

The Angiogenic Makeup of Human Hepatocellular Carcinoma Does Not Favor Vascular Endothelial Growth Factor/Angiopoietin-Driven Sprouting Neovascularization

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Quantitative data on the expression of multiple factors that control angiogenesis in hepatocellular carcinoma (HCC) are limited. A better understanding of the mechanisms underlying angiogenesis in HCC will improve the rational choice of anti-angiogenic treatment. We quantified gene and protein expression of members of the vascular endothelial growth factor (VEGF) and angiopoietin systems and studied localization of VEGF, its receptors VEGFR-1 and VEGFR-2, Angiopoietin (Ang)-1 and Ang-2, and their receptor, in HCC in noncirrhotic and cirrhotic livers. We employed real-time reverse transcription polymerase chain reaction (RT-PCR), western blot, and immunohistology, and compared the outcome with highly angiogenic human renal cell carcinoma (RCC). HCC in noncirrhotic and cirrhotic livers expressed VEGF and its receptors to a similar extent as normal liver, although in cirrhotic background, VEGFR-2 levels in both tumor and adjacent tissue were decreased. Ang-1 expression was slightly increased compared with normal liver, whereas Tie-2 was strongly down-regulated in the tumor vasculature. Ang-2 messenger RNA (mRNA) levels were also low in HCCs of both noncirrhotic and cirrhotic livers, implying that VEGF-driven angiogenic sprouting accompanied by angiopoietin-driven vascular destabilization is not pronounced. In RCC, VEGF-A levels were one order of magnitude higher. At the same time, endothelially expressed Ang-2 was over 30-fold increased compared with expression in normal kidney, whereas Ang-1 expression was decreased. **Conclusion:** In hepatocellular carcinoma, tumor vascularization is not *per se* VEGF/angiopoietin driven. However, increased CD31 expression and morphological changes representative of sinusoidal capillarization in tumor vasculature indicate that vascular remodeling is taking place. This portends that therapeutic intervention of HCC at the level of the vasculature is optional, and that further studies into the molecular control thereof are warranted. (HEPATOLOGY 2008;48: 1517-1527.)

Abbreviations: Ang, Angiopoietin; HCC, hepatocellular carcinoma; mRNA, messenger RNA; RCC, renal cell carcinoma; RT-PCR, reverse transcription polymerase chain reaction; SEC, sinusoidal endothelial cells; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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Hepatocellular carcinoma (HCC) is the fifth most common tumor worldwide, and the third cause of cancer-related death.¹ The grave prognosis of HCC is a consequence of the absence of adequate curative options. Therapeutic possibilities are seriously incapacitated by the fact that most HCC develops in patients with cirrhotic livers, which is the end stage of a chronic liver disease.² Liver transplantation, partial hepatectomy, or local ablation are the only intentionally curative treatment options but are restricted to highly selected patient categories, and the overall survival in general remains poor.^{3,4} In addition, chemotherapeutic possibilities are limited.⁵ Advances in tumor biology have, however, in recent years led to the development of novel therapeutics, among others agents that affect tumor-controlled angiogenesis, several of which are currently under clinical trials.⁶ Evidence suggests that in different tumor types differential angiogenic activity can be present and that angiogenic activity is also dependent on the site of growth in different host organs. As a consequence, responsiveness to anti-angiogenic therapy can be differentially controlled.⁷ Insight into the molecular angiogenic features of HCC is therefore pivotal for selection of the proper drug class and treatment regimen tailored for the disease.

HCC is regarded as a hypervascular tumor, a property that is applied as a radiological characteristic in diagnosing HCC.⁸ Furthermore, numerous studies reported high microvessel density counts and the expression of vascular endothelial growth factor (VEGF) and other angiogenic factors.^{9,10} It remains unclear however, whether these parameters reflect an active, pro-angiogenic phenotype of HCC. In a previous study, we found that most microvessels in HCC consist of mature vessels, as evidenced by pericyte coverage, with a concurrent low rate of endothelial cell proliferation and apoptosis of the tumor microvessels.¹¹ These findings brought us to hypothesize that active angiogenesis in HCC is less robust than formerly assumed. Because HCC is a malignant transformation in which proliferation of malignant hepatocytes takes place in a well-vascularized niche, abundant vascularization of HCC could result from co-option or remodeling of the preexisting hepatic sinusoidal network, rather than from neo-vessel formation. Knowledge regarding the true angiogenic status is of essential importance in the choice of therapy.

The current working model of angiogenic sprouting describes VEGF-induced VEGF-Receptor (VEGFR)2 signaling concomitant with vessel destabilization via the angiopoietin/Tie-2 system. In this model, overexpression of angiopoietin (Ang)-2 as the dynamic component of the Ang/Tie-2 system, competes with Ang-1 for Tie-2 binding, resulting in inhibition of Tie-2 phosphorylation. As a

consequence, the endothelium becomes prone to VEGFR-induced signaling.¹² To determine the angiogenic status of human HCC in cirrhotic and noncirrhotic livers, we investigated gene and protein expression levels of VEGF-A, VEGFR-1, VEGFR-2, Ang-1, Ang-2, and their receptor tyrosine kinase Tie-2 using quantitative (real-time) reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis in tumors, adjacent liver, and normal liver tissues. The cellular localization of the proteins was by immunohistochemistry assigned to designated cell types. We compared the outcome with expression of the same angiogenic factors in renal cell carcinoma (RCC), which represents one of the most angiogenic human tumors known.¹³

Patients and Methods

Patients and Tissue Samples. All procedures and use of (anonymized) tissue were performed according to recent national guidelines. Tissue samples of 55 HCCs obtained from 21 noncirrhotic and 34 cirrhotic livers were included. Demographic data are detailed in Table 1. HCC samples were taken at the periphery of a tumor nodule to avoid necrotic areas, and separate from adjacent nontumorous tissue. The 34 cirrhotic samples were harvested from explanted livers after orthotopic liver transplantation. The 21 noncirrhotic patients underwent a partial hepatectomy. Samples of histologically normal liver (n = 9), obtained from surplus donor liver, or partial liver resection for benign disorders, were used as controls. Both for immunohistochemistry to localize protein expression, and for real-time RT-PCR and western blot to quantify gene protein expression, the same tissue blocks were used, except for CD31 and CD34 immunohistochemistry, which was performed on paraffin sections.

