Human and mouse VEGFA-amplified hepatocellular carcinomas are highly sensitive to sorafenib treatment

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Abbreviations: HCC - Hepatocellular Carcinoma, VEGFR - VEGFA Receptor, HGF - Hepatocyte Growth Factor, Mdr2+/− - Multiple drug resistance variant 2 deficient mice, aCGH - array Comparative Genomic Hybridization, qPCR - quantitative PCR, CISH - Chromogenic In Situ Hybridization, Mbp - Mega base pairs, FISH - Fluorescent In Situ Hybridization, Amppos - VEGFA amplification positive tumor, Ampneg - VEGFA amplification negative tumor, TAMs - Tumor Associated Macrophages, AST - Aspartate Aminotransferase, pHH3 - phosphorylated Histone H3.

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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 biological characteristics. Unlike common tumor amplifications, this one appears to work via heterotypic paracrine interactions: stromal VEGF receptors (VEGFRs), 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.

Statement of 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 frontline treatment of advanced HCC, which has an overall moderate therapeutic efficacy.
Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide, with the highest increase rate in northern 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, patient’s 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 none 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 K-RAS 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: B-Raf, c-Raf, PDGFR2, c-Kit, and the VEGFR receptors (VEGFRs) (10, 16). While 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’s 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 that 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 the VEGFRs (22, 23). Moreover, VEGF-A also has non-angiogenic 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 utilized 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 comparative genomic hybridization (aCGH) to 10 HCCs obtained from 16 months old Mdr2−/− mice (Supplementary Fig. 1A). We detected several amplifications and deletions, including an amplification in the qB3 band of murine chromosome 17 (Chr17qB3, Supplementary Table 1) 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. 1B) and chromogenic in situ hybridization (CISH, Fig. 1A); 13 out 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 to 48.5 mega base pairs (Mbp) from the chromosome 17 start. This minimal amplified region spanned 53 genes (Supplementary Table 2).

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 7-40% (29-33). Accordingly, through
fluorescent in situ hybridization (FISH) we found VEGFA amplifications and chromosome 6 polysomies in 11% of human HCCs we tested (21/187, Fig. 1C and Supplementary Table 3).

To elucidate the tumor relevance of these chromosomal gains we analyzed the expression of mouse Chr17qB3 amplicon genes in amplicon positive (herein Amp\textsuperscript{pos}) and negative (Amp\textsuperscript{neg}) tumors. Matched increases in mRNA and DNA levels were found for VEGFA, Tjap1 and Exportin 5 (Fig. 1D and Supplementary Fig. 2A). We further found a correlation between Chr17qB3 amplification and VEGF-A protein levels in both tumor extracts and serum (Fig. 1E and Supplementary Fig. 2B). Furthermore, double immunostaining for VEGF-A and E-cadherin in Amp\textsuperscript{pos} 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.

**Amp\textsuperscript{pos} tumors display distinct histological features**

Tumor cell proliferation is a strong and consistent marker of poor prognosis in human HCC (34). BrdU immunostaining revealed that Amp\textsuperscript{pos} mouse HCCs displayed a 2-fold higher proliferation index compared to Amp\textsuperscript{neg} tumors (Fig. 2A and B, upper panels). No differences were noted in apoptosis (Supplementary Fig. 2C) or in the presence of neutrophils or fibroblasts (data not shown). Other histological features, differing between Amp\textsuperscript{pos} and Amp\textsuperscript{neg} mouse HCCs, include a 6-fold higher vessel density (Fig. 2A and B, middle panels) and 4-fold higher macrophage content (Fig. 2A and B, lower panels).
Moreover, several signature genes of tumor associated macrophages (TAMs) including Arginase 1, TGFβ, and Ym1 (35, 36), were elevated in the Amp\textsuperscript{pos} group (Fig. 2C), while markers of classically activated macrophages, including TNFα, iNOS, CXCL10, and IL12p40 were similar in both tumor groups (Fig. 2C and Supplementary Fig. 3A). Along with these, immunofluorescent stain for the pro-tumorigenic macrophage marker Mrc1, revealed a higher presence of Mrc1 expressing cells in Amp\textsuperscript{pos} 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 Amp\textsuperscript{pos} tumors as a distinct subgroup of HCCs characterized by enhanced presence of specific microenvironmental components and increased proliferation rate.

