An inducible hepatocellular carcinoma model for preclinical evaluation of anti-angiogenic therapy in adult mice

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Abstract

The limited availability of experimental tumor models that faithfully mimic the progression of human tumors and their response to therapy remains a major bottleneck to the clinical translation and application of novel therapeutic principles. To address this challenge in hepatocellular carcinoma (HCC), one of the deadliest and most common cancers in the world, we developed and validated an inducible model of hepatocarcinogenesis in adult mice. Tumorigenesis was triggered by intravenous adenoviral delivery of Cre recombinase in transgenic mice expressing the hepatocyte-specific albumin promoter, a loxP-flanked stop-cassette, and the SV40 large T-antigen (iAST). Cre recombinase-mediated excision of the stop cassette led to a transient viral hepatitis and resulted in multinodular tumorigenesis within 5 to 8 weeks. Tumor nodules with histological characteristics of human HCC established a functional vasculature by cooption, remodeling and angiogenic expansion of the pre-existing sinusoidal liver vasculature with increasing signs of vascular immaturity during tumor progression. Treatment of mice with Sorafenib rapidly resulted in the induction of vascular regression, inhibition of tumor growth, and enhanced overall survival. Vascular regression was characterized by loss of endothelial cells leaving behind avascular type IV collagen-positive empty sleeves with remaining pericytes. Sorafenib treatment led to transcriptional changes of Igf1, Id1 and cMet over time, which may reflect the emergence of potential escape mechanisms. Taken together, our results established the iAST model of inducible hepatocarcinogenesis as a robust and versatile preclinical model to study HCC progression and validate novel therapies.

Precis

The study validates a versatile inducible model of hepatocellular carcinoma (HCC) in adult mice for the study of multinodular HCC tumorigenesis and response to therapy during tumor progression.
**Introduction**

Hepatocellular Carcinoma (HCC) is the fifth most common cancer in men and the seventh in woman, and overall the third leading cause of death from cancer worldwide (1). It commonly develops from cirrhotic livers due to chronic hepatitis B and C infections (2, 3) or a history of alcohol consumption. Multifocal intrahepatic tumor development is common (4) and the prognosis is poor due to a limited response to systemic chemotherapy and radiotherapy, and lack of other treatment options. Early diagnosed patients are eligible for tumor resection or transplantation if cirrhosis is present. However, the incidence of tumor recurrence is very high. A widely accepted technique to treat HCC is transarterial chemoembolization (TACE), during which tumor-associated arterial vessels are selectively obstructed resulting in tumor necrosis (5).

Recently, Sorafenib, a small multikinase inhibitor, has shown some efficacy as mono-therapy in patients with late stage hepatocellular carcinoma (6). Yet, the overall clinical benefit of Sorafenib in terms of time-to-progression (TTP) and overall survival (OS) is rather modest (7), highlighting the need to mechanistically better understand beyond the molecular target specificity how Sorafenib is actually interfering with tumor growth and what limits its clinical efficacy. As a multikinase inhibitor targeting the Raf/MEK/MAPK, VEGFR and PDGFRβ signaling pathways (8), Sorafenib exerts direct anti-tumorigenic as well as anti-angiogenic effects, which have been demonstrated in various animal models as well as in patients (9, 10). Yet, the detailed functional mechanism-of-action *in vivo* has not been analyzed in much detail.

The better mechanistic understanding of the effects of Sorafenib on HCC progression is also of general interest because angiogenesis in HCC is different from angiogenesis in other solid tumors as a consequence of the unique vascular properties of the liver: The intrahepatic vasculature consists of a dual vascular system, the arterial/venous and the sinusoidal endothelium. Both are believed to contribute to HCC development by changing from a venous towards a capillary endothelial cell-like (EC) phenotype with expression of markers of continuous, non-fenestrated EC (11). Indeed, early intra-tumoral blood supply appears to primarily depend on the sinusoidal vasculature with later
involvement of the hepatic arterial system (12, 13). HCC angiogenesis depends on the activation, proliferation and migration of EC as well as of pericytes, events that are also found in other tumors with high angiogenic potential (14). Clinical studies have solidly established a correlation of the angiogenic activity with a high risk of vascular invasion, metastasis and poor prognosis (15, 16).

A number of animal models have been established for the study of HCC progression. These include subcutaneous and orthotopic grafting models, genetically engineered mouse models as well as chemically-induced carcinogenesis models (17). While all of these models resemble key features of the pathogenesis of HCC, many of them are also somewhat limited by either the too global induction of tumorigenesis in the liver which hardly mimics the multinodular development of HCC as it occurs in humans or the long duration until tumor development, which in some of the genetic models may take up to two years (18). Likewise, none of the established models has been optimized for the analysis of HCC angiogenesis, which is one of the reasons for the hitherto poorly characterized effects of Sorafenib in experimental models of HCC.

