (Heidelberg, Germany). Clinical information included age at diagnosis, tumor diameter, gender, and survival time information. Examination of tumor hematolin and eosin (H&E) sections was performed by an expert liver pathologist (O. Pappo). Samples from Heidelberg were also reviewed by Heidelberg patholo- gists (C. Mogler and P. Schirmacher). The study was approved by each of the institutions’ ethics committees—numbered 206/05 (Heidelberg), 660-2010 (Hannover), and EKB820 (Basel). Required cohort size was computed by power analysis to yield a power of at least 90% with an α value of 0.05. Construction of the tissue microarray (TMA) was performed as follows: tissue samples were fixed in buffered 10% formaldehyde and embedded in paraffin. H&E-stained sections were made from each selected primary block (named donor blocks) to define representative tissue regions. Tissue cylinders (0.6 mm in diameter) were then punched from the region of the donor block with the use of a custom-made precision instrument (Beecher Instruments). Afterward, tissue cylinders were transferred to a 25 mm × 35 mm paraffin block to produce the TMAs. The resulting TMA block was cut into 3-μm sections that were transferred to glass slides by use of the Paraffin Sectioning Aid System (Instrumedics). Sections from the TMA blocks were used for FISH analysis.

Mice

Male and female Mdr2+/− mice on FVB background were held in specific pathogen-free conditions. Experiments on Mdr2+/− mice were performed in cohorts of 10 to 20 mice each time; results show the combined data from at least four different experiments. Wild-type (WT) controls were age-matched FVB mice. Mouse body weights during the experiments were 30 to 45 g. Sorafenib (Xingcheng Chemp- harm Co., Ltd.) was administered daily (50 mg/kg) by oral gavage. Cremophor EL, Sigma)ethanol/water (1:1) was used as a vehicle. Two hours before sacrifice, mice were injected with 10 μL BrdUrd (Cell Proliferation labeling reagent; Amersham) per 1 g body weight. Mice were anesthetized with ketamine and xylazine and the liver was perfused via the heart with PBS–Heparin solution followed by 4% formaldehyde. Following perfusion, livers were removed and sub- jected to standard histlogic procedures. Xenograft experiments were performed by subcutaneous injection of transduced Hep3B cells (2.5 × 10⁶) suspended in 100 μL PBS and 100 μL Matrigel (Becton Dickin- son) into NOD/SCID mice. Tumor volumes were assessed by external measurement with callipers. All animal experiments were performed in accordance with the guidelines of the institutional committee for the use of animals for research. In all mouse experiments, the different groups were housed together in the same cages.

Viral Vectors and Cultured Cells

Adenoviral vectors encoding GFP or GFP and sFLT—a kind gift from David Curiel (Washington University, St. Louis, MO) and Yosef Havi (Hadassah Hospital, Jerusalem, Israel)—were prepared in GH354 cells using standard procedures. A titer of 10⁶ transducing units was injected into mice tail veins. Mice whose livers did not yield minimal 60% adenovector transduction efficiency (by tissue staining) were excluded. Lentiviral-based vectors were prepared by subcloning the PCR products of the human VEGFA165 gene [from cDNA of decidual natural killer (NK) cells; a kind gift from Ofer Mandelboim, Hebrew University, Jerusalem, Israel] into the pSfC-B plasmid using the Strata- Clone Kit (Stratagene), subsequently digested with BamHI and NotI and subcloned into the self-inactivating lentiviral vector PHAGE (gift of Gustavo Mostoslavsky, Boston University School of Medicine, Boston, MA) digested with BamHI and NotI. Lentivectors were produced by cotransfection of the backbone vector plasmid with the gag-pol and PMD2.G plasmids and using standard procedures. Hep3B cells (obtained from the ATCC) were grown in Dulbecco’s Modified Eagle Medium (DMEM; 10% FBS). Cells were tested free of Mycoplasma before transduction and injection. Lentivector transduction efficiency was assessed by fluorescent microscopy and was estimated as 80%. In vitro proliferation was determined through XTT assay (Biological Industries) using the manufacturer’s protocol. Murine recombinant VEGF-A (R&D Systems) was used at the concentration of 100 ng/mL.