Histological analysis of human HCCs revealed several characteristic traits of HCCs harboring VEGFA gains. A higher incidence of vascular invasion was found in the Amp\textsuperscript{pos} group (9/20 Amp\textsuperscript{pos} tumors vs. 1/20 Amp\textsuperscript{neg} tumors, p<0.01, Supplementary Table 4). A lower incidence of fibrosis within tumor tissue was found in the Amp\textsuperscript{pos} group as well. Few additional traits nearly reaching statistical significance were identified (Supplementary Table 4). We found no differences in several clinical characteristics of HCC including underlying disease, gender or tumor size (Supplementary Fig. 4A-C). Altogether, these results indicate that in both murine Mdr2\textsuperscript{-/-} and human HCCs, tumors that harbor genomic gains in the VEGFA locus are distinct from those that do not.
Macrophage-tumor cell crosstalk in Amp^pos^ tumors

Previous studies have delineated a hepatocyte-endothelial crosstalk taking place in non-neoplastic liver, wherein VEGF-A stimulates endothelial cells to secrete several mitogens including HGF (24, 25). We hypothesized that Amp^pos^ HCCs exploit this interaction for promoting tumor cell proliferation. Following this notion, we detected a 3-fold elevation of HGF mRNA levels in Amp^pos^ vs. Amp^neg^ mouse tumors (Fig. 3A). We did not find significant changes in other angiocrine-produced molecules (24, 25) – Wnt2, IL-6 and HB-EGF (data not shown). Immunostaining detected HGF expression only in Amp^pos^ tumors, exclusively in the non-neoplastic stromal cells (Fig. 3B). Immunofluorescent staining for 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. 3B), a time point signified by marked dysplasia, yet no overt HCC formation (27). mRNA profiling of these fractions portrayed that VEGFRs (FLT1 and KDR) and coreceptors [Neuropilin (Nrp)1 and 2] were higher in macrophages while the HGF receptor (c-MET) was more abundant in hepatocytes (Fig. 3E). This aligns with previous work showing that hepatocytes are inert to direct activation with VEGF-A (24). Immunostaining for KDR in Amp^pos^ 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 to the increase in mRNA levels of recruited macrophage and endothelial
markers Msr1 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 Amp<sup>pos</sup> 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**

