

ORIGINAL ARTICLE

Microvessel density and clinicopathological characteristics in hepatitis C virus and hepatitis B virus related hepatocellular carcinoma

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Aims: To compare intratumorous microvessel density (MVD) and clinicopathological features in two different groups of hepatocellular carcinoma (HCC), namely: hepatitis B virus (HBV) related HCC (B-HCC) and HCV related HCC (C-HCC).

Methods: Fifty consecutive cases each of B-HCC and of C-HCC were studied. Microvessel numbers were assessed by staining for the antigen CD34; in each case, three areas with the highest numbers of microvessels were counted in both the intratumorous and the surrounding non-tumorous tissue; the mean value represented the final MVD.

Results: Patients with B-HCC were significantly younger than those with C-HCC (mean age, 60.1 (SD, 4.1) v 66.4 (4.3) years); no significant differences were seen for sex or Child's class distribution. The tumour diameter was larger in B-HCCs than in C-HCCs (mean, 5.6 (SD, 1.8) v 3.8 (1.8) cm). Tumour microsatellite formation was significantly higher in C-HCCs (12 v 4 cases). No differences were found for histological subtype, degree of differentiation, tumour encapsulation, and vascular invasion. The mean MVD value was significantly higher in tumorous (mean, 54 (SD, 13.8) v 38 (8.9)) and in the surrounding non-tumorous liver tissue (mean, 15 (SD, 4.3) v 7 (3.1)) of C-HCCs.

Conclusions: C-HCCs present as smaller tumours in older patients, with a higher incidence of tumour microsatellite formation and higher MVD values both in the tumorous and the non-tumorous areas, suggesting a link between HCV infection, angiogenesis, and hepatocarcinogenesis.

Chronic hepatitis B and C are thought to be the major causes of cirrhosis and of hepatocellular carcinoma (HCC). Hepatitis B virus (HBV) related hepatocarcinogenesis has been studied intensively and largely clarified during the past decade,^{1–3} but the carcinogenetic mechanism of hepatitis C virus (HCV) still remains unclear.^{4–5} Furthermore, the clinicopathological features of resected HCC with HBV and HCV infections can differ,^{6–17} thus suggesting different mechanisms of carcinogenesis for these two viruses.

“The carcinogenetic mechanism of hepatitis C virus still remains unclear”

Angiogenesis is of crucial importance to tumour growth and the metastasis of solid tumours.^{18–19} The importance of angiogenesis for tumour growth is supported by the observation that an avascular tumour rarely grows larger than 2–3 mm², but once a tumour becomes vascularised, tumour growth is rapid.^{20–21} HCC is a hypervascular tumour, but unlike other solid tumours an inverse correlation between angiogenesis and tumour size has been found.^{22–24} The importance of neovascularisation in the progression of HCC has been highlighted in recent studies,^{25–26} which showed that microvessels increase gradually from cirrhotic nodules through low grade and high grade dysplastic nodules, with the greatest numbers recorded in HCC. To date, few reports have compared microvessel density (MVD) in HBV and HCV associated HCC, and they have found no differences.^{22–27–28} However, non-neoplastic livers infected with HCV showed a higher MVD than those infected with HBV,^{28–29} and a possible active role for HCV in angiogenesis has been suggested.^{28–30}

To clarify these apparently conflicting data we evaluated angiogenesis, as measured by microvessel counting, in two different groups of HCC, namely: HBV related (B-HCC) and HCV related HCCs (C-HCC) and in the surrounding liver tissue. We also carried out a comparative study of the clinicopathological features of both groups of tumours.

MATERIAL AND METHODS

Case selection

Between January 1997 and December 2002, 219 surgically resected HCCs were collected in the department of human pathology and oncology at the University of Florence, Italy. For the purposes of the study we selected 50 consecutive cases of B-HCC and C-HCC that met the following criteria: (1) cirrhosis Child's class A or B; (2) hepatic resection was considered curative; (3) the tumours presented as single nodular lesions; (4) no other treatments for HCC; (5) no apparent distant metastases. Complete preoperative serological tests were available for each case. HBV infection was confirmed by the detection of the HBV surface antigen and HBV core antigen; HCV infection was established using commercial enzyme linked immunosorbant assays for anti-HCV antibodies. Patients with evidence of co-infection were excluded from our study, as were those with an associated history of alcohol abuse. In all cases, multiple sections of the tumour and the surrounding tissue were available.