Samples of RCC (n = 5) were included as reference for a tumor type that is considered highly angiogenic. Similar to HCC livers, adjacent nontumorous renal tissue and samples of normal renal tissue were included (n = 2). The latter were obtained from discarded donor kidneys.

Quantitative RT-PCR for Messenger RNA Analysis. Total RNA was isolated with the RNeasy Mini Kit (Qiagen, Leusden, Netherlands) with subsequent DNA removal using the RNase-free DNase set (Qiagen), both according to the protocol of the manufacturer. RNA was analyzed qualitatively by gel electrophoresis and quantitatively by Nanodrop ND-100 spectrophotometry (NanoDrop Technologies, Rockland, DE) and was consistently found to be intact and protein free. Reverse transcription and real-time PCR were performed as described previously,¹⁴ using 1 μ g total cellular RNA for the synthesis of first-strand complementary DNA and 10 ng

Table 1. The Demographic Data of Patients with HCC Studied

	HCC in Noncirrhotic Liver	HCC in Cirrhotic Liver	P Value
Male/female	13/8	27/7	NS
Age	56.9 ± 17.5	54.5 ± 14.9	NS
Main tumor diameter 0-5 cm versus >5 cm	2/19	25/9	<0.01
Venous invasion Yes versus No	15/6	15/19	NS
Edmondson grade I and II versus III and IV	13/8	25/9	NS
	α1-Anti-trypsin	0	1
	Neonatal hepatitis	0	1
	Cryptogenic	0	3
	Glycogen storage disease	0	1
	HCV	0	9
	HBV/HCV	0	3
Liver disease on which HCC developed	HBV	0	7
	Primary biliary cirrhosis	0	1
	Tyrosinemia	0	1
	Alcohol	0	4
	Non-alcoholic steatohepatitis	0	1
	Budd-Chiari	0	1
	Hemochromatosis	0	1

complementary DNA for each PCR reaction. Exons overlapping primers and minor groove binder probes for real-time RT-PCR were purchased as assay-on-demand from Applied Biosystems (Nieuwekerk a/d IJssel, The Netherlands): housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (assay ID Hs99999905_m1), VEGF (Hs00173626_m1), VEGFR-1 (Hs00176573_m1), VEGFR-2 (Hs00176676_m1), Tie2 (assay ID Hs00176096_ml), Ang-1 (assay ID Hs00181613_ml), Ang-2 (assay ID Hs00169867_ml). All PCR reactions were performed in triplicates. Control samples of distilled water and isolated RNA not subjected to reverse transcription were consistently found to be negative. TaqMan quantitative RT-PCR was performed in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). Gene expression was normalized to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase, yielding the relative gene expression value.

Quantification of Angiogenic Proteins by Western Blot. Of each frozen tissue block, 20 samples of 5- μ m-thick tissue slices were lysed in radioimmunoprecipitation assay buffer [50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% nonidet P-40, 0.25% Na-deoxycholate, 1 mM ethylene diamine tetraacetic acid, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 100 g/mL phenylmethylsulfonyl fluoride, 1 g/mL aprotinin (Sigma), and 1 g/mL leupeptin (Roche), and 1 g/mL pepstatin (Roche)]. Cell debris was removed by centrifugation at 10,000g for 15 minutes, and protein concentration was measured using pyrogallol red-molybdate solution. Twenty to forty micrograms protein lysates were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (0.45 μ m, Bio-Rad laboratories; Hercules, CA). The membranes were next probed with primary antibodies at the indicated dilutions (Table 2) followed by incubation with horseradish peroxidase–

Table 2. Antibodies Used in Western Blot (WB) and Immunohistochemistry (IHC)

Antibody	Dilution for WB*	Dilution for IHC	Company	Code Number
Rabbit anti-VEGF-A (A-20)	1/1000	1/100†	Santa cruz	sc-152
Goat anti-Ang-1 (N-18)	1/2000	1/100†	Santa cruz	sc-6319
Goat anti-Ang-2 (F-18)	1/3000	1/50†	Santa cruz	sc-7017
Rabbit anti-Tie2 (C-20)	1/300	1/50†	Santa cruz	sc-324
Rabbit anti-VEGFR-1		1/100†	Abcam	ab2350
Rabbit anti-VEGFR-2		1/100†	Abcam	ab2349
Mouse anti- β -actin	1/3000		Abcam	ab8226
Mouse anti-CD34		Ready to use	Immunotech	1185
HRP-conjugated rabbit anti-mouse Ig	1/1000	1/100‡	Dako	P0260
HRP-conjugated goat anti-rabbit Ig	1/1000	1/100‡	Dako	P0448
HRP-conjugated rabbit anti-goat Ig	1/1000	1/100‡	Dako	P0160

*Diluted in 5% nonfat milk/0.1% TBST; †Diluted in 1% BSA/PBS; ‡Diluted in 1% BSA/PBS+1% human albumin. Abbreviations: HRP, horseradish peroxidase; Ig, immunoglobulins.