In order to prove the functional role of *VEGFA* in this genomic amplification, we set to inhibit VEGF-A in Mdr2<sup>−/−</sup> tumors. To this end, we injected intravenously adenoviral vectors encoding GFP alone or GFP and the soluble VEGFR1 (sFLT), a potent inhibitor of VEGF-A (38), into 56 tumor-harboring Mdr2<sup>−/−</sup> mice aged 14 to 18 months and sacrificed them ten days following injection (Supplementary Fig. 5A). *VEGFA* amplification status was determined after sacrifice by both DNA qPCR and VEGF-A mRNA expression (Fig. 4C and data not shown). Liver damage, measured through plasma Aspartate Aminotransferase (AST) activity, was similar in all groups (Supplementary Fig. 5B). Immunostaining for BrdU, Ki67, and phospho-histone H3 (pHH3) revealed that blocking VEGF-A markedly inhibited tumor cell proliferation in Amp<sup>pos</sup> tumors, but not in Amp<sup>neg</sup> ones (Fig. 4A and B and Supplementary Fig. 5C and D). 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. 5C and D and data
not shown). Macroscopic and histological analyses revealed multiple foci of coagulative necrosis only in sFLT treated Amp<sup>pos</sup> tumors (3/6 vs. 0/10 in Amp<sup>neg</sup>, p<0.05 Fig. 4D). In these specific tumors we also found an elevation of the HIF1α target genes Glut1 and PGK1 (Fig. 4C), indicative of tissue hypoxia. Immunostaining for vWF revealed a trend of decrease in vasculature only in VEGF-A blocked Amp<sup>pos</sup> tumors, particularly in the hypoxic tumors (Supplementary Fig. 5C and D). These data denote Amp<sup>pos</sup> 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 macrophage HGF - heterotypic circuit in tumor growth, we injected human Hep3B HCC cells transduced with a lentivirus overexpressing human VEGF-A into immune-deficient mice (Supplementary Fig. 6A). The *in vivo* growth rate of VEGF-A overexpressing cells was higher than control vector transduced cells (Fig. 5A and Supplementary Fig. 6B). This correlated with higher proliferation rate and increased HGF expression (Fig. 5B-D and Supplementary Fig. 6C and D), phenocopying the Amp<sup>pos</sup> Mdr2<sup>−/−</sup> 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 PHD3 and LDHA in these xenografts, lending further support to a non-angiogenic role for VEGF-A in HCC (Supplementary Fig. 6E). Immunofluorescence
confirmed VEGF-A expression in transduced tumor cells and revealed HGF expression in macrophages (Supplementary Fig. 7A).

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 and endothelium specific genes, Msr1 and CD105 respectively, confirmed successful separation of cell populations (Supplementary Fig. 7B). While VEGFA and control tumor groups did not show significant differences in the tumor associated macrophage genes TGFβ, Ym1, TNFα and iNOS (Supplementary Fig. 7C), we found a twofold 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 macrophages, and leads to increased proliferation of tumor cells.

Profiling proangiogenic-factor expression in Mdr2^−/− HCC

As Amp^neg^ tumors did not respond to sFLT treatment, we profiled other proangiogenic factors that can support tumor vascularization. mRNA qPCR Analysis of Angiopoietin 1 and 2, Angiopoietin like 2, FGF 1 and 2, PDGF-A, -B and -C, PLGF and VEGF-B revealed that several of these factors were overexpressed in Mdr2^−/−^ HCCs compared with normal livers, irrespectively of the amplicon status. Notably, PDGF-C levels were significantly higher (2.4 fold) in Amp^neg^ compared with Amp^pos^ 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 Amp<sup>neg</sup> tumors in response to sFLT.

**Murine Amp<sup>pos</sup> 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 frontline treatment for these patients (3). As sorafenib inhibits VEGFRs and B-Raf - 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 Amp<sup>pos</sup> tumors. We treated 58 Mdr2<sup>-/-</sup> mice aged 14 to 18 months with sorafenib or vehicle alone for three days after which they were sacrificed and tumor tissue was analyzed (experimental design depicted in Supplementary Fig. 5A). Amplification status was assessed after sacrifice by DNA qPCR and verified by VEGF-A mRNA expression (Fig. 7C and data not shown). Immunostaining for BrdU and pHH3 demonstrated decreased proliferation in sorafenib treated mice in Amp<sup>pos</sup> but not Amp<sup>neg</sup> HCCs (Fig. 7A and B and Supplementary Fig. 8A and B). Similarly to VEGF-A inhibition, HGF levels were decreased only in sorafenib-treated Amp<sup>pos</sup> tumors (Fig. 7C). While no effects were observed on tumor macrophage density (Supplementary Fig. 8A and B), expression of the tumor associated macrophage marker TGFβ was decreased in sorafenib-treated Amp<sup>pos</sup> tumors (Supplementary Fig. 8C). Blood vessel density changes or signs of hypoxia were absent (Supplementary Fig. 8A-C), possibly due to the short treatment duration, implying that
the early inhibitory effect of sorafenib in VEGFA amplified tumors may be independent of angiogenesis. Amp\textsuperscript{neg} 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. Similarly to the Mdr2\textsuperscript{-/-} 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. 9A-D). Despite the longer duration of treatment, we still could not detect changes in macrophage or blood vessel densities (Supplementary Fig. 10A-C). Of note, a differential response to sorafenib was not evident \textit{in vitro}, emphasizing the importance of microenvironmental factors (Supplementary Fig. 10D).