The RipTag model of endocrine pancreas tumorigenesis (RipTag = rat insulin promoter – SV40 large T-antigen) has over the years proven to be a powerful system for the spatiotemporal analysis of intratumoral angiogenesis during tumor initiation and tumor progression (19-20). SV40 large T-antigen-driven tumorigenesis has also been employed for the induction of liver tumorigenesis (21). While global constitutive liver TAg induction may yield limited insights into HCC pathogenesis, inducible models of TAg-driven hepatocarcinogenesis may be particularly suitable to mechanistically dissect the intricate tumor-stromal crosstalk during HCC progression including the induction of angiogenesis. We have in the present study taken advantage of a recently developed inducible variant of TAg-driven hepatocarcinogenesis, in which tumorigenesis is induced through the adenoviral delivery of Cre recombinase (22). The unprecedented versatility, robustness, and temporal properties of this model provide unique opportunities to spatiotemporally study the progression of HCC and the response of HCC to therapy.
Materials and Methods

Animals and tumor induction. Cre-inducible AST-transgenic mice (22) bred in the C57/Bl6 background were housed in the institutional animal facility. Neoplastic transformation was induced in 8 to 12 weeks old mice by tail vein injection of $10^9$ infectious units of adenovirus expressing Cre recombinase (Vector BioLabs). Genotype of the transgene was confirmed by PCR. All experiments were approved by the institutional and governmental Animal Care and Use Committees [RP Karlsruhe (35-9185.81/G-228/10)].

In vivo studies. Mice were treated daily with 20 mg/kg, 40 mg/kg, 60 mg/kg or 100 mg/kg of Sorafenib delivered by oral gavage. Sorafenib, p-Toluenesulfonate (LC Laboratories), was diluted in 1:1 Cremaphor EL (Sigma)/50% ethanol. Vehicle-treated animals received 1:1 Cremaphor EL/50% Ethanol solution. For perfusion experiments, 150 μl of 1 mg/ml lectin (FITC-labeled Bandeiraea simplicifolia; Sigma); for leakage analysis, 150 μl of 60 mg/kg Dextran (TRITC-Dextran, low molecular weight; 65-85 kDa, Sigma) and for hypoxia 150 μl of 60 mg/kg pimonidazole was injected into the tail vein 20 min prior to sacrifice.

Computed tomography. Mice were anaesthetized using a mixture of isoflurane (1.5%) and oxygen (0.5 l/min). 100 μl Fenestra LC contrast agent (LC-133, Art, Canada) was injected i.v. in the tail vein. Respiratory-gated VCT imaging was performed on a prototype flat-panel equipped volumetric computer tomograph (Volume CT, Siemens) with the following parameters: tube voltage, 80 kV; tube current, 50 mA; scan time, 80 seconds; frames per second, 120; rotation speed, 10 seconds; Kernel H80a; matrix 512 x 512. Images were processed with the program OsiriX v.5.5.1 (23). The program was used with GPU engine and active shading at default settings. Image series were imported and processed with the function “3d volume rendering”.

Magnetic resonance imaging. Mice were anaesthetized using a mixture of isoflurane (1.5%) and oxygen (0.5 L/min). Magnetic resonance images were acquired on a 1.5-T clinical MR scanner (Symphony, Siemens, Germany). A home-built coil for radiofrequency excitation and detection was used and designed as a cylindrical volume resonator with an inner diameter of 83 mm and a usable
length of 120 mm. T2-weighted images were acquired using a turbo spin-echo sequence (orientation axial, TR 3240 ms, TE 81 ms, matrix 152 x 256, resolution 0.35 x 0.35 x 1.5 mm³, 3 averages, 15 images, scan time 3:40 min). Images were processed with “The Medical Imaging Interaction Toolkit” (MITK version 0.10.0, 2008, freeware). Image series were imported and tumor nodules were marked using the “interactive segmentation” tool. Subsequently, nodule volume was determined in ml using the volumetry function of the same tool. The five largest nodules were analyzed to assess tumor progression over time. Tumor volumes were averaged per mouse and per time from vehicle: N=8 and Sorafenib: N=9 treated animals, respectively. Values were grouped in three time points starting with the treatment initiation (week 0) and the respective follow-ups (week 1 and week 2).

**Immunohistochemistry.** Paraffin-embedded liver samples were dehydrated followed by antigen retrieval using citrate buffer, pH 6.0 (DAKO). Prior to antibody incubation, slides were subjected to 3% H₂O₂ to inactivate endogenous peroxidase activity and blocked in 4% BSA/2% normal goat serum diluted in 1x TBS/Tween-20 (0.5%) (AppliChem). The following primary antibodies were used: anti-mouse Glypican3 antibody (1:200; Abcam); rabbit anti-mouse HSP70 (1:100; Cell Signaling); polyclonal rabbit anti-human CD3 antibody (1:300, Dako); polyclonal rabbit-anti IGF1Rß antibody (1:300, Cell Signaling); polyclonal goat anti-mouse VEGF₁₆₄ antibody (1:10; R&D Systems), and secondary goat anti-rabbit or goat anti rat IgG biotinylated antibody (1:200, Vector Laboratories) or donkey anti-goat IgG biotinylated antibody (1:200, Jackson Dianova). Antibody detection was visualized by DAB after ABC-HRP incubation and sections were counterstained with Mayer’s hematoxilin. Hypoxia was assessed using Hypoxyprobe-1 Plus kit (hpi) including FITC conjugated mouse IgG (1:50) and rabbit HRP-conjugated anti-FITC secondary antibody (1:100), antigen retrieval was performed in Citric-EDTA buffer. Human HCC nodules were detected with two different mouse monoclonal antibodies: anti-human Glypican3 antibody Clone 1G12 (1:100; Biomosaics); anti-human HSP70 antibody (1:250; Santa Cruz); and rabbit-anti-mouse biotinylated IgG (1:250 Dako). Staining was performed using an automated immunohistochemical stainer (Techmate 500, DakoCytomation) and ChemMate detection kit in accordance with the manufacturer’s protocol.
Cryosections: Cryosections (7 μm) were Methanol-fixed and the tumor vasculature was detected by rat anti-mouse CD31 staining (1:100; BD Pharmingen) and goat anti-rat IgG conjugated with Alexa 488 (1:500; Invitrogen). Tumor pericytes were detected with two different antibodies: rabbit anti-Desmin polyclonal antibody (1:200; Abcam) and goat anti-rabbit IgG biotinylated antibody (Invitrogen), which was detected by Streptavidin conjugated with Alexa 546 (1:250; Invitrogen), and a mouse monoclonal anti-human/mouse alpha-smooth muscle actin (αSMA) directly labeled with Cy3 antibody (1:250; Sigma). Basement membrane component collagen type IV was detected with rabbit anti-collagen type IV polyclonal antibody (1:200; Serotec); apoptosis was detected with rabbit anti-mouse cleaved caspase3 (1:100; Cell Signaling); sinusoidal endothelial cells were detected with rabbit anti-mouse Lyve1 (1:200; Reliatech); and goat anti-rabbit IgG conjugated with Alexa 546 (1:500; Invitrogen) as well as directly biotinylated monoclonal mouse anti-mouse Stabilin2 (1:500). Details of morphological and vascular analyses as well as whole liver imaging and perfusion analysis are outlined in the Online Supplement.