Abbreviations: B-HCC, hepatitis B virus hepatocellular carcinoma; C-HCC, hepatitis C virus related hepatocellular carcinoma; COX-2, cyclooxygenase-2; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; iNOS, inducible nitric oxide synthase; MVD, microvessel density; PD-ECGF, platelet derived endothelial cell growth factor

Pathological examination

The morphological and histological features of all cases were reviewed. An adequate number of sections from each tumour (six on average; range, 4–12 sections depending on tumour diameter) and surrounding tissue were available for our study. Tumour size was measured as the maximal diameter of the tumour by gross examination. The presence of a tumour capsule, evidence of vascular invasion, and tumour microsatellite formation were assessed by microscopic examination. Smaller neoplastic nodules clearly separated from the main lesion were considered to be tumour microsatellite nodules. Microsatellite formations were divided into multifocal and metastatic groups. Multifocal HCC was defined according to the histological criteria of Tsuda *et al.*³¹ Satellite nodules consisting of Edmondson's grade II, III, or IV HCCs and exhibiting a similar or lower grade of differentiation in comparison with the main tumour were categorised as intrahepatic metastases.³²

Tumours were histologically subtyped according to the World Health Organisation classification system.³³ The degree of differentiation was determined according to Edmondson and Steiner,³⁴ and was stratified into those tumours with better (Edmondson grade I and II) and poorer (Edmondson grade III and IV) cellular differentiation.

Immunohistochemistry

Immunohistochemical staining was performed for CD34 on 4 µm thick, formalin fixed, paraffin wax embedded sections, using the streptavidin–biotin immunoperoxidase technique. A monoclonal anti-CD34 antibody (Immunotech, Marsiglia, France) was used at a 1/100 dilution. Antigen retrieval consisted of microwave treatment with citrate buffer, pH 6.0, for 10 minutes. As a negative control for each case, the primary antibody was replaced with normal rabbit serum. We chose CD34 because it is more sensitive than other markers for liver endothelial cells.^{24 27 28 35–37}

Evaluation of MVD

MVD was assessed by light microscopy using the counting method introduced by Weidner *et al.*³⁸ CD34 was used to identify and count intratumorous and extratumorous vessels. Tumorous and non-tumorous tissue sections were scanned at low magnification ($\times 40$ and $\times 100$) to find the areas that showed the most intense vascularisation (hot spots). Individual microvessels were counted in three fields at $\times 200$ magnification ($\times 20$ objective lens and $\times 10$ ocular lens; 0.7386 mm²/field). The final MVD was the mean value obtained from the counts of the three fields. MVD was expressed as mean (SD) (vessels/mm²). Any immunostained endothelial cells or endothelial cell clusters that were clearly separated from the adjacent microvessels, tumour cells, and other connective tissue elements were considered to be single and countable microvessels. Vessel lumens were not necessary for a structure to be defined as a microvessel, and red blood cells were not used to define a vessel lumen.³⁸ The evaluation of MVD was performed without knowledge of the clinicopathological data.

Table 1 Comparison of clinical data between B-HCC and C-HCC

Variable	B-HCC	C-HCC	p Value
Mean (SD) age in years	60.1 (4.1)	66.4 (4.3)	<0.001
Sex (M/F)	40/10	42/8	NS
Child's class (A/B)	34/16	31/19	NS

B-HCC, hepatitis B virus related hepatocellular carcinoma; C-HCC, hepatitis C virus related hepatocellular carcinoma; NS, not significant.

Statistical analysis

Statistical analysis was performed using the SPSS software program (version 9.0 for Windows; SPSS Inc, Chicago, Illinois, USA). The Wilcoxon test and χ^2 test were used to compare the findings between the two groups (B-HCC and C-HCC). Tests were considered significant when their p values were less than 0.05.