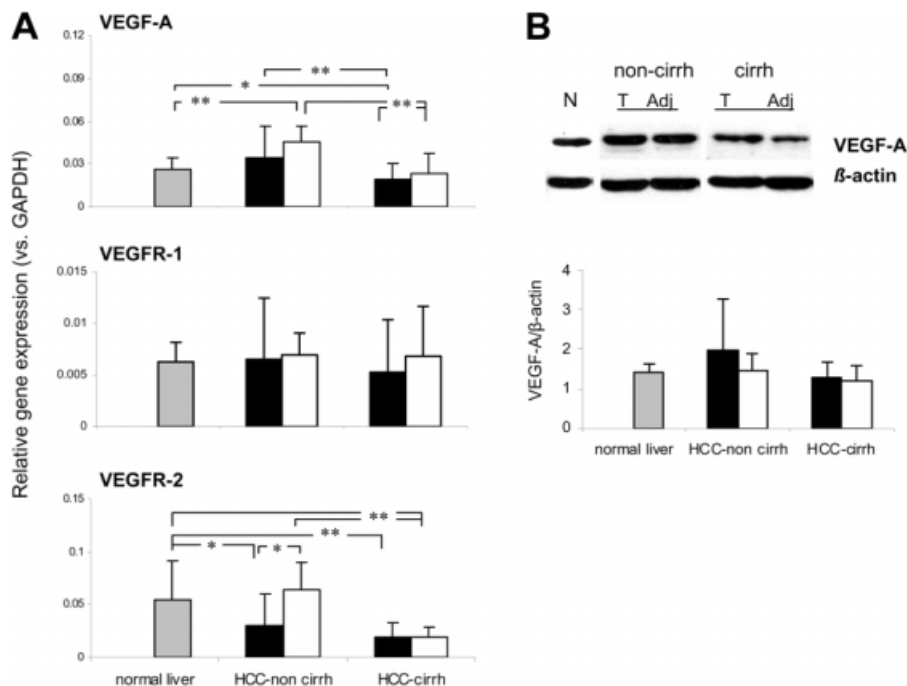


Fig. 1. VEGF and its receptors are not up-regulated in HCC. (A) Relative gene expression of VEGF-A, VEGFR-1, and VEGFR-2 in normal livers ($n = 9$), in HCC in noncirrhotic livers ($n = 21$) and their respective adjacent tissues ($n = 11$), and in HCC in cirrhotic livers ($n = 34$) and their respective adjacent liver tissues ($n = 23$), as determined by quantitative RT-PCR. (B) Upper graph: representative VEGF-A western blot of five different samples and their β -actin loading controls (N, normal liver; T, tumor; Adj, corresponding adjacent nontumorous liver). Lower graph: relative VEGF-A protein levels in normal livers ($n = 9$), HCC in noncirrhotic livers and their adjacent livers ($n = 11$), HCC in cirrhotic livers and their adjacent livers ($n = 22$), as detected by western blot. Values are given as mean; error bars represent the standard deviations. * $P < 0.05$; ** $P < 0.01$; gray bars, normal liver; black bars, tumor; white bars, adjacent to tumor.

labeled secondary antibodies and treated with an enhanced chemiluminescent substrate for detection (Amersham Life Science, London, UK). After detection of protein of interest, the membranes were stripped with stripping buffer (200 mM glycine, 1% sodium dodecyl sulfate, pH 2.0) and stained for β -actin as loading control. Protein bands (intensity \times mm²) were quantified using image analysis software (Quantity One, Bio-Rad). The volume of each sample was divided by the volume of β -actin, yielding the protein expression value presented in the figures. Some HCC and liver tissue adjacent to tumor samples were left out from the analysis because insufficient amounts of protein were isolated for follow-up by western blot.

Immunohistology to Identify Cellular Location of Protein Expression. All antibodies and concentrations used for immunohistology are summarized in Table 2.

For VEGF-A, VEGFR-1, VEGFR-2, Ang-1, Ang-2, and Tie2, 5- μ m sections of frozen tissues were mounted on slides and dried overnight at room temperature. After fixation in acetone for 10 minutes, slides were incubated with primary antibodies at 4°C overnight. Then endogenous peroxidase was blocked with 0.08% H₂O₂ for 30 minutes, followed by incubation with horseradish peroxidase-conjugated secondary and tertiary antibodies. 3-Amino-9-ethylcarbazole was used to develop the staining reaction, and sections were counterstained with hematoxylin, then sections were mounted with Kaiser's glycerine-gelatin. CD34 and CD31 were stained on formalin-fixed tissue using clone Qbend 10 (Immunotech Marseille, France), JC/70A (DAKO, Glostrup, Den-

mark), in a method as described previously,¹⁵ with a 30-minute protease pretreatment for CD31.

Statistics. Quantitative data were expressed as mean \pm standard deviation. For data that did not show a normal distribution, logarithmic transformation of the data was performed. Independent-samples t test was used to compare the mean values between groups, and paired-samples t test was used to compare the mean values between tumors and adjacent tissues. The chi-squared test was used to analyze the relationship between categorical variables. For all statistical analyses, the level of significance was set at 0.05. SPSS 15.0 statistical software for windows (SPSS Inc., Chicago, IL) was used for all analyses.

Because of the small number of normal kidney samples available, a statistical analysis could not be performed on normal kidney and RCC data generated.

Results

In HCC Neither VEGF-A Nor VEGFRs Are Up-Regulated. Results of quantitation of messenger RNA (mRNA) and protein levels of the VEGF system as analyzed by real-time RT-PCR and western blot, respectively, are summarized in Fig. 1. In HCC of both cirrhotic and noncirrhotic livers, VEGF-A and its receptors were present, and their levels of expression were only moderately different from those in normal livers (VEGFR-1 and VEGFR-2 western blot; data not shown). When comparing within the cirrhotic group HCC in viral-infected background with HCC in non-viral-infected back-