Biochemical and molecular biological techniques. Protocols for standard qPCR, microarray analysis, Western blot analysis, and ELISA measurements are summarized in the Online Supplement.

Statistical analysis. GraphPad Prism software was used. Data were analyzed by unpaired Student’s t test unless otherwise indicated. Values were expressed as mean ± SD. P < 0.05 was considered statistically significant.
Results

Adenoviral Cre-driven hepatocellular carcinogenesis leads to the formation of multinodular tumors

Inducible AST transgenic mice (iAST) express the SV40 TAg oncogene under the control of the albumin promoter. Yet, the oncogene is not transcribed due to the insertion of a floxed stop cassette between the promoter and the oncogene (22). Oncogenic transformation of hepatocytes is induced by excision of the floxed stop cassette, which is mediated by the hepatotropism of tail vein injected adenovirus expressing Cre recombinase (22).

Adenoviral Cre recombinase delivery induced acute hepatitis and parenchymal destruction at 1 week post-injection (pi) (Supplementary Figure S1A). Oncogenic transformation with atypical hepatocytes appeared at 2 weeks pi (Fig. 1A, left image) accompanied by amelioration of the acute hepatitis with persistent immune cell infiltration (Fig. 1A, right image). The nuclear-cytoplasmic ratio shifted at 4 weeks pi with the emergence of nodular structures and fully developed HCC nodules at 9 weeks pi (Supplementary Figure S1A).

Non-invasive CT imaging identified multifocal liver tumor nodules of varying sizes after 5 to 8 weeks (Fig. 1B and C). CT data were validated by histological analyses of sectioned liver samples (Fig. 1D, left image). SV40 TAg was expressed within the tumor nodules as well as in single cells, indicating an early tumorous transformation of hepatocytes (Fig. 1D, right image and Supplementary Figure S1B to B). Non-parenchymal cells did not express SV40 TAg. Notably, SV40 TAg-expressing lesions were positive for markers of malignant hepatocellular transformation in humans, including glypican 3 (GP3) and HSP70 (24) (Fig. 1E, F). Taken together, the iAST-induced tumorigenesis proved to be a versatile mouse model of hepatocarcinogenesis enabling temporal (time of virus delivery), and spatial (albumin promoter) induction of hepatocarcinogenesis in adult mice resembling the multifocal tumorigenesis observed in human HCC.
Tumor nodules establish a functional neovasculature with increasing immaturity during tumor progression

Human HCC is a highly vascularized tumor. Increased circulating levels of angiogenic factors such as VEGF and Angiopoietin2 (Ang2) have been reported in HCC patients and correlate with poor prognosis (25-28). Correspondingly, tumor-bearing iAST mice revealed signs of active angiogenesis: Circulating levels of VEGF were upregulated in iAST-induced tumor-bearing mice (Fig. 2A). VEGF-A expression was restricted to hepatocytes, as previously described (29), in normal livers and upregulated in non-transformed hepatocytes outside the tumorigenic area as well as in cells within larger nodules at later stages (Supplementary Figure S2). Ang1 and even more so Ang2 mRNA and protein levels were strongly upregulated in tumorigenic livers of iAST mice (Fig. 2B, C). To quantitatively assess the tumor angiogenic response in relation to tumor progression, tumor nodules were categorized in three groups based on different nodule size, namely small (histologic surface area < 0.1 mm\(^2\)), intermediate (0.1-0.2 mm\(^2\)), and large (> 0.2 mm\(^2\)). Surprisingly, no changes in total tumor vessel area within tumor nodules were observed during tumorigenesis. Notably, even the smallest nodules contained microvessels (Fig. 2D), suggesting that tumor angiogenesis did not occur from expansively growing avascular nodules. Instead, tumor cells in growing nodules coopted and remodeled preexisting sinusoidal vessels, similar to the recently reported cooptive growth of liver metastasizing tumors (30). This conclusion was also confirmed by histological analyses, which revealed a grossly altered tumor microvascular architecture with enlarged, strongly CD31-positive intratumoral microvessels (Fig. 2E), compared to the low CD31 expression of liver sinusoidal endothelial cells (LSEC) as has similarly been observed in a constitutive SV40-TAg HCC model (31). These CD31-positive microvessels were well embedded in a type IV collagen-positive basement membrane in tumor nodules of different sizes (Fig. 2E, F). In addition, tumor vessels at all stages were well perfused and tumor nodules showed little to no signs of necrosis (Supplementary Figure S3), indicating the presence of a functional vascular network at all analyzed tumor stages.
Analysis of intratumoral vessel maturation by staining for mural cell markers identified profound changes during tumorigenesis: Microvessels in smaller nodules stained prominently for the pan-pericyte marker Desmin (55-60%), whereas microvessel in larger nodules were characterized by lower pericyte coverage indices (20%-25%) (Fig. 2G, H). In contrast, the fraction of microvessels positive for the pericyte activation marker αSMA was significantly larger in big nodules compared to small nodules (20% vs. 10%; Fig. 2I, J). This pattern of Desmin high / αSMA low in small nodules and Desmin low / αSMA high in large nodules reflected an increasing immaturity of the tumor microvasculature during tumor progression. The same marker combination has also been observed in Lewis lung carcinomas (32). Taken together, the vascular analysis of growing tumors identified a functional vascular network in early stages of tumorigenesis with signs of increasing microvascular immaturity during tumor progression.