RESULTS

Clinicopathological features

Table 1 shows the distribution of the clinical data in our study population. The patients with B-HCC (mean age, 60.1; SD, 4.1 years) were significantly younger than those with C-HCC (mean, 66.4; SD, 4.3) ($p < 0.001$). No significant differences were found with regard to sex and Child's class distribution. Among the tumour related factors, the tumour diameter of the B-HCC group (mean, 5.6; SD, 1.8 cm) was significantly greater than that found in C-HCC (mean, 3.8; SD, 1.8 cm) ($p < 0.001$). Tumours larger than 5 cm were found more frequently in the B-HCC group; no differences were found for small or moderate sized tumours, although a trend towards a higher number of tumours ≤ 2 cm was seen in the C-HCC group (table 2). Tumour microsatellite formation was seen significantly more often in the C-HCC group (12 cases) than in the B-HCC group (four cases) ($p = 0.03$). Multifocal HCC was found in nine of the 12 C-HCC cases and in one of the four B-HCC cases with tumour satellite nodules; these differences were significant ($p < 0.007$). Three patients showed intrahepatic metastases in both groups; differences were not significant ($p = 0.67$).

No significant differences between the two groups were found when evaluating histological subtype, cellular differentiation, tumour encapsulation, and vascular invasion (table 2).

Microvessel density

Microvessels were found to be heterogeneously distributed within the tumour, and maximal density was seen at the periphery of the lesion, near the borders (fig 1). The mean value of tumour MVD was 47 (SD, 11.4; range, 18–78). When MVD was correlated with clinicopathological features, significantly higher MVD values were associated with

Table 2 Comparison of pathological features between B-HCC and C-HCC

Variable	B-HCC N	C-HCC N	p Value
Tumour size			
≤ 2 cm	2	6	NS
2.1–5 cm	30	37	NS
> 5 cm	18	7	0.01
Architectural pattern			
Trabecular	36	35	NS
Pseudoglandular	10	9	
Compact	3	5	
Clear cell	1	1	
Edmondson grade			
G1–G2	29	27	NS
G3–G4	21	23	
Microsatellite formation			
Absent	46	38	0.03
Present	4	12	
Fibrous capsule			
Absent	45	43	NS
Present	5	7	
Vessel involvement			
Absent	33	35	NS
Present	17	15	

B-HCC, hepatitis B virus related hepatocellular carcinoma; C-HCC, hepatitis C virus related hepatocellular carcinoma; NS, not significant.

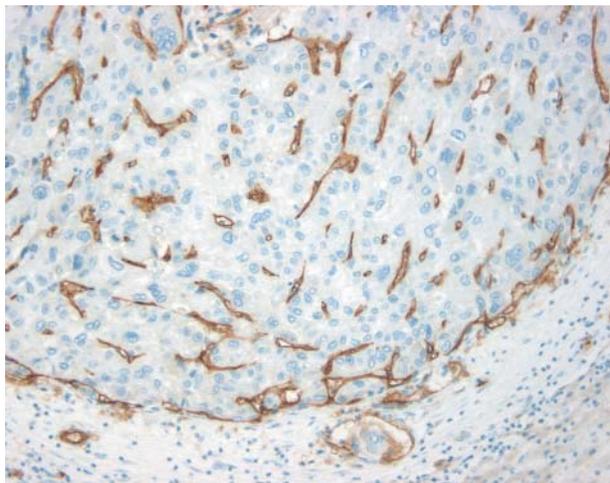


Figure 1 Area of hepatitis C virus related hepatocellular carcinoma showing high density of microvessels as assessed by anti-CD34 immunostaining. Original magnification, $\times 200$.

Child's class B, HCV infection, and tumour size (tables 3 and 4). The mean tumour MVD was 38 (SD, 8.9; range, 18–50) in the B-HCC group and 54 (SD, 13.8; range, 28–78) in the C-HCC group. Differences were significant ($p < 0.01$) (table 3). The mean MVD was significantly higher in the C-HCC group when different Child's class disease and tumour size were taken into account (table 5).