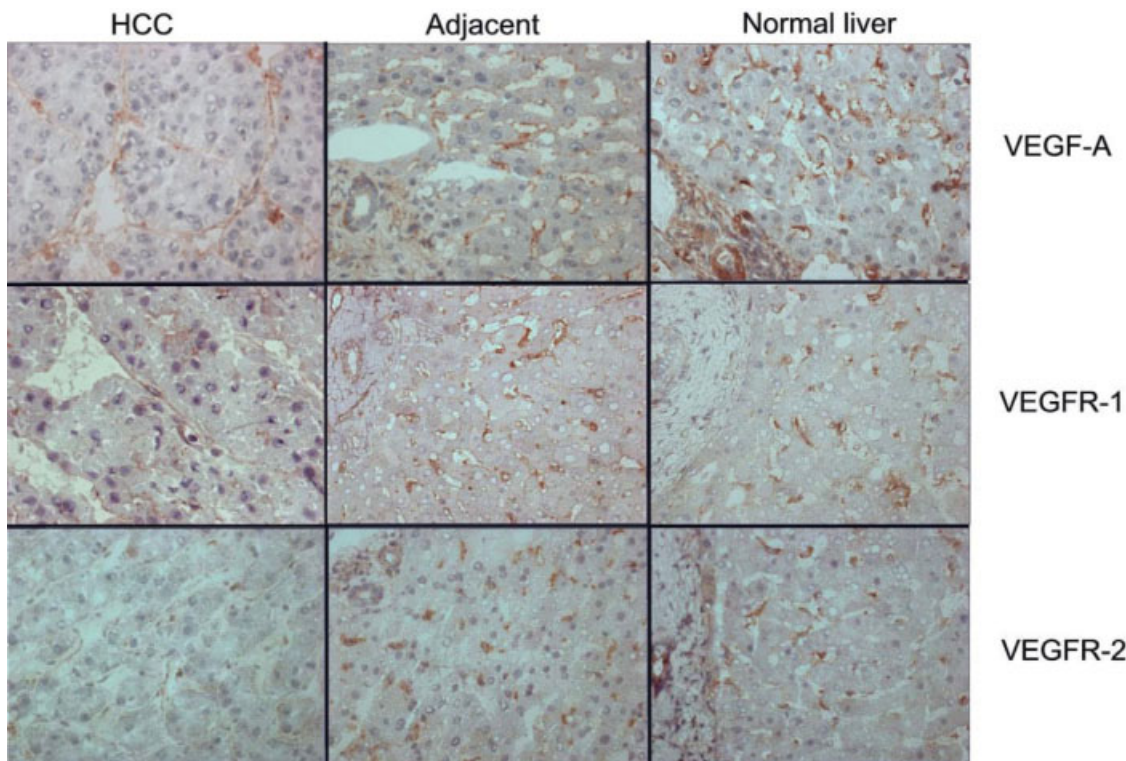


Fig. 2. Localization of VEGF and VEGFR expression in HCC. Immunostaining of VEGF-A, VEGFR-1, and VEGFR-2 in HCC, its adjacent nontumor liver tissue, and normal liver (original magnification 100 \times).

ground, no differences in gene and protein expression levels could be detected (Supporting Fig. 1).

Immunohistologically, VEGF-A positive staining in normal livers was seen in endothelial cells of portal veins, hepatic arteries, terminal hepatic venules, and sinusoidal endothelial cells, as well as in the wall of hepatic arteries and in bile duct and ductular epithelial cells, but not in hepatocytes. In HCC, VEGF-A was located in endothelial cells within the tumor, although the staining intensity was less pronounced compared with that in normal liver and nontumorous parenchyma adjacent to the tumor (Fig. 2). Tumor cells were negative.

In normal livers the expression pattern of VEGFR-1 was similar to that of VEGFR-2; both were mainly associated with Kupffer cells (Fig. 2). Only a weak expression was observed in endothelial cells including sinusoidal endothelial cells as well as in vessel walls. In HCC, VEGFR-1 and VEGFR-2 were only weakly expressed in some tumor endothelial cells whereas all other structures were negative. Nontumorous liver tissue adjacent to HCC both in noncirrhotic and cirrhotic livers showed a similar pattern as in normal liver. In cirrhotic cases, stromal cells in fibrous septa expressed VEGFR-1 and VEGFR-2.

Ang-1 and Ang-2 Expression Are Modestly Changed in HCC. The Ang-1 mRNA expression level was

slightly, statistically significantly higher in HCC of both noncirrhotic and cirrhotic livers and adjacent liver tissue in cirrhotic liver as compared with control livers with normal histology (Fig. 3A). Accordingly, Ang-1 protein levels were slightly higher in noncirrhotic HCC (Fig. 3B). There was no significant difference between HCCs and adjacent liver tissues at either mRNA or the protein level (Fig. 3A, B). Although Ang-2 mRNA expression levels in HCC of cirrhotic livers were not significantly altered as compared with the adjacent liver and normal liver tissues, a statistically significant yet small increase in mRNA of Ang-2 was observed in HCC of noncirrhotic livers compared with adjacent and normal control liver (Fig. 3A). Also, the Ang-2 mRNA level was higher in HCC in noncirrhotic liver as compared with HCC in cirrhotic liver. In all liver and HCC samples, Ang-2 protein was below the western blot detection limit (Fig. 5). Comparison of virus-infected versus non-virus-infected HCC in a cirrhotic background revealed a threefold lower mRNA expression level of Ang-1 in virus-infected HCC, whereas Ang-2 mRNA did not differ between groups. The lower Ang-1 mRNA level was not paralleled by lower protein expression (Supporting Fig. 1).

Immunohistologically, Ang-1-positive cytoplasmic staining was seen in HCC cells and hepatocytes in adjacent and normal livers, with weak staining in vascular