**Sorafenib treatment inhibits tumor growth, improves vascular architecture and enhances survival**

The multikinase inhibitor Sorafenib has some efficacy as monotherapy in late stage HCC patients (6). We consequently examined its therapeutic efficacy in the preclinical iAST model of hepatocarcinogenesis. To this end, mice were staged by magnetic resonance imaging (MRI) and treatment was initiated as soon as tumors became detectable. Daily treatment with Sorafenib (100 mg/kg) dramatically reduced tumor growth compared to vehicle-treated animals (Fig. 3A-D). To assess the microvasculature in the different treatment groups, livers were perfused through the portal vain with microfil cast. Sorafenib treatment resulted in the maintenance of the typical hierarchical branching structures similar to control-treated mice (Fig. 3E).

Next, we examined the preclinical efficacy of a lower dose of Sorafenib (60 mg/kg) as maintenance therapy that had previously been studied as long-term treatment (33). Early initiation of Sorafenib maintenance therapy (5 weeks after tumor initiation) resulted on average in a four-fold extended overall survival compared to the vehicle control group (Fig. 3F). Of note, mice in the Sorafenib treatment group died or were sacrificed because of poor health condition and not as a direct consequence of tumor growth. These mice displayed severe weight loss, partial necrosis and
hemorrhage in the liver, and lung bleeding after 80 days of treatment, effects that can similarly be observed in human patients (Supplementary Figure S4). A delayed initiation of Sorafenib treatment (upon MRI detection of tumor nodules) still resulted in a significant extension of overall survival, albeit with lower efficacy (Fig. 3F). The analysis of transcriptomic changes during Sorafenib treatment identified the downregulation of Hbegf and Hgf expression, whereas cMet was upregulated (Fig. 3G). The data demonstrate a potent preclinical efficacy of Sorafenib in the iAST model of hepatocarcinogenesis with reduced tumor growth and a largely preserved organ vascular architecture. The therapeutic response closely resembled the treatment outcome in human patients with earlier Sorafenib administration resulting in improved clinical efficacy.

The anti-tumorigenic effect of Sorafenib results from its anti-angiogenic activity

Although Sorafenib has been shown to inhibit tumorigenic Raf-signaling, treatment of iAST mice had only minimal effects on bRaf and cRaf as well as p44/42 MAPK (Fig. 4A-C). In contrast, Akt phosphorylation was prominently reduced upon Sorafenib treatment (Fig 4D). Moreover, expression and phosphorylation of the angiogenic receptor tyrosine kinases VEGFR2 and PDGFRβ was strongly downregulated in the livers of Sorafenib-treated tumor bearing iAST mice (Fig. 4E-H). This anti-angiogenic effect resulted in the downregulation of Ang2 mRNA and protein and stabilized expression of the Angiopoietin receptor Tie2 despite a downregulation of Tie2 mRNA levels (Fig. 4F, H). VEGF expression was prominently upregulated upon Sorafenib treatment, both, at the mRNA and protein level in serum and liver lysates (Fig. 4H-J) as well as in the tumorigenic areas in the liver (Supplementary Figure S5), possibly due to hypoxia (Supplementary Figure S6), suggesting a compensatory mechanism in response to treatment. Sorafenib treatment had no effect on the resting organ vasculature outside tumor nodules as evidenced by unaltered expression of the sinusoidal EC markers Lyve1 (34) and Stabilin2 (35) (Supplementary Figure S7). Together, these data show that Sorafenib exerted its anti-tumorigenic effect in the iAST model of hepatocarcinogenesis primarily at the microvascular level as an inhibitor of tumor-associated angiogenesis.
Sorafenib treatment inhibits pericyte recruitment and leads to intratumoral microvessel regression

The anti-angiogenic effects of Sorafenib could be due to effects on EC, pericytes or both. Based on the prominent downregulation of PDGFRß expression upon Sorafenib treatment (Fig. 4B), we performed additional qPCR and Western blot analyses to examine the effect of Sorafenib on intratumoral pericytes. Expression of most pericyte markers was downregulated upon Sorafenib treatment (Desmin, αSMA, neurogenin2, CD248) (Fig. 5A).