IHC, Immunofluorescence, and ELISA

Antibodies used for tissue immunostaining throughout the work were—vWF (dilution at 1:300; Dako), pH3 (1:800; Upstate), cleaved caspase-3 (1:200; Cell Signaling Technology), F4/80 (1:300; Serotek), HGF (1:100; R&D Systems), BrdUrd (1:200; NeoMarkers), KDR (1:800; Cell Signaling Technology), Ki67 (1:100; NeoMarkers), E-cadherin (1:100; Cell Signaling Technology), and VEGF (1× as supplied; Spring). IHC was performed on 5-μm paraffin sections. Antigen retrieval was performed in a decloaking chamber (Bioscience Medical) in citrate buffer for all antibodies except vWF and F4/80, for which retrieval was performed with promise (Sigma). Horseradish peroxidase (HRP)-conjugated secondary antibodies for all IHC antibodies used were Histone (Nichirei Biosciences), except for anti- mouse–derived antibodies that were detected with Envision (Dako). 3,3′-Diaminobenzidine (DAB; Lab Vision) was used as a chromogen. Immunohistochemical stainings were quantitated when indicated using an Ariol SL-50 system (Applied Imaging). For quantification of nuclear immunostaining, the ki-sight module of the Ariol-SL50 robotic image analysis system was applied. This system designates classifiers for positive (red-brown) and negative (azure) nuclei defined by color intensity, size, and shape. Each tumor cell nucleus (distinguished by morphology) was designated as either positive or negative by these parameters. The fraction of positive cells was calculated from counting at least five randomly selected fields in each tumor.

Immunofluorescence was performed on snap-frozen tissue embed- ded in OCT gel (Sakura Finetek) and sectioned to 8-μm slices. Slides were incubated at 37°C and fixed with both acetone and 4% paraformaldehyde sequentially. Fluorophore-conjugated secondary antibodies used were donkey anti-goat Alexa Fluor 647 (Invitro- gen), donkey anti-rabbit Cy2/Cy5, donkey anti-mouse Cy3, and goat anti-rat Cy3 (The Jackson Laboratory). Hoechst 33342 was used as a nuclear marker (Invitrogen). Antibodies used for flow FACS sort- ing were CD45-Pacific blue, F4/80-PE (both 1:50; BioLegend), and Meca32-biotin (1:50; BioLegend) used with streptavidin APC-Cy7 (BD Biotemc). Flow cytometry–based cell sorting was performed in a FACSaria III cell sorter (BD Biosciences). VEGF-A ELISA was performed using Quantikine mouse ELISA kit (R&D Systems).

DNA In Situ Hybridizations

Probes for CISH analysis of mouse tumors were prepared from the BAC clones RP24-215A3 for the murine Chromosome 17 (BACPAC resources center). BAC clones were labeled with DIG using Nick-Translation mix (Roche). Mouse Cot-1 DNA (Invitrogen) and soni- cated murine genomic DNA were added to the probe for background block. Tissues were prepared by boiling in pretreatment buffer and digestion with pepsin (Zymed). Hybridization was performed at 37°C.
overnight after denaturation at 95°C for 5 minutes. The SpvT-Light Detection Kit (Invitrogen) was used for anti-DIG antibody and HRP-conjugated secondary antibody.