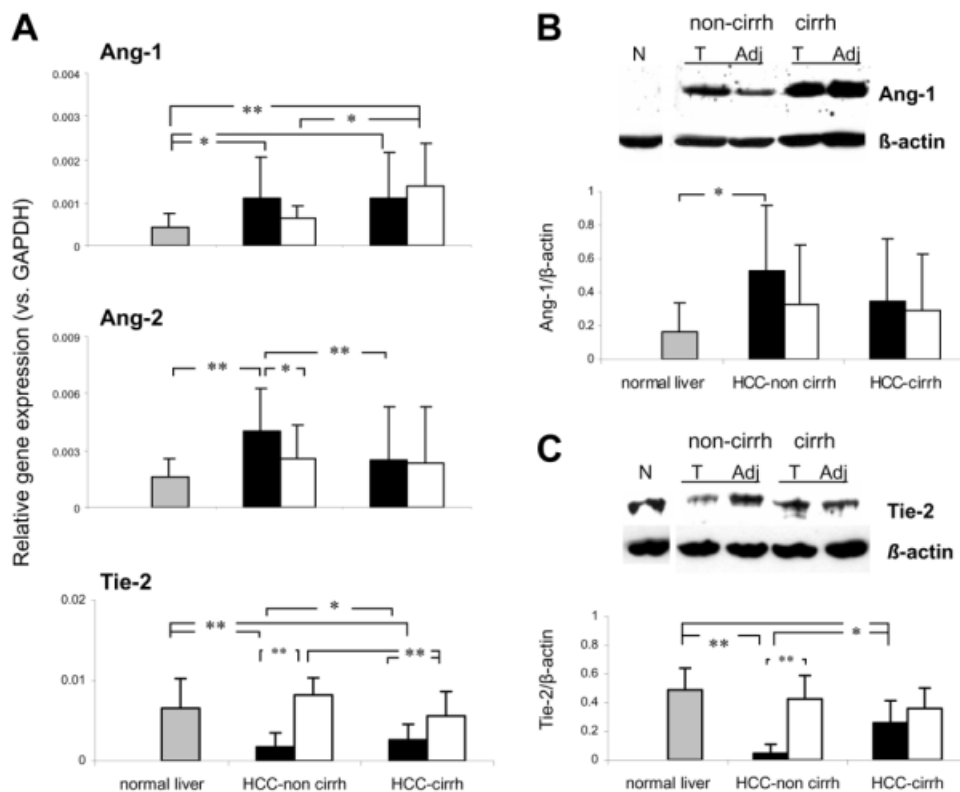


Fig. 3. Tie-2 is down-regulated in HCC while Angiopoietin (Ang)-1 and Ang-2 are slightly induced compared with normal liver. (A) Relative gene expression of Ang-1, Ang-2, and Tie-2 in normal livers (n = 9), HCC in non-cirrhotic livers (n = 21), and their adjacent tissues (n = 11), and HCC in cirrhotic livers (n = 34), and their adjacent liver tissues (n = 23), as determined by quantitative RT-PCR. (B and C) Western blot of Ang-1 and Tie-2, respectively (N, normal liver; T, tumor; Adj, corresponding adjacent nontumorous liver). Upper graphs: representative western blot of five different samples and their β -actin loading controls; Lower graphs: Quantitation of western blot analysis of all samples. Number of samples included: normal livers n = 9, HCC in non-cirrhotic livers and their adjacent livers, n = 11, HCC in cirrhotic livers and their adjacent livers n = 22. Values are given as mean; error bars represent the standard deviations. * $P < 0.05$; ** $P < 0.01$; gray bars, normal liver; black bars, tumor; white bars, adjacent to tumor.

endothelium. Ang-2 staining was hardly detectable in HCC: only occasionally scattered Ang-2-expressing tumor-associated endothelial cells were observed (Fig. 4). In contrast, in adjacent and normal livers, Ang-2-positive sinusoidal endothelial cells (SEC) presented in a zonal distribution around terminal hepatic venules, whereas vascular endothelium was weakly expressing Ang-2.

In addition, the expression of the Angiopoietin receptor Tie-2 was significantly lower in HCC compared with its expression in normal adjacent liver and normal liver, evidenced by both quantitative RT-PCR and western blot analysis (Fig. 3A, C). This difference was also visible by immunohistochemistry (Fig. 4), with a sinusoidal pattern of Tie-2 expression in normal liver and tissue adjacent to the tumor, and a more scattered pattern in HCC. Differences in Tie-2 mRNA expression between virus-infected and non-virus-infected HCC were visible, yet small, and could not be substantiated with western blot analysis (Supporting Fig. 1).

Expression Levels of Members of the VEGF and Angiopoietin/Tie-2 System in RCC. RCC is considered to be a tumor type with prominent angiogenic sprouting activity as a means to support tumor cell demands for nutrients and oxygen. We analyzed RCC in a similar way as we analyzed HCC, to enable a direct comparison with the molecular features of HCC. A pattern of significant up-regulation of components of the VEGF system was observed in RCC in comparison with normal kidney (Fig.

6). Besides a 4.5-fold increase in VEGF-A mRNA in RCC compared with normal kidney, VEGFR-1 expression was also increased. VEGFR-2 mRNA expression levels were at a similar level in RCC and in normal kidney.

In addition to these changes in the VEGF system, the angiopoietins showed a remarkable shift in balance in RCC compared with normal kidney (Fig. 6). Although Ang-1 mRNA levels in the tumor were approximately 25% less compared with their levels in normal kidney tissue, Ang-2 mRNA was increased over 30-fold in the tumor. A similar shift in angiopoietin balance was recently reported in RCC by Baldewijns et al.¹⁶ Although mRNA levels of different genes cannot be directly compared, the change in Ang-1/-2 mRNA ratio implies a shift toward a vessel destabilization phenotype. The increased mRNA levels of Ang-2 in RCC were corroborated by western blot analysis, showing significant amounts of Ang-2 protein in this tumor type. Also by immunostaining, RCC presented as a tumor rich in Ang-2-positive vessels (Fig. 5), in contrast to HCC.