To analyze the structural features of EC and pericytes upon treatment in more detail, we assessed vessel characteristics by immunofluorescence (IF) stainings for CD31, αSMA, Desmin as well as the basement membrane component type IV collagen. Sorafenib treatment resulted in a significant reduction of intratumoral microvessel area (Fig. 5B) and a reduced number of vascular branchpoints compared to vehicle-treated animals (Fig. 5C). Whole mount image analysis revealed that the overall architecture of larger vessels was preserved in the Sorafenib treatment group, whereas the vascular tree in the tumors of control mice was characterized by disorganized and chaotropic vessels (Fig. 5D). On the microvascular level, Sorafenib treatment resulted in a strong reduction of αSMA- (Fig. 5E-G) and Desmin-covered microvessels (Fig. 5H, I). Concomitantly, significantly increased numbers of αSMA-positive and Desmin-positive pericytes without microvascular association (i.e., no CD31 co-localization) were detected in tumor nodules of Sorafenib-treated iAST mice (Fig. 5G, J) demonstrating that Sorafenib treatment potently blocked intratumoral microvessel maturation by inhibiting pericyte recruitment. Furthermore, Sorafenib treatment resulted in a decrease of type IV collagen deposition around intratumoral microvessels (Fig. 5K-M). However, we identified a strong increase in the number of type IV collagen-positive empty sleeves upon Sorafenib treatment indicating active regression of sprouting neovessels presumably of non-pericyte covered immature capillaries. Collectively, the detailed vascular analyses identified a dual anti-vascular mechanism of action of Sorafenib, which was characterized by an inhibition of pericyte recruitment to EC and regression of immature microvessels.
Sorafenib leads to rapid intratumoral microvessel regression

The pronounced anti-angiogenic effects of Sorafenib in the iAST model prompted us to analyze in more detail the temporal kinetics of the observed vessel regression phenotype. No detectable microvascular changes were observed within 12 h and 16 h of Sorafenib treatment (Fig. 6A). Thereafter, massive vascular regression was rapidly initiated after 24 h of therapy induction resulting in a vessel phenotype comparable to the 14-day endpoint (Fig. 6A). At this time point, Desmin-positive pericytes were left within type IV collagen-positive empty sleeves (Fig. 6B). After 6 days of treatment, the dense network of intratumoral microvessels as seen in the control group was remodeled to a pruned tree of larger diameter vessels in the Sorafenib treatment group (Fig. 6C, Supplementary Figure S8). The temporal analysis of key regulators of the angiogenic cascade by qPCR in the different treatment groups revealed a significant time-dependent decrease in \( \text{Vegfr2} \) and \( \text{Tie2} \) expression most likely reflecting the reduction of the absolute numbers of EC upon treatment. \( \text{Vegfa} \) expression increased gradually over time indicating a compensatory upregulation mechanism in response to the anti-angiogenic effects. Surprisingly, the Tie2 ligands Ang1 and Ang2 were most dynamically regulated being strongly downregulated as rapidly as 24 h after initiation of therapy (Fig. 6D).

IGF1/IGF1R/IRS signaling is a potential driver of tumor progression during Sorafenib treatment

We performed microarray analyses of iAST tumors from the different experimental groups in order to identify additional mechanisms of action to Sorafenib treatment over time (Supplementary Figure S9; original data deposited in the Gene Expression Omnibus (GEO) database; accession no. GSE54857). Sorafenib treatment led to an increase of insulin-like growth factor 1 (\( \text{Igf1} \)) (Fig. 7A, B) and \( \text{cMet} \) expression (Fig. 7B), whereas \( \text{Hgf} \) expression was decreased after 14 d as well as after long-term treatment (Fig. 7B). Correspondingly, following a sharp decline of circulating IGF1 upon tumor induction, Sorafenib treatment led to an increase of circulating IGF1 over time (Fig. 7C). Concomitantly, analysis of IGF1R receptor expression in the iAST model revealed a prominent gradual increase of IGF1R in tumor nodules during long-term Sorafenib treatment (Fig. 7D). Activated Insulin...
Receptor Substrate (IRS), the substrate for active IGF-1R signaling, was correspondingly upregulated in the long-term Sorafenib-treated samples compared to the 14d Sorafenib-treated samples as evidenced by Western blot analysis of tumorigenic liver lysates (Supplementary Figure S10). Similarly, phospho-Akt was upregulated in the LT-Sorafenib-treated samples indicating that the tumorigenic areas in these samples regained proliferative potential. To validate the increase in IGF1 as a putative mechanism of Sorafenib to overcome tumor growth reduction, lower doses of Sorafenib (20 mg/kg and 40 mg/kg) were given to late stage mice (upon MRI detection). While 20 mg/kg resulted on average in a survival advantage of 6 days, 40 mg/kg resulted in a more significant increase in survival (Fig. 7E). Mice receiving 40 mg/kg Sorafenib were divided in two groups: One group (progressing) was analyzed at end stage, when the abdomen was increased in size and the second group (slow progressing) was analyzed before end stage (no visible swelling of the abdomen). Side effects such as severe weight loss were not observed in these two groups (Supplementary Figure S11). As evidenced in Fig. 7F and G, Igf1 mRNA and protein from whole liver lysates were analyzed by qPCR and ELISA, respectively, and revealed an increase in Igf1 mRNA and protein level in the ‘progressing’ group and more so in the ‘slow progressing’ group. Taken together, the data provide evidence for a possible role of the IGF1/IGF1R/IRS signaling as a possible escape mechanism during continuous Sorafenib treatment.
Discussion

The dramatic developments in cancer genome analysis lead to the discovery of tumor targets at an unprecedented pace. In turn, the translation of preclinical target identification and validation research into novel therapies is slow and as inefficient as never before. Preclinical mouse tumor models play a central role in the clinical translation of such targets. Yet, the limited availability of mouse models that truthfully mimic the course and progression of human tumors and their response to therapy has emerged as one of the most rate-limiting bottlenecks of translational oncology research.