**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 HCC patients that underwent tumor resection. This retrospective cohort was collected from 3 different centers. To assess the correlation between VEGFA gain and survival, we analyzed human tumor samples by FISH (Fig. 1C). Survival of HCC patients that did not receive sorafenib was independent of the VEGFA status (Fig. 7E). However, a markedly improved survival was seen in the VEGFA-gain group compared to the non-gain group in sorafenib treated patients (indefinable median survival from sorafenib treatment start and 11 months, respectively, pLog-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 histological appearance, rate of proliferation and microenvironmental content. We delineated a cytokine-based heterotypic crosstalk between malignant Amp\textsuperscript{pos} hepatocytes and tumor stromal cells. Importantly, we show that mouse Amp\textsuperscript{pos} 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 Mdr2\textsuperscript{2-/-} 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). While 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-pos tumors harbor higher numbers of TAMs compared with Amp-neg 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); 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 pro-tumorigenic functions of VEGF-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 VEGF-A and extent of amplification in human HCC (29). Amplifications in 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 7-30% of human HCCs (29-33). Our interventional studies in the Mdr2−/− model indicate that VEGFA is a major driver of this amplicon, which minimally harbors 53 genes in Mdr2−/− murine tumors and 11 genes in humans (32). 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 vivo and that the tumor growth advantage gained through this overexpression can be negated by sorafenib. Nevertheless, we cannot completely exclude contribution of other amplicon genes to tumorigenesis.

The first line of treatment for advanced HCC is the multikinase inhibitor sorafenib, which prolongs median survival by 10-12 weeks (3, 4). The response to sorafenib appears 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 B-Raf - 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. Based on our small-scale retrospective study, we show that VEGFA gains may predict response to sorafenib in HCC, thus enabling to tailor treatment only to those patients who may benefit from this side effects-prone therapy. Notably, the same amplification
was found also in lung, colorectal, bone and breast cancers (44-48), thus it is plausible that similar considerations could be applied to other tumors harboring \textit{VEGFA} gains.
Methods

Human tissue samples and tissue microarray

Human HCC tissues were obtained from resected patients from the institutes of Basel University Hospital, Switzerland; Hannover Medical School Hospital and Heidelberg University Hospital, Germany. Clinical information included age at diagnosis, tumor diameter, gender, and survival time information. Examination of tumor H&E sections was performed by an expert liver pathologist (O.P.). Samples from Heidelberg were also reviewed by Heidelberg pathologists (C.M., P.S.). The study was approved by each of the institutions ethics committee – numbered 206/05 (Heidelberg), 660-2010 (Hannover), and EKBB20 (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 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, Silver Spring, USA). Afterwards, tissue cylinders were transferred to a 25x35 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, Hackensack, USA). 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 performed on Mdr2-/- mice were performed in cohorts of 10-20 mice each time, results show the combined data from at least 4 different experiments. WT controls were aged matched FVB mice. Mouse body weights during the experiments were 30-45 grams. Sorafenib (Xingcheng Chempharm Co., Ltd Taizhou, China) was administered daily (50 mg/kg) by oral gavage. Cremophor EL (Sigma)/ethanol/water; (1:1:6) was used as vehicle. Two hours prior to sacrifice, mice were injected with 10 μg BrdU (Cell Proliferation labeling reagent, Amersham) per 1 gram body weight. Mice were anesthesized 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 subjected to standard histological procedures. Xenografts experiments were performed by subcutaneous injection of transduced Hep3B cells (2.5×10⁶) suspended in 100 μl PBS and 100 μl Matrigel (Becton Dickinson) into NOD/SCID mice. Tumor volumes were assessed by external measurement with caliper. 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) and Yosef Haviv (Hadassah Hospital, Israel) were prepared in GH354 cells using standard procedures. A titer of 10⁹ 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 VEGFA<sub>165</sub> gene (from cDNA of decidual NK cells, a kind gift from Ofer Mandelboim, Hebrew University, Jerusalem) into pSC-B plasmid using the StrataClone 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) digested with BamHI and NotI. Lentivectors were produced by cotransfection of the backbone vector plasmid with the gag-pol and pMD.G plasmids and using standard procedures. Hep3B cells (obtained from the ATCC) were grown in DMEM (10% fetal bovine serum). Cells were tested free of mycoplasma prior to 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, Beit Haemek) using the manufacturer’s protocol. Murine recombinant VEGF-A (R&D systems) was used at the concentration of 100ng/ml.