HCC-Associated Endothelium Gains CD31 and CD34 Expression. In a liver with normal histology, the sinusoidal endothelial cells express the endothelial marker gene CD31 and CD34 to a limited extent, in the periportal areas (Figs. 4, 7A), corroborating previously published data.¹⁷ However, although the mean vascular density between the healthy sinusoidal network and the tumor vasculature did not dramatically change, tumor endothelium

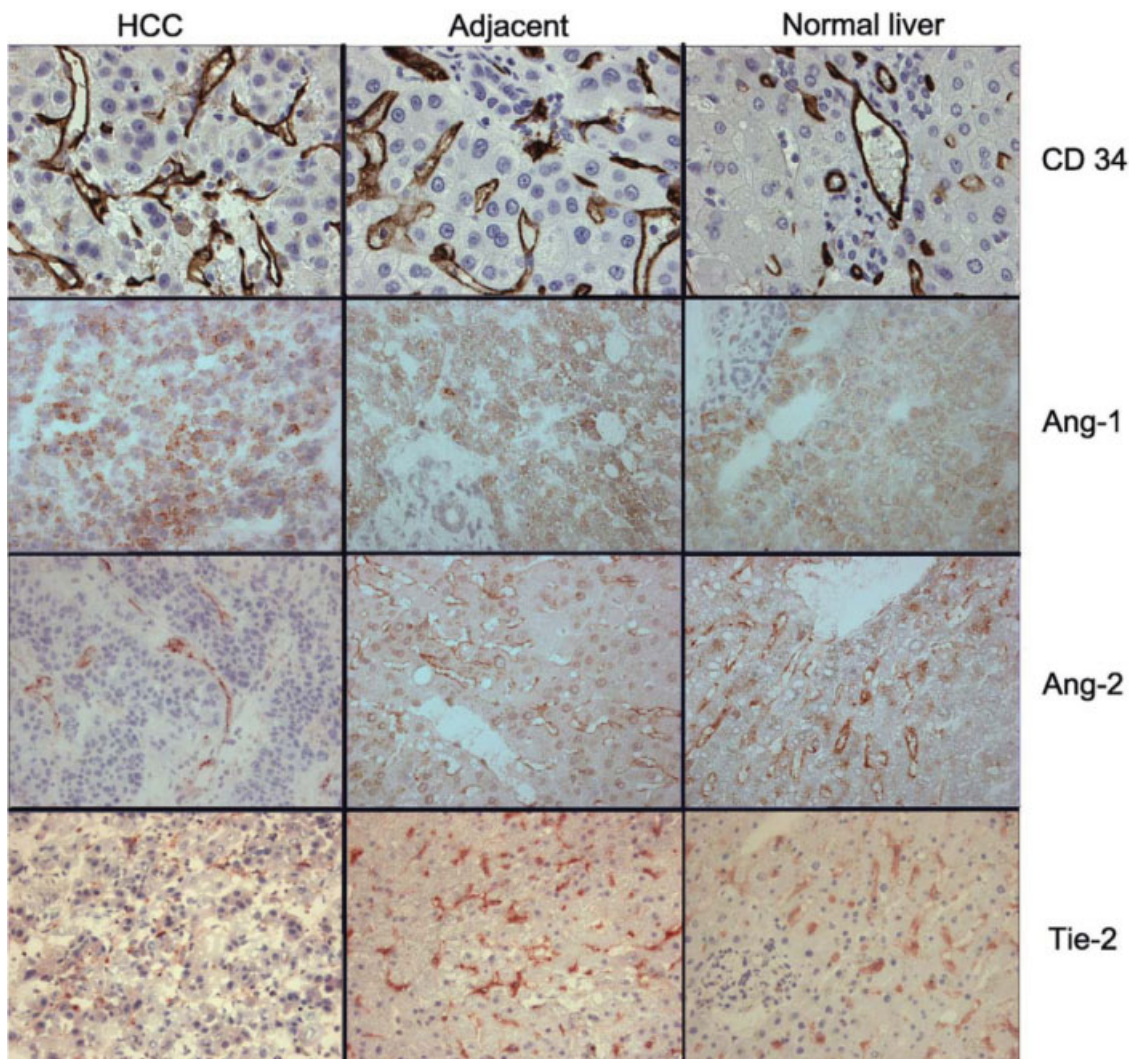


Fig. 4. Localization of Ang-1, Ang-2, and Tie-2 expression in highly vascularized HCC. Morphological appearance of the vasculature in HCC, its adjacent nontumor liver tissue, and normal liver, as detected by CD34 immunohistochemical staining of formalin-fixed tissues, and respective immunostaining of Ang-1, Ang-2, and Tie-2 in HCC in frozen materials (original magnification 400 \times). Although Ang-2 is expressed in a sinusoidal distribution pattern in normal liver and normal liver adjacent to the tumor, its expression in HCC is scattered (picture) and mostly absent from tumor-associated vasculature.

gained CD31 and CD34 expression (Figs. 4, 7B), indicative of a phenotypical change in endothelial cell behavior taking place under the influence of tumor-related (growth) factors.

Discussion

The current study investigated the status of angiogenic sprouting in primary human hepatocellular carcinoma as represented by Ang/Tie-2–based vascular destabilization and VEGF-driven angiogenic activation. We quantitatively and qualitatively compared the results with human RCC, which is a highly angiogenic tumor because of a mutation in one allele of the Von Hippel Lindau tumor suppressor gene, as a consequence of which several genes involved in angiogenic sprouting are overexpressed.¹³ We

demonstrated that in HCC no major differences in VEGF/VEGFR and Ang/Tie-2 gene and protein expression exist compared with normal liver. In contrast, in RCC a strongly increased expression of Ang-2 was paralleled by a decrease in Ang-1 and a significant increase in VEGF-A and VEGFR-1. These data imply that in HCC, regardless of whether they originate in a noncirrhotic or a cirrhotic liver, and irrespective of whether they are from a viral or nonviral background, the tumor blood supply is regulated in a VEGF–Ang/Tie-2–independent manner. This observation has important implications for therapeutic decision making for anti-angiogenesis–based therapy of HCC.