There is also need for better mouse tumor models because the information flow is increasingly not going uni-directional from bench to bedside, but in circle from the bedside back to the bench when clinical observations require mechanistic analysis in defined experimental models (36). Likewise, the advancement of personalized medicine has promoted the concept of co-clinical trials, which similarly depends on the availability of robust and reliable preclinical tumor models (37).

The bench-to-bedside-and-back paradigm has prototypically shaped the field of anti-angiogenesis research. Anti-angiogenic therapy was originally pioneered in preclinical tumor models to starve tumors to death by driving the intratumoral vasculature into regression (38). Yet, only the clinical translation of anti-angiogenic therapy has revealed that vessel normalization by selective pruning of immature tumor vessels and subsequent facilitation of chemotherapy is a major determinant of anti-angiogenic therapy in human tumors (39). Today, the clinical efficacy of anti-angiogenic tumor therapy continues to be rather modest and further advancements such as the rational, mechanism-based implementation of anti-angiogenic combination therapies are hampered by the limited availability of relevant tumor models.

We have in the present study characterized the angiogenic properties of the recently established Cre recombinase-inducible albumin promoter - SV40 T-antigen transgenic model (iAST) of hepatocellular carcinogenesis (22) and exploited it for the study of the anti-angiogenic and anti-tumorigenic
properties of Sorafenib on HCC. Tumor growth is in this model initiated by the intravenous injection of Cre recombinase-expressing adenovirus. The adenoviral transfer selectively targets hepatocytes, in which Cre recombinase excises a floxed stop cassette, leading to expression of the T-antigen under the control of the albumin promoter. Unlike constitutive T-antigen HCC models, this approach gives the model a number of unique properties that give it robustness and versatility: First, the inducible character enables a unique temporal versatility allowing tumor initiation even in aged or in pretreated mice (e.g., tumor initiation on top of CCl$_4$-induced fibrosis). Second, titration of adenoviral dosage allows calibration of the system leading to tumor development with 100% penetrance in a multifocal manner resembling the development of human HCC. Third, the multifocal character facilitates comparison of different tumor stages in a single tumorigenic liver. Fourth, combined with non-invasive imaging techniques, the model can conveniently be employed for therapy experiments.

Considering the intense vascularization of HCC, we focused our temporal analysis of the iAST model on vascular changes during tumor progression. As the key driver of angiogenesis in most tumor models, VEGF expression was upregulated during tumor progression. Furthermore, both, Ang1 and Ang2 were remarkably upregulated in tumorigenic livers. The intense angiogenic activity was associated with distinct processes of vessel cooption, remodeling and vascular expansion during tumor progression with accompanying phenotype changes of the angiogenic vasculature. Previous work had shown that EC transdifferentiation is a major pathogenic event in human HCC and in the iAST model in the transition from cooptive to angiogenic tumor vascularization characterized by the loss of differentiation markers of LSEC (40). The present study identified an increasing vascular immaturity during tumor progression characterized by distinct pericyte populations with an immature gene expression signature (Desmin low/αSMA high), which has previously been reported in other tumor models including the RipTag model (32, 41). Interestingly, these changes occurred in the absence of major changes in intratumoral microvessel density. Moreover, unlike many other tumor models, intratumoral vessels in the iAST model were well perfused and not leaky during early nodule formation.
Sorafenib treatment of iAST tumor nodules resulted in very rapid anti-vascular effects evident as early as 24 h after initiation of therapy. Sorafenib treatment appeared to drive intratumoral EC into rapid regression leaving behind type IV collagen-positive empty sleeves, which were still filled with pericytes. Selective VEGFR blockade in a spontaneous RipTag2 mouse model has similarly been shown to result in EC-depleted vessels sparing the pericytes (42). Yet, Sorafenib targets pericytes by inhibiting PDGFRβ, which has been demonstrated in vitro before (43). This led to altered expression of the pericyte markers αSMA, Desmin, NG2 and CD248. These transcriptional changes could reflect drop-out or reduced motility of pericytes (44). The number of pericytes decreased throughout the 14-day treatment. Yet, the overall effect was less severe compared to EC. Recently, inhibition of pericytes in addition to EC in the tumor vasculature has been shown to exert beneficial therapeutic effects (45). It needs further analysis to what extend the remaining pericytes might contribute to the development of resistance, metastasis or other pathways that could stimulate tumor regrowth.

Sorafenib is currently the only systemic treatment for non-resectable late stage HCC patients. Yet, the clinical efficacy is limited and resistance eventually develops as it has been observed in preclinical models (32). It is noteworthy that pro-oncogenic factors such as IGF1/IGF1R (46), cMet (47) and Id1 (48) were up-regulated in the iAST model upon Sorafenib treatment. Currently, several clinical trials inhibiting cMet and VEGFR together are underway (49) and results await the outcome regarding tumor behavior and patient survival. Importantly, IGF1 levels increased gradually during the response to treatment before end stage and before developing severe side effects. As inhibition of the IGF1/IGF1R pathway has been shown to reduce HCC growth in vivo (50), it is an important finding concerning Sorafenib therapy. The iAST model may therefore be suitable to further advance combination therapies and to systematically screen for emerging resistance mechanisms during therapy.