**Immunohistochemistry, immunofluorescence and ELISA**

Antibodies used for tissue immunostaining throughout the work were - vWF (dilution 1:300, Dako), phosphorylated Histone H3 (pHH3, 1:800, Upstate), cleaved Caspase 3 (1:200, Cell Signaling), F4/80 (1:300, Seroteq), HGF (1:100, R&D), BrdU (1:200, NeoMarkers), KDR (1:400, Cell Signaling), Ki67 (1:100, NeoMarkers), e-cadherin (1:100, Cell Signaling) and VEGF (1X as supplied, Spring). IHC was performed on 5 μm paraffin sections. Antigen retrieval was performed in a decloaking chamber (Biocare...
Medical) in citrate buffer for all antibodies except vWF and F4/80 for which retrieval was performed with Pronase (Sigma). HRP conjugated secondary antibodies for all immunohistochemistry antibodies used were Histofine (Nichirei Biosciences), except for anti mouse derived antibodies that were detected with Envison (Dako). 3,3′-Diaminobenzidine (DAB, Lab Vision) was used as 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 5 randomly selected fields in each tumor.

Immunofluorescence was performed on snap frozen tissue embedded 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 647 (Invitrogen), Donkey anti Rabbit Cy2/Cy5, Donkey anti mouse Cy3 and Goat anti-Rat Cy3 (Jackson Laboratories). Hoechst 33342 was used as a nuclei marker (Invitrogen). Antibodies used for flow FACS sorting were – CD45-pacific blue, F4/80-PE (both 1:50, BioLegend) and Meca32-biotin (1:50, BioLegend) used with streptavidin APC-Cy7 (BD biosciences). 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 sonicated 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 in 95°C for 5 minutes. The Spot-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 RPCIB753M0921Q (imaGENES, Berlin, Germany), 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 *Alu* restriction enzyme (Invitrogen) and labelled with Cy3-dUTP (GE Healthcare) using the BioPrime Array CGH Kit (Invitrogen). Labeling reaction was assessed by Nanodrop (Nanodrop, Wilmington, DE, USA). Labeled DNA was purified with the FISH Tag DNA Kit (Invitrogen). Tissue microarrays 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 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. 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 (2× SSC, 0.3% NP40, pH 7–7.5) and counterstained with DAPI I solution (1000 ng/ml; Vysis Abbott Molecular). As reference, a Spectrum Green-labeled chromosome 6 centromeric probe (Vysis Abbott Molecular) was used. Images were obtained with a Zeiss fluorescence microscope using a 63× 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, amplified - VEGFA/Chr6 ratio higher than 2.2, as proposed by the ASCO/CAP 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 2.26-3.75 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 that had no access to the clinical data (L.T.).
Array-based comparative genomic hybridization and qPCR