In non-tumorous liver tissue, immunostaining for CD34 was sparse and more pronounced in the C-HCC group (mean MVD, 15; SD, 4.3; range, 3–24) than in the B-HCC group (mean MVD, 7; SD, 3.1; range, 0–12); differences were significant ($p < 0.01$).

DISCUSSION

We found certain clinicopathological differences in relation to the aetiology of HCC. In accordance with previous reports,^{6–17} patients in the C-HCC group were significantly older than those with HBV infection. This is mainly thought to be the result of differences in the origin and duration of infection.⁹ HBV infection often occurs via perinatal transmission from the mother, whereas HCV infection is more often acquired in adulthood, via blood transfusions, intravenous drug use, or contaminated instruments.^{16 39}

The average tumour diameter was significantly larger in the B-HCC group than in the C-HCC group. This finding is consistent with most studies, which show a higher incidence of more advanced cancers in patients with HBV infection

Table 3 Comparison between MVD and clinical features in the 100 hepatocellular carcinomas studied

Variable	N	Mean (SD) MVD	p Value
Mean age			
≤ 63.9	57	46 (7.6)	NS
> 63.9	43	51 (6.9)	
Sex			
Male	82	49 (10.2)	NS
Female	18	46 (9.3)	
Child's class			
A	65	39 (7.1)	<0.01
B	35	55 (8.8)	
Hepatitis virus status			
Hepatitis B positive	50	38 (8.9)	<0.01
Hepatitis C positive	50	54 (13.8)	

MVD, microvessel density.

Table 4 Comparison between MVD and pathological features in the 100 hepatocellular carcinomas studied

Variable	N	Mean (SD) MVD	p Value
Tumour size			
≤ 2 cm	8	35 (5.6)	<0.03
2,1–5 cm	67	56 (9.8)	
> 5 cm	25	46 (10.4)	
Architectural pattern			
Trabecular	71	45 (9.3)	NS
Pseudoglandular	19	44 (8.8)	
Compact	8	46 (5.8)	
Clear cell	2	48 (2.0)	
Edmondson grade			
G1–G2	56	47 (8.4)	NS
G3–G4	44	44 (7.2)	
Microsatellite formation			
Absent	84	46 (6.8)	NS
Present	16	50 (7.8)	
Fibrous capsule			
Absent	88	44 (8.1)	NS
Present	12	49 (7.6)	
Vessel involvement			
Absent	68	48 (7.6)	NS
Present	32	43 (8.2)	

MVD, microvessel density; NS, not significant.

than in those with HCV infection.^{11 12 14–16} This is probably because HCV infected patients are closely followed up for chronic liver dysfunction, whereas HBV positive patients are comparatively young, asymptomatic, and have blood test results within normal ranges, so they seldom undergo imaging examination.

Tumour microsatellite formation was significantly higher in the C-HCC group; most satellite nodules in the C-HCC group were thought to be of multifocal origin, whereas in the B-HCC group, satellite nodules were intrahepatic metastases. These data are in accordance with the results of Miyagawa *et al.*¹⁰

When all cases were considered, MVD was significantly higher in patients with advanced liver disease and in tumours of intermediate size. These findings are in accordance with those of El-Assal *et al.*²² A possible explanation for the relation between MVD and HCC size may be found in the characteristics of the tumour microcirculation, which change as the tumour grows.²² El-Assal *et al* have hypothesised that angiogenesis plays a fundamental role in the tumour proliferation of HCCs between 2 and 5 cm in diameter, whereas the importance of neovascularisation is reduced as the tumour becomes larger.