In conclusion, we describe in the present study the unique properties of the inducible iAST HCC tumor model as a versatile experimental system for the study of HCC progression and the response to therapy.
Authors’ contribution


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References


Figure legends

Figure 1: Formation of tumor nodules of varying sizes following tumor induction by adenoviral Cre delivery. (A) Representative images of HE and the T-cell marker CD3 stainings of paraffin-embedded liver sections showing tumorous transformation (arrows) as well as an acute viral hepatitis with T-cell infiltrations (arrowheads) 2 weeks after adenoviral Cre infection. (B) Representative 2D CT images of mouse livers 7 (left) and 9 weeks (right) after adenoviral Cre infection (L, normal liver tissue; St, stomach; n, tumor nodule). (C) 3D reconstruction of a CT image series in (B); arrows indicate small nodules at 7 weeks after tumor induction that have increased in size after 9 weeks (S, spleen). (D) HE staining of paraffin embedded liver section (left) showing heterogeneous nodules of different sizes (dotted lines); note clear cell and basophilic nodules that are similarly observed in human HCC. Green fluorescently stained SV40 TAg in cryosection (right) showing positive labeled transformed hepatocytes (dotted lines) in tumorigenic iAST livers, small box with higher magnification. Note, non-tumorigenic cells (arrows) and the typical flat shaped nucleus of endothelial cells (arrowheads) are void of SV40 TAg staining. (E) Glypican3, and (F) HSP70 staining of mouse (left) and human (right) paraffin-embedded liver sections showing positive staining (arrowheads) in the tumorigenic areas of mouse and human HCC nodules (T, tumorigenic area; CV, central vein).

Figure 2: Expression of angiogenic factors and vascular characteristics during iAST-induced hepatocellular carcinoma progression. (A) VEGFA ELISA showing increased levels of circulating VEGF in the serum of tumor-bearing mice. (B) qPCR showing upregulated expression of Ang1 and Ang2 in livers with tumor nodules (N=3-4 per group). (C) Western blot analysis displaying increased protein levels of Ang1 and Ang2 in tumorigenic whole liver lysates. (D) Quantitative analysis of the intratumoral microvasculature revealing no changes in tumor vascular area in different size tumor nodules (<0.1, 0.1-0.2 and >0.2 mm²) (N=5-6, n=10-20 nodules per experimental group). (E) Representative images of cryosections double-stained for CD31 (green) and type IV collagen (red). (F) Quantitative analysis of type IV collagen and CD31 co-stained areas (N=5-6, n=20-40 nodules per experimental group). (G) Representative images of cryosections double-stained for CD31 (green) and
Desmin (red). (H) Quantitative analysis of Desmin-covered microvessels of total intratumoral CD31-positive microvessels (N=5-6, n=10-20 nodules per experimental group). (I) Representative images of cryosections double-stained for CD31 (green) and αSMA (red). (J) Quantitative analysis of αSMA-covered microvessels of total intratumoral CD31-positive microvessels (N=5-6, n=10-20 nodules per experimental group). Scale bar: 200 μm; ELISA data were analyzed by Mann-Whitney U test (N=4-5).

* p<0.05, ** p<0.01, *** p<0.001; error bar: SD; n, tumor nodule; CV, central vein.

**Figure 3: Analysis of the effect of Sorafenib on tumor growth in the iAST model.** (A) Representative MR images of livers with detectable tumor nodules (arrowheads) at the start of Sorafenib treatment. (B) Representative images of livers from normal non-treated mice, vehicle- and Sorafenib-treated mice 14 days after treatment initiation (asterisk in A and B shows the same nodule). (C) Macroscopic images of livers from normal non-treated, vehicle- and Sorafenib-treated mice obtained 14 days after initiation of Sorafenib. Note reduced overall tumor burden in the Sorafenib-treated liver. (D) Quantitative analysis of tumor growth in vehicle- and Sorafenib-treated mice (100 mg/kg) (N=7-8 per group). (E) Microcast of livers from normal non-treated, vehicle- and Sorafenib-treated mice displaying the vascular architecture of the liver vascular tree. (F) Kaplan-Meier curve of vehicle-treated mice (black line), mice with early onset of Sorafenib treatment (blue line; initiation of therapy 5 weeks after adenoviral Cre delivery) and late onset of treatment (red line; initiation of therapy upon MRI detection of tumor nodules) (60 mg/kg) (N=7-11 per group). (G) qPCR of Hbegf, Hgf, and cMet in normal mice, as well as in vehicle-treated and Sorafenib-treated tumor bearing mice (N=5 per group). *p<0.05, **p<0.01, ***p<0.001; error bar: SD; L, left; R, right; Sp, spinal cord; St, stomach; A, aorta; Gb, gall bladder.

**Figure 4: Changes of angiogenic growth factor and receptor expression upon Sorafenib treatment.**

(A-G) Western blot analysis of bRaf (A), cRaf (B), p44/42 MAPK (C), Akt (D), VEGFR2 (E), Ang1, Ang2, Tie2 (F), and PDGFRβ (G) expression in whole liver lysates from normal mice as well as vehicle- and Sorafenib-treated tumor-bearing mice. Sorafenib treatment had minor effects on bRaf, cRaf and p44/42 MAPK expression. Yet, phospho-Akt levels were strongly reduced. Likewise, phospho-VEGFR2...
and phospho-PDGFRβ levels were strongly reduced as well as the expression of total VEGFR2, Ang1 and Ang2, and total PDGFRβ upon Sorafenib treatment. (H) qPCR analysis of Vegfa, Vegfr2, Ang1, Ang2 and Tie2 expression in normal mice, as well as vehicle- and Sorafenib-treated tumor-bearing mice demonstrating reduced expression of Vegfr2, Ang2 and Tie2 mRNA upon Sorafenib treatment, whereas Vegfa was significantly upregulated upon treatment (N=5 per group). The upregulation of VEGFA upon Sorafenib treatment was validated on the protein level in the serum (I) as well as in lysates of liver tissue (J) (N=5-7 per group). *p<0.05, **p<0.01, ***p<0.001; error bar: SD;