Genomic DNA was isolated using the QIAGEN DNAeasy Tissue kit. Samples were hybridized to mouse CGH 60-mer oligonucleotides microarrays (Agilent Technologies), washed and scanned according to Agilent Technologies instructions. Data was analyzed using Feature Extraction software V8.1 (Agilent), GeneSpring GX V7.3.1 and CGH Analytics V3.4.27 (Agilent) software. RNA was extracted from tissues by mechanical grinding in TriReagent (Sigma) with a Polytron tissue homogenizer (Kinematica) at maximum speed. cDNA was prepared with MMLV reverse transcriptase (Invitrogen). qPCR analyses were carried out with SYBR green (Invitrogen) in 7900HT Fast Real-Time PCR System (Applied BioSystems). Results were analyzed using the qBase v1.3.5 software. Primer sequences are available in Supplementary Table 5. In the Xenografts experiment, murine HGF mRNA levels were assessed with Taqman probe (Life Sciences). Human HPRT and UBC were used as reference genes in the xenograft experiment. HPRT and PPIA combined were used as reference genes in all murine analyses except for the Hepatocyte vs. Macrophage comparison in which UBC, β2M and TBP were additionally applied. Primers detecting the murine chromosome 17 pericentromeric 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 50xg for 2 minutes, and after 3 washes were frozen immediately in
liquid nitrogen for RNA preparation. Non parenchymal cells were pelleted by centrifugation at 150xg 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 3 times and immediately frozen in liquid nitrogen. Purity of hepatocytes and macrophage fractions was determined by Hematoxylin & Eosin staining of cytospin preparations and always exceeded 90%. Dissociation of cells from tumor xenografts was performed using the gentleMACS dissociator (Miltenyi Biotech) according to manufacturer’s protocol.

**Statistics**

Data was analyzed using a paired two tails Student’s T-Test at p<0.05. Histological differences were analyzed using Pearson’s χ² test at p<0.05. Data was 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 standard error of the mean (SEM).

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Dan Lehmann, Rachel Horwitz and Chamutal Gur for expert advice and kind assistance. We are indebted to Dr. Daniel Goldenberg for supplying aged Mdr2

-/- mice. We are grateful to Drs. Christoffer Gebhardt, Robert Goldstein, Moshe Biton, Zvika Granot and Tzachi Hagai for fruitful discussions.
References


Figure legends

**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<sup>pos</sup> tumor. Thin line represents non-amplified regions, 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. 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 WT livers, Amp<sup>neg</sup> and Amp<sup>pos</sup> Mdr2<sup>−/−</sup> tumors in matching pairs show a correlation between the increase in mRNA and protein levels. **(F)** Confocal microscopy images of an Mdr2<sup>−/−</sup> Amp<sup>pos</sup> tumor immunostained for E-cadherin and VEGF-A. Hoechst 33342 marks nuclei. Scale bars: 20 μm.

**Figure 2.** Amp<sup>pos</sup> tumors are a distinct tumor subpopulation. **(A)** Representative IHC photomicrographs for BrdU, vWF, and F4/80. Scale bars: 50 μm (BrdU) 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 (pro-tumorigenic) and classically activated (anti-tumorigenic) macrophage markers. Each dot represents a
different tumor. Cross line signifies geometric mean (n.s.= not significant, **p<0.01, ***p<0.001). (D) Immunofluorescent stain for Mrc1 on Amp\textsuperscript{neg} and Amp\textsuperscript{pos} tumors. Scale bars: 40 μm. (E) Quantification of Mrc1 immunofluorescence. Bars represent geometric mean, **p<0.01.