In our study, MVD was significantly higher in the C-HCC group, even when our cases were stratified according to tumour diameter and Child's class disease, thus suggesting a

Table 5 MVD in B-HCC and C-HCC in relation to Child's class and tumour diameter

Variable	Mean (SD) MVD		p Value
	B-HCC	C-HCC	
Child class			
A	35 (6.9)	47 (8.2)	<0.03
B	48 (8.4)	59 (9.8)	<0.01
Tumour diameter			
≤ 2 cm	30 (4.1)	44 (7.2)	<0.04
2,1–5 cm	45 (9.3)	62 (11.2)	<0.001
> 5 cm	41 (8.2)	54 (9.8)	<0.001

B-HCC, hepatitis B virus related hepatocellular carcinoma; C-HCC, hepatitis C virus related hepatocellular carcinoma; MVD, microvessel density.

particular link between angiogenesis and HCV infection. This result differs from the findings of previous studies.^{22 27 28} El-Assal *et al* failed to demonstrate a significant correlation between MVD and HCC associated with either HCV or HBV infection. Nevertheless, differences between MVD results in our study and the above mentioned one may be attributable to differences in the study designs, the study populations, and the different antibodies used to highlight microvessels; El-Assal *et al* used a factor VIII related antibody, whereas we used anti-CD34, which is highly sensitive and specific for labelling microvessels in HCCs.^{24 27 28 35–37} Nevertheless, no differences in MVD values in HBV and HCV related HCC were found by Tanigawa and colleagues²⁷ or Yamamoto and colleagues²⁸ when angiogenesis was evaluated with anti-CD34. A possible explanation may be found in the different characteristics of the study populations, especially in relation to the number of HBV and HCV infected patients and the clinicopathological features of each series. Interestingly, Yamamoto *et al* found a significantly higher MVD in the surrounding non-neoplastic tissue of C-HCCs compared with the surrounding liver of the patients with B-HCC. This last observation is consistent with our results. Moreover, Yamamoto *et al* reported significantly higher expression of platelet derived endothelial cell growth factor (PD-ECGF), a well known angiogenic factor, in C-HCC compared with B-HCC, whereas no differences were found in the surrounding tissue. PD-ECGF expression was found to correlate with MVD in the surrounding liver tissue but not within HCC. From these data the authors speculated that PD-ECGF may play a role in the angiogenesis of the surrounding liver but not in HCC; nevertheless, they emphasise that there may be cooperation between HCV and PD-ECGF in hepatocarcinogenesis mediated by the angiogenic pathway.

“Microvessel density was significantly higher in the hepatitis C virus (HCV) related hepatocellular carcinoma group, even when our cases were stratified according to tumour diameter and Child’s class disease, thus suggesting a particular link between angiogenesis and HCV infection”

A recent study by Rahman *et al* also found that angiogenesis was particularly important in HCV associated HCC.³⁰ The authors found that inducible nitric oxide synthase (iNOS) expression was significantly higher only in the hepatitis C virus positive HCCs and significant correlations between iNOS, cyclooxygenase-2 (COX-2) and MVD were also found in the same tumour group. Therefore, the upregulation of iNOS and the induction of COX-2 expression may play a role in tumour angiogenesis in C-HCCs.

There is some evidence that other angiogenic factors, such as vascular endothelial growth factor, angiopoietins, and tissue factor, may play an important role in the development and progression of HCC^{40–42}; however, to date little is known about the relation between HCV infection and the regulatory mechanisms of angiogenesis in human HCC.

In conclusion, our results highlight the fact that the clinicopathological profiles of B-HCC and C-HCC may differ. C-HCCs were found to present as smaller tumours in older patients, with a higher incidence of tumour microsatellite formation, thus indicating differences in the tumorigenic potential of HBV and HCV infection. Furthermore, we found higher MVD values in both the HCCs and the surrounding liver tissue of HCV positive cases, suggesting that angiogenesis may be especially linked to HCV infection, and may play a more important role in tumour progression in C-HCC than in B-HCC. Further research in this direction may help to elucidate the role of HCV in the induction of angiogenesis in

Take home messages

- Hepatitis C virus (HCV) related hepatocellular carcinomas (C-HCCs) present as smaller tumours in older patients, with a higher incidence of tumour microsatellite formation and higher microvessel density values both in the tumorous and non-tumorous areas
- These findings suggest a link between HCV infection, angiogenesis, and hepatocarcinogenesis
- Further research may help to elucidate the role of HCV in the induction of angiogenesis in HCC and may form the basis for future novel therapeutic strategies

HCC and may form the basis for future novel therapeutic strategies.

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