FISH analysis for human HCCs was performed as follows. The genomic BAC clone RPCB753M0921Q (imaGENES), which covers the human VEGFA gene region, was used for preparation of the FISH probe. BAC-DNA was isolated using the Large-Construct Kit (Qiagen) according to the instructions of the manufacturer. Isolated BAC-DNA (1 µg) was digested with Alul restriction enzyme (Invitrogen) and labeled with Cy3-UTP (GE Healthcare) using the BioPrime Array CGH Kit (Invitrogen). The labeling reaction was assessed by NanoDrop. Labeled DNA was purified with the FISH Tag DNA Kit (Invitrogen). TMA's and whole-tissue sections were deparaffinized in xylene for 20 minutes and subsequently washed with 100%, 96%, and 70% ethanol followed by a wash with tap water (2 minutes each step). Slides were air dried at 75°C for 3 minutes. Slides were then boiled in pretreatment buffer [70% formamide, 2x saline-sodium citrate buffer (SSC)] at 100°C for 15 minutes followed by a wash with tap water. Tissue was then subjected to Proteinase K (Sigma) treatment at 37°C for 70 minutes followed by a wash in tap water (2 minutes). Dehydration of slides was performed by serial immersion of slides in 70%, 96%, and 100% ethanol (2 minutes each step). Slides were then air dried at 75°C for 3 minutes. The FISH probe was applied and slides were sealed with rubber cement. Following a denaturation step (10 minutes at 75°C), slides were incubated overnight at 37°C. Slides were washed in Wash Buffer (2x SSC, 0.3% NP40, pH 7.7–8.0) and counterstained with 4',6-diamidino-2-phenylindole (DAPI) I solution (1,000 ng/mL; Vysis Abbott Molecular). As reference, a Spectrum Green-labeled chromosome 6 centromere probe (Vysis Abbott Molecular) was used. Images were obtained with a Zeiss fluorescence microscope using a 63x objective (Zeiss) and the Axiovision software (Zeiss).

FISH results were evaluated according to (i) absolute VEGFA gene copy number and chromosome 6 copy number and (ii) VEGFA gene/chromosome 6 copy number ratio. The following classification was used: not amplified—VEGFA/Chr6 ratio of less than 1.8; equivocal/borderline—VEGFA/Chr6 ratio between 1.8 and 2.2; and amplified—VEGFA/Chr6 ratio higher than 2.2, as proposed by the ASCO/CAP (American Society of Clinical Oncology/College of American Pathologists) guidelines for HER2 amplification in breast cancer. High polysomy was defined as >3.75 copies of the CEP6 probe, low polysomy was defined as cases displaying between 2.26 and 3.75 copies of the CEP6 probe (52,53). All cases displaying either amplification or polysomy were collectively defined as VEGFA gain. FISH quantification and classification were done by an expert molecular pathologist who had no access to the clinical data (L. Tornillo).

aCGH and qPCR

Genomic DNA was isolated using the QiAGEN DNAeasy Tissue Kit. Samples were hybridized to mouse CGH 60-mer oligonucleotide microarrays (Agilent Technologies), washed, and scanned according to the Agilent Technologies’ instructions. Data were analyzed using Feature Extraction software version 8.1 (Agilent). GeneSpring GX V7.3.1, and CGH Analytics V3.4.27 (Agilent) software. RNA was extracted from tissues by mechanical grinding in TriReagent (American) and classifying in which anthine phosphoribosyltransferase (Ppia) combined were used as reference genes in all murine analyses except for the hepatocyte versus macrophage comparison in which U6, B2m, and Tbp were additionally applied. Primers detecting the murine chromosome 17 pericentric region were used as references in DNA qPCR analyses.

Cell Separation

Hepatocytes and macrophages were isolated from Mdr2−/− mice livers essentially as described by Kamimura and Tsukamoto (54). Briefly, livers were digested enzymatically with pronase and collagenase (Sigma) by in situ perfusion. Hepatocytes were isolated by centrifugation at 50 × g for 2 minutes, and after three washes were frozen immediately in liquid nitrogen for RNA preparation. Nonparenchymal cells were pelleted by centrifugation at 150 × g for 8 minutes, laid on top of a four-density gradient of Larcoll (Sigma) and centrifuged at 20,000 rpm at 25°C for 30 minutes using a SW41Ti rotor (Beckman). Liver macrophages were recovered from the interface between 8% and 12% Larcoll, washed three times and immediately frozen in liquid nitrogen. Purity of hepatocytes and macrophage fractions was determined by H&E staining of cytospin preparations and always exceeded 90%. Dissociation of cells from tumor xenografts was performed using the gentleMACS dissociator (Miltenyi Biotec) according to the manufacturer’s protocol.