**Figure 3.** A macrophage-tumor cell cross talk within Amp\textsuperscript{pos} tumors. (A) qPCR analysis of HGF mRNA in WT livers, Amp\textsuperscript{neg} and Amp\textsuperscript{pos} tumors. Cross line signifies geometric mean (**p<0.001). (B) Representative photomicrographs of IHC for HGF on Amp\textsuperscript{neg} and Amp\textsuperscript{pos} tumors. Scale bars: 100 μm. (C) Representative confocal microscopy images of Amp\textsuperscript{pos} tumor immunostained for vWF, F4/80 and HGF. Hoechst 33342 marks nuclei. Scale bars: 40 μm. (D) Representative cytospin preparations of macrophage and hepatocyte fractions from Mdr2\textsuperscript{-/-} livers. (E) qPCR analysis of hepatocyte and macrophage fractions (n=11 different mice), isolated from livers of Mdr2\textsuperscript{-/-} mice. Bars represent geometric mean (**p<0.001, ***p<0.0001). (F) Representative photomicrograph of IHC for the VEGF receptor KDR in Amp\textsuperscript{neg} and Amp\textsuperscript{pos} tumors. Note that expression of KDR is confined to endothelial and stromal cells. Scale bars: 100 μm. (G) mRNA qPCR analysis for the VEGFRs KDR and FLT1 and the macrophage and endothelium markers Msr1 and CD105 on tissue lysates from the indicated groups. Each dot represents a different tumor. Cross line represents geometric mean (**p<0.0001). (H) Peritoneal macrophages were cultured under serum free conditions followed by 8h exposure to recombinant murine VEGF-A (100ng/ml). HGF expression was measured by qPCR. Bars represent geometric mean, n≥3, *p<0.05.
Figure 4. VEGF-A inhibition impedes proliferation in Amp<sup>pos</sup> tumors. Mdr2<sup>-/-</sup> mice were treated with adenovectors expressing either GFP alone or GFP and sFLT for 10 days. (A) Representative photomicrographs of IHC for BrdU. Tumor infiltrating cells remain proliferative. Scale bars: 100 μm. (B) BrdU immunostaining was quantified using automated image analysis. (n≥6, *p<0.05). (C) mRNA qPCR analysis of the indicated genes in Amp<sup>neg</sup> and Amp<sup>pos</sup> tumors treated with the indicated adenovectors. Each dot represents a different tumor. Cross line signifies geometric mean (**p<0.0001). (D) Left panel - histological section stained with H&E depicting necrosis, representing three out of the six sFLT treated Amp<sup>pos</sup> tumors. Scale bar: 500 μm. Right panel - a macroscopic picture of a tumor with hemorrhagic necrosis. Scale bar: 0.5 cm.

Figure 5. VEGF-A overexpression enhances tumor cell proliferation. Immune deficient 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 BrdU in control or VEGF-A lentivector-transduced xenografts. Scale bars: 100 μm. (C) BrdU 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 lentivector-transduced xenografts. Bars represent geometric mean (n≥4, *p<0.05, **p<0.01). (E) XTT in vitro proliferation assay of the indicated lentivector-transduced cultured cells. (F) Expression
of HGF in macrophage and endothelial fractions from tumors overexpressing VEGFA 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.

**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. Cross line signifies geometric mean, significance marks immediately above each group refer to comparison with normal livers. Top most 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).

**Figure 7.** Amppos tumors are uniquely sensitive to sorafenib. (A) Representative BrdU immunostains. Scale bars: 100 μm. (B) BrdU immunostaining was quantified using automated image analysis (n≥6, *p<0.05). (C) qPCR analysis of Ampneg and Amppos tumors treated as indicated. Each dot represents a different tumor. 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 non-treated (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 (pLog-Rank>0.05). (F) Kaplan-Meier curves showing survival of HCC patients treated with
sorafenib, negative (n=47, 10 months median) or positive for VEGFA gain (n=7, median undefined, pLog-Rank=0.029). (G & 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 tumor associated macrophages expressing the mitogen HGF (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 with decreased HGF signaling and blood supply, impeding tumor growth.
Figure 1

A  Murine HCCs

<table>
<thead>
<tr>
<th>Tumor #1</th>
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B  Murine Amp<sup>pos</sup> HCCs

Mbp from Chromosome 17 start

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<tr>
<td>Aæv2</td>
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<tr>
<td>Mibx</td>
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<tr>
<td>Cap11</td>
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<tr>
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<tr>
<td>Mepk3</td>
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C  Human HCCs

normal  polysomic  amplified

D

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<tr>
<th>Gene</th>
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E