Several studies reported on the expression of members of the VEGF system in HCC,^{18,19} and the members of the

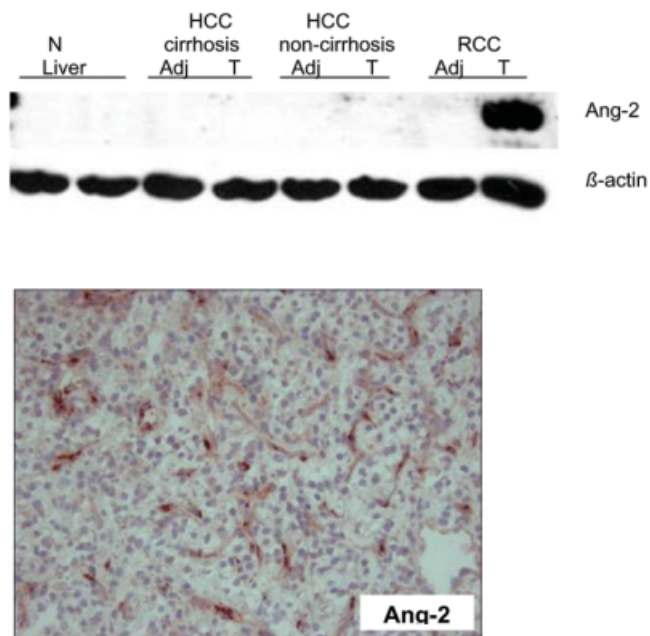


Fig. 5. Angiopoietin-2 is absent in HCC, while being abundantly expressed in RCC. Top: Western blot analysis of Ang-2 and β -actin of HCC from cirrhosis and noncirrhosis background, and RCC (N, normal liver; T, tumor; Adj, corresponding adjacent nontumorous tissue). Lower: Representative pattern of immunostaining of Ang-2 in RCC (original magnification 200 \times).

Angiopoietin family have been (semi-) quantitatively analyzed and related to VEGF and microvascular density.^{20,21} Our data corroborated previous observations that no significant differences in VEGF-A expression exist between HCC and adjacent liver,²² and that Ang-1 was increased in HCC compared with normal liver.²³ However, some discrepancies became apparent. For example, in the study by Zhang et al.,²¹ all four angiogenes analyzed, that is, Ang-1, Ang-2, Tie-2, and VEGF, were expressed at a similar level.²¹ In contrast, we found that VEGF expression in HCC was one order of magnitude higher than that of the Angiopoietins and Tie-2. The lower Tie-2 mRNA expression in HCC was substantiated by western blot (protein) and illustrated by immunohistochemistry. Although it is difficult to quantitatively

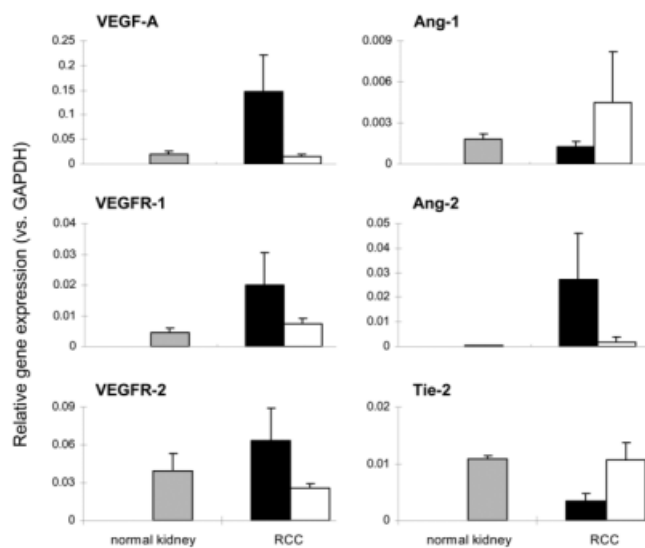


Fig. 6. Gene expression levels of the members of the VEGF and Tie-2 system in RCC. Relative gene expression of VEGF-A, VEGFR-1, VEGFR-2, Ang-1, Ang-2, and Tie-2 in normal kidney ($n = 2$), RCC ($n = 5$), and their adjacent tissues ($n = 2$), as determined by quantitative RT-PCR. Data are presented as mean values and their respective standard deviations. Gray bars, normal kidney; black bars, RCC; white bars, adjacent to tumor.

compare quantitative RT-PCR-based mRNA and western blot-based protein levels, all data provided in our study point to a lower expression of Tie-2 in HCC than in normal liver. Amaoka and colleagues²⁴ recently reported a relation between VEGF protein expression in HCC as determined by enzyme-linked immunosorbent assay and clinicopathological features. One important difference in analysis between their study and ours is the site from which the HCC samples were obtained. Amaoka et al. retrieved tumor samples from non-necrotic HCC central areas, whereas we retrieved our samples from the periphery of the tumor nodules. Possibly, in more central tumor areas, hypoxia is more prominent, resulting in higher VEGF levels and hence tumor/normal VEGF ratios exceeding 1, which was the case in 18 of 28 cases.²⁴

The relation between VEGF/angiopoietin expression and the angiogenic phenotype of HCC has been exten-

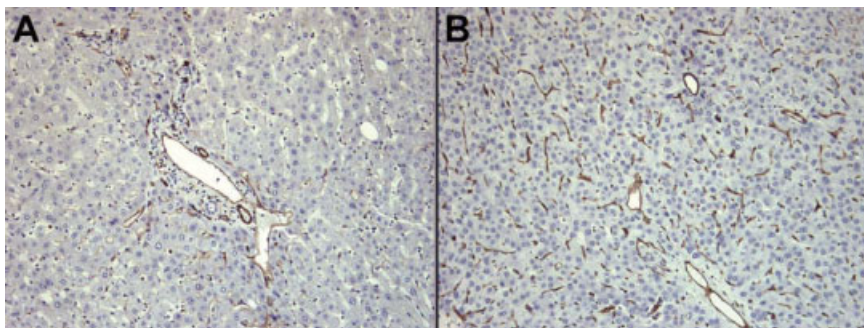


Fig. 7. HCC tumor vasculature gains CD31 expression. Immunostaining of CD31 in normal adjacent liver (A) and in HCC (B), demonstrating a phenotypical as well as morphological difference between normal sinusoidal endothelial cells and tumor endothelial cells (original magnification 100 \times).