Statistical Analysis

Data were analyzed using a paired two-tailed Student t test at P < 0.05. Histologic differences were analyzed using the Pearson χ test at P < 0.05. Data were processed using Microsoft Excel 2007. Graphs were generated using either GraphPad Prism 5.0 or Excel software. Kaplan–Meier calculations and graphs were performed in GraphPad Prism 5.0. Log-rank (Mantel–Cox) was used to determine survival P value. Throughout the work, error bars represent 1 SEM.

Disclosure of Potential Conflicts of Interest

R. Koschky has received travel grants from Bayer Company. E. Pikarsky has filed for a provisional patent application based on this article. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: E. Horwitz, I. Stein, J. Hess, P. Angel, Y. Ben-Neriah, E. Pikarsky

Development of methodology: E. Horwitz, I. Stein, M. Andreozzi, R.M. Porat, M. Grunewald

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Horwitz, I. Stein, J. Nemeth, A. Shoham, O. Pappo, N. Schweitzer, L. Tornillo, N. Kanarek, L. Quagliata, F. Zrek, R.M. Porat, R. Finkelstein, R. Koschky, T. Ganten, C. Mogler, O. Shibolet, K. Breuhahn, M. Grunewald, P. Schirmacher, A. Vogel, L. Terracciano

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Horwitz, A. Shoham, O. Pappo, N. Schweitzer, L. Quagliata, H. Reuter, C. Mogler, O. Shibolet, J. Hess, A. Vogel, L. Terracciano, P. Angel, Y. Ben-Neriah, E. Pikarsky

Writing, review, and/or revision of the manuscript: E. Horwitz, I. Stein, N. Schweitzer, R. Koschky, T. Ganten, C. Mogler, O. Shibolet, J. Hess, P. Schirmacher, P. Angel, Y. Ben-Neriah, E. Pikarsky

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E. Horwitz, A. Shoham, N. Kanarek, L. Quagliata, T. Ganten, J. Hess, P. Schirmacher, A. Vogel, L. Terracciano

Study supervision: Y. Ben-Neriah, E. Pikarsky

Acknowledgments

The authors thank Rivka Ben-Sasson, Reba Condioti, Shaffika El-Kawasmi, Etti Avraham, Mohamad Juma’, and Drs. Hila Giladi, Gustavo Mostoslavsky, David Curiel, Yosef Haviv, Shemuel Ben-Sasson,
and Sharon Elzur for providing expertise and reagents. The authors also thank Drs. Jacob Hannah, Hidekazu Tsuchimoto, Ofer Mandelboim, Noam Stern-Ginzor, Noa Stanietzky, Rachel Yamin, Seth Salpeter, Temima Schnitzer-Perlman, Dan Lehmann, Rachel Horwitz, and Chamtal Gaur for expert advice and kind assistance. The authors are indebted to Dr. Daniel Goldenberg for supplying aged Mdr2−/− mice and are grateful to Drs. Christoffer Gebhardt, Robert Goldstein, Moshe Biton, Zvika Granot, and Tsachi Hagai for fruitful discussions.

Grant Support

The research leading to these results has received funding from the European Research Council under the European Union’s Seventh Framework Programme (FP/2007-2013)/ERC Grant Agreements 281738 to E. Pikarsky and 294390 to Y. Ben-Neriah; the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (AMRF), the German Research Foundation (DFG, SFB-TR77), the Cooperation Program in Cancer Research of the Deutsches Krebsforschungszentrum (DKFZ), and Israel’s Ministry of Science, Culture and Sport (MOST) to E. Pikarsky, Y. Ben-Neriah, and P. Angel; the Israel Science Foundation (ISF) Centers of Excellence to E. Pikarsky and Y. Ben-Neriah; and an ISP Project Grant to I. Stein and O. Pappo.

Received October 21, 2013; revised March 26, 2014; accepted March 26, 2014; published OnlineFirst March 31, 2014.

REFERENCES