VEGF<sub>a</sub> mRNA (qPCR)

WT liver  Amp<sup>neg</sup>  Amp<sup>pos</sup>

VEGF<sub>a</sub> Protein (ELISA)

Mdr2<sup>-/-</sup> tumors

F

Hoechst  E-cadherin  VEGFA  Merge

Amppos Tumor
Figure 2

A

\( \text{Amp}^{\text{neg}} \text{Tumor} \)  \( \text{Amp}^{\text{pos}} \text{Tumor} \)

B

\( \text{BrdU} \)

<table>
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\( \text{vWF} \)

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\( \text{F4/80} \)

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C

Pro-tumorigenic markers

- \( \text{Arginase 1} \)
- \( \text{TGF}\beta \)
- \( \text{Ym1} \)

Anti-tumorigenic markers

- \( \text{TNF}\alpha \)
- \( \text{IROS} \)
- \( \text{CXCL10} \)

D

\( \text{Amp}^{\text{neg}} \text{Tumor} \)  \( \text{Amp}^{\text{pos}} \text{Tumor} \)

E

\( \text{Mrc1} \)

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Figure 3

A

B

C

D

E

F

G

H

**Figure 3**

(A) Relative expression of FLT1, KDR, Nrp1, Nrp2, and c-Met in WT liver, Amp<sup>neg</sup> Tumor, and Amp<sup>pos</sup> Tumor. 

(B) Immunohistochemical staining of HGF in Amp<sup>neg</sup> Tumor and Amp<sup>pos</sup> Tumor.

(C) Immunofluorescence staining of vWF, F4/80, and HGF in Amp<sup>pos</sup> Tumor, with merge image.

(D) H&E staining of macrophages and hepatocytes.

(E) Relative expression of KDR and FLT1 in Hepatocytes and Macrophages.

(F) Immunohistochemical staining of KDR in Amp<sup>neg</sup> and Amp<sup>pos</sup>.

(G) Relative expression of KDR, FLT1, Msr1, and CD105 in WT liver, Amp<sup>neg</sup> Tumor, and Amp<sup>pos</sup> Tumor.

(H) Relative expression of HGF in NT and 8h vVEGFA-treated peritoneal macrophages.
Figure 4

A

BrdU

B

BrdU

percent positive nuclei

C

VEGF-A

HGF

D

Amp\textsuperscript{pos} sFLT

Glut1

PGK1

H&E

Research.
Figure 6

Ampneg Amppos Ampneg Amppos Ampneg Amppos Ampneg Amppos Ampneg Amppos

Angiopoietin 1 Angiopoietin 2 Angptl 2 FGF 1 FGF 2

Ampneg Amppos Ampneg Amppos Ampneg Amppos Ampneg Amppos Ampneg Amppos

PDGF-A PDGF-B PDGF-C PLGF VEGF-B

n.s. n.s. n.s. n.s. * 

n.s. n.s. n.s. n.s. n.s. *** 

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n.s. n.s. n.s. n.s. **

n.s. n.s. n.s. n.s. **

n.s. n.s. n.s. n.s. **
Figure 7

A. Amp\textsuperscript{neg} Vehicle \hspace{1cm} Amp\textsuperscript{neg} Sorafenib \hspace{1cm} Amp\textsuperscript{pos} Vehicle \hspace{1cm} Amp\textsuperscript{pos} Sorafenib

B. BrdU

C. VEGF-A, HGF

D. Tumor volume (mm\textsuperscript{3})

E. Resected HCC patients

F. Resected patients treated with sorafenib

G. VEGF-A amplified malignant hepatocyte

H. VEGF-A amplified malignant hepatocyte
Human and mouse VEGFA-amplified hepatocellular carcinomas are highly sensitive to sorafenib treatment

Elad Horwitz, Ilan Stein, Mariacarla Andreozzi, et al.

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