sively addressed in recent years, yet the data remain difficult to interpret. In mouse models, it was convincingly shown that development of HCC intraperitoneally or in the subcutaneous, avascular pocket was dependent on the VEGF/angiopoietin system.^{25,26} That these factors are present in HCC in humans has been extensively reported^{18,20,27}; however, their mere presence does not shed light on the question of whether they represent active angiogenesis in the microenvironment of the tumor in the human liver. By relating our data on HCC to the expression levels of the same genes in human RCC, which is an established model for active angiogenesis in humans, we placed the expression levels in HCC in broader perspective. One important finding in this respect was that in HCC the expression level of VEGF was only 20% of the level observed in RCC. Moreover, we identified a striking difference in Ang-1/Ang-2 mRNA ratios between the two tumor types. Although this ratio dramatically dropped from approximately 2 in normal kidney to 0.06 in RCC as a result of overexpression of Ang-2 concomitant with a decreased expression of Ang-1, in HCC it was approximately 0.3 in noncirrhotic and cirrhotic HCC, which is in the same order of magnitude as reported previously in a small number of HCCs in a cirrhotic background.²³ In virus-associated HCC, the ratio was clearly lower (approximately 0.1) than that in non-virus-associated HCC (approximately 1), which is attributed to both a reduced expression of Ang-1 and a slightly induced expression of Ang-2. Whether the virus-associated HCC-related changes in Ang-1/Ang-2 balance in the absence of induced VEGF-A production can give rise to tumor growth-associated angiogenic sprouting is unclear and should be a subject of future study, for example, by analysis of phospho-VEGFR-2 or phospho-Tie-2/CD34 immunofluorescence double staining.

The liver sinusoids may represent a specialized vascular bed that preferentially facilitates pathology-related demands by vascular remodeling via mechanisms other than angiogenic sprouting. Recently, Straub and colleagues²⁸ showed that prolonged exposure of mice to arsenic (III) initiated vascular remodeling by SEC capillarization, which was paralleled by increased sinusoidal CD31 expression, similar to our observation in HCC.²⁸ In this respect, our previous observation of increased numbers of alpha-smooth muscle actin-positive cells adjacent to CD34 expressing sinusoidal cells in HCC,¹¹ along with our current finding of increased Ang-1 in HCC, support the concept of sinusoidal remodeling in which recruitment of pericytes is stimulated by Ang-1.²⁹ Additionally, some tumors acquire their vasculature by vessel cooption instead of angiogenic sprouting.^{30,31} We currently lack a vascular marker for (sinusoidal) endothelial cooption and

hence cannot test the hypothesis that HCC is also nourished by cooption more than by angiogenic sprouting. However, the finding that HCC can grow in the absence of increased expression of VEGF and a destabilized Tie-2 phenotype puts forward an intriguing premise that the permissive role of SEC may be a general feature for tumor growth in the liver. The recent observations that the expression of VEGF-A in primary colorectal carcinoma is higher than in colorectal carcinoma metastases in the liver³² and that primary pancreatic cancer expresses twice as much VEGF as compared with its metastasis in the liver³³ are compelling indications that the liver niche indeed represents a microenvironment that can actively influence tumor growth-associated processes.

In light of the current findings, the recently reported clinical effects of sorafenib and sunitinib in patients with HCC seem contradictory.³⁴⁻³⁶ However, they can be explained by the fact that sorafenib and sunitinib are multikinase inhibitors that do not only affect VEGFR-mediated signal transduction but also platelet-derived growth factor receptor, B-Raf and C-Raf, cKit, and flt3 activity. Raf-mediated enhanced activation of MEK (mitogen-activated protein kinase [MAPK]/extracellular signal-regulated kinase [ERK] kinase) and downstream targets is implicated in proliferative and migratory capacity of HCC tumor cells.³⁷ Inhibition of the phosphorylation of MEK/ERK in combination with MEK/ERK independent molecular changes, resulted in tumor cell proliferation inhibition and apoptosis induction. *In vivo* in the PLC/PRF/5 xenograft model, the sorafenib-associated tumor cell apoptosis induction coincided with a reduced microvessel density, but direct tumor vascular effects were not reported.³⁸ It is not unlikely, therefore, that these multikinase inhibitors primarily affect tumor cells and their production of various growth factors, leading to indirect vascular effects.

Knowledge on the exact status of the molecular processes underlying vascular remodeling supporting tumor outgrowth is essential for therapeutic intervention. From our data, HCC growth in both noncirrhotic and cirrhotic liver is not predominantly VEGF/angiopoietin driven. Still, the tumor microvascular capillaries are distinctly different from SEC, both morphologically and phenotypically. The trabeculae and pseudoglands of HCC are covered by endothelial cells which phenotypically differ from SEC as visualized in our study by the expression of CD31, as well as CD34, antigens that are expressed to a limited extent or even absent on normal SECs. Apart from the sinusoidal-like vasculature, HCC also contains so-called unpaired arteries, which are haphazardly distributed small arteries without accompanying bile duct. Possibly, epidermal growth factor family mem-

bers including Betacellulin and epidermal growth factor receptor,^{39,40} or other (angiogenic) factors and their respective receptors such as platelet-derived growth factor/platelet-derived growth factor receptor alpha, can influence tumor vascularization processes.^{41,42} Of interest in this respect is furthermore the recent publication by Lai and colleagues,⁴³ in which they showed that the enzyme sulfatase-2 positively affected the binding and subsequent downstream signal transduction of fibroblast growth factor 2.⁴³ Because sulfatase-2 was overexpressed in approximately 60% of HCCs studied, it is tempting to speculate that HCC-associated vascular remodeling can be driven by fibroblast growth factor 2 without fibroblast growth factor 2 being overexpressed. Further detailing on the exact molecular control of HCC-driven vascularization is needed to provide a rationale for vasculature-directed therapy⁴⁴ with or without combination with tumor-directed therapeutics.

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