**Figure 5: Sorafenib treatment results in vascular regression with loss of endothelial cells and maintained pericytes.** (A) qPCR analysis of livers from non-treated normal mice as well as vehicle- and Sorafenib-treated mice showing a significant downregulation of the pericyte markers αSMA, Desmin, PDGFRβ, NG2 and CD248 (N=5 per group). (B, C) Quantitative analysis of the vasculature in livers of vehicle- and Sorafenib-treated mice showing significant reduction of vascular area (B) and branchpoints (C) upon Sorafenib treatment (N=5-6 per group). (D) Representative OPT Bioptonic images of livers stained by immunofluorescence for αSMA showing a preserved architecture of the large vascular trees upon Sorafenib treatment. (E, H, K) Representative images of liver cryosections from vehicle- or Sorafenib-treated mice double-stained for CD31 (green) and αSMA (red) (E), CD31 (green) and Desmin (red) (H), and CD31 (green) and type IV collagen (red) (K). The arrowheads mark EC-free type IV collagen-positive sleeves (K). (F, G, I, J, L, M) Quantitative analysis of αSMA-covered CD31-positive intratumoral microvessels (F, G), Desmin-covered CD31-positive intratumoral microvessels (I, J), and relative ratio of type IV collagen staining to CD31 staining (L, M) (N=5-6 per group). The relative ratio of αSMA-positive (F), Desmin-covered (I), and type IV collagen-positive (L) intratumoral microvessels was significantly reduced upon Sorafenib treatment. Yet, the percentage of αSMA-positive (G) and Desmin-positive (J) cells not in contact with CD31-positive EC was significantly increased upon Sorafenib treatment. Likewise, the number of EC-empty type IV collagen-positive sleeves was significantly increased upon Sorafenib treatment (M). Scale bar: 200 μm (E, H, K); 1 mm (D). *p<0.05, **p<0.01, ***p<0.001; error bar: SD; T, tumorigenic area.
Figure 6: Rapid vascular changes upon Sorafenib treatment. (A-C) Confocal images of CD31/cleaved caspase3 double stained cryosections from tumorous livers 12, 16, and 24 h after induction of Sorafenib treatment showing vascular regression with little evidence of apoptotic EC death. (B) Confocal images of triple-stained cryosections (CD31/Desmin/type IV collagen) from tumorous livers of vehicle-treated and 24 h Sorafenib-treated mice showing a reduction of microvessel density with remaining Desmin-positive pericytes in type IV collagen-positive empty sleeves (arrowheads). (C) Confocal images of CD31-stained cryosections from tumorous livers of vehicle-treated and 6 d Sorafenib-treated mice showing stable vessels with vascular pruning and few capillary sprouts (arrowheads) following treatment. (D) qPCR analysis of Vegfa, Vegfr2, Ang1, Ang2, and Tie2 at different times after vehicle and Sorafenib treatment (N=5 per group). Scale bar: 50 μm. *p<0.05, **p<0.01, ***p<0.001; error bar: SD; n, tumor nodule.

Figure 7: IGF1 evolves as potential tumor escape mechanisms upon low dose and long-term Sorafenib treatment. (A) Microarray heat map showing Igf1 upregulation upon long-term Sorafenib treatment. (B) qPCR analysis of Igf1, Id1, Hgf, and cMet mRNA expression showing upregulation of Igf1 and Id1 expression upon long-term Sorafenib treatment. In turn, Hgf was downregulated upon Sorafenib treatment and expression of cMet was transiently increased (N=4-5 per group). (C) ELISA determination of IGF1 serum concentrations from 14 days- and long-term Sorafenib-treated mice confirming the time-dependent increase of IGF1 expression compared to vehicle controls (N=4-6 per group). (D) Expression of IGF1R in liver sections from vehicle-treated as well as 24 h, 14 d and long-term Sorafenib-treated mice showing increased IGF1R expression in the 14 day- and long-term Sorafenib-treated samples (arrowheads; ‘*’, Central vein). (E) Kaplan-Meier survival curve of vehicle-treated mice (N=4; black line), mice with 20 mg/kg (N=17; red line) and 40 mg/kg (N=14; blue line) Sorafenib treatment (initiation of therapy upon MRI detection of tumor nodules). (F) qPCR analysis of Igf1 mRNA expression showing upregulation of Igf1 expression in progressing (end stage) and higher Igf1 expression in slow progressing (before end stage) 40 mg/kg Sorafenib-treated mice. (G) IGF1 ELISA of liver lysates confirming the increase of IGF1 expression in the slow progressing tumorigenic
mice upon 40 mg/kg Sorafenib treatment. Scale bar: 500 μm; Microarrays were performed on Illumina chips (N=3-4 per group) and analyzed using Chipster software. *p<0.05, **p<0.01, ***p<0.001; error bar: SD; T, tumorigenic area.
Runge et al. - Fig. 5
**A**

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**Runge et al. - Fig. 6**

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An inducible hepatocellular carcinoma model for preclinical evaluation of anti-angiogenic therapy in adult mice

Anja Runge, Junhao Hu, Matthias Wieland, et al.

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