IMMUNOHISTOCHEMICAL EXPRESSION OF VEGF IN HEPATOCELLULAR CARCINOMA AND SURROUNDING LIVER TISSUE

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ABSTRACT

Introduction: Angiogenesis is a fundamental process involved in malignant transformation and progression of cancer, in tumor growth and metastasis. VEGF plays an essential role in the angiogenesis of hepatocellular carcinoma (HCC). *Method:* We evaluated VEGF expression in 16 resected HCCs and in the surrounding liver tissue using the IHC staining method and monoclonal antibody anti-VEGF, clone VG1. The results were statistically analyzed using the Epi Info 6.04 program; we considered as significant the value < 0.05. *Results:* Positive VEGF expression was observed in 14 (87.7%) of the 16 patients with HCC and in 62.5% of the surrounding liver tissue (cirrhotic or non-cirrhotic), without significant differences between HCC and non-Tumor liver (0.11). Capsular infiltration (p=0.242), vascular invasion (p=0.383) and intrahepatic metastases (p=0.13) were observed more frequently in patients with positive VEGF expression. *Conclusions:* VEGF expression was higher in HCC than in cirrhotic nodules; our results suggest that VEGF can play an important role in the angiogenesis and prognosis of HCC, as well as in the angiogenesis of liver cirrhosis.
Key words: angiogenesis, VEGF, HCC, surrounding liver tissue

INTRODUCTION

Angiogenesis is an important process involved in the development, prognosis, growth and metastasis of malignant tumors (Folkman J, 1990; Zetter BR, 1998). The vascular endothelial growth factor (VEGF) is the most potent between the angiogenic factors involved directly in tumor progression (Dvorak HF, 1995).

VEGF plays an essential role in the angiogenesis of hepatocellular carcinoma (HCC) (Mise M, 1996; Park YN, 2000). It has been demonstrated that HCC expresses many angiogenic factors, including VEGF (Yamaguchi R, 1998; Yamaguchi R, 2006) and angiogenin (Hisai H, 2003). In 60-70% of human HCCs, elevated levels of VEGF expression can be found (Mise M, 1996; Suzuki K, 1996). In patients with HCC, tumor expression and serum level of VEGF were correlated with tumor size, level of invasion, capacity of metastasis and prognosis of HCC (Jinno K, 1998; Pang R, 2006).

Neovascularization is critical for the growth and progression of highly vascularized solid tumors like HCC. Primary intrahepatic and lung metastases are developed mainly by hematogenous dissemination, a process in which VEGF plays an important role (Li XM, 1998; Jeng KS, 2004).

Increased expression of VEGF receptors in HCC was demonstrated on different levels, including mRNA and protein (Shimamura T, 2000; Yamaguchi R, 2000). The patterns of VEGF expression (mRNA or protein expression) in HCC and surrounding liver tissue are still controversial. Most of the studies report that the mRNA VEGF expression level is higher in HCC than in the surrounding hepatic tissue tor (Yao DF, 2005; Miura H, 1997); the expression of VEGF protein is inconsistent, demonstrating a higher level either in HCC or in the surrounding liver tissue (El-Assal ON, 1998; Yamaguchi R, 1998).

In this study we investigated *the level of VEGF expression in HCC and the surrounding liver tissue;* we evaluated the relationship between the VEGF expression and the morpho-clinical factors of patients with liver cancer from the studied group.

MATERIAL AND METHODS

Clinical features of CHC patients

The study included 16 patients (10 women and 6 men) who had undergone curative hepatic resection for HCC. The age of patients ranged from 21 to 69 years (mean 56.4).

Preoperatively, the tumors were diagnosed using biochemical tests, ultrasonographical and angiographical investigations, as well as computed tomography (CT) scan. None of the selected patients were exposed to preoperative chemotherapy or embolization therapy. After the surgery the patients were followed-up for at least 3 years and recurrences were diagnosed by ultrasonography, angiography, CT and α-fetoprotein (α-FP) evaluation.

Light microscopy and immunohistochemistry

In this study we did not use biopsy material. In all cases, clinical diagnosis was confirmed on conventional histological sections stained HE. All sections included both tumor tissue (HCC) and cirrhotic or non-cirrhotic surrounding liver tissue.

For the correct interpretation of VEGF immunostaining, we selected tissue blocks that contained HCC and surrounding liver tissues.

Tumors were classified according to Edmondson-Steiner classification system as well- differentiated (grade I), moderately differentiated (grade II) and poorly differentiated (grade III or IV) carcinomas.

The clinic-pathologic parameters that we analyzed included gender and age of the patients, associated liver pathology (infection with B or C hepatic virus; hepatitis, cirrhosis), size of the tumor (<5 cm vs. ≥5 cm), tumor differentiation (well, moderately, poorly), capsule formation (present vs. absent), capsule infiltration (present vs. absent), vascular invasion (including vascular invasion and/or tumor trombi in portal or hepatic vein) and intrahepatic metastases (present vs. absent); all clinic-pathologic data are presented in table 2.

The patients included in this study presented single or multiple tumor nodules with sizes varying between 1 and 7.5cm. The histological size of the tumor was calculated as the sum of all tumor nodules identifies grossly and microscopically in the resected liver.

EVALUATION OF VEGF IMMUNOSTAINING

For the immunohistochemical (IHC) study of VEGF expression we used the anti-VEGF monoclonal antibody, clone VG1, type IgG (Novus Biologicals), LSAB+ technique. For antigen retrieval, sections were pretreated by boiling in the microwave (MW) oven in retrieval solution pH9 (DAKO), for 15 minutes. Then, the sections were incubated with the primary antibody diluted 1:25 for 0ne hour. The system of visualization that we used included DAB and counterstain with modified Lillie hematoxylin.

The VEGF positive staining had a cytoplasmic localization. The percentage of positive VEGF cells was assessed by examining 10 microscopic fields at high magnification (x400) from each section. The IHC expression of VEGF was evaluated/graded using a semiquantitative score, according to the sum of two parameters: the percentage of positive cells and the intensity of immunostaining.

- *The percentage of positive cells*:
  - 0 = 0% immunopositive cells;
  - 1 = < 25% positive cells;
  - 2 = 26-50% positive cells;
  - 3 = > 50% positive cells;
- *The intensity of immunostaining*:
  - 0 = negative immunoreaction;
  - 1 = weak intensity;
  - 2 = moderate intensity;
  - 3 = strong intensity.

By summing up the two parameters we obtained a final score that varies between 0 and 6. In our study we considered:

- Negative immunoreaction (-) for a score between 0 and 2;
- Weakly positive immunoreaction (+) for a score between 3 and 4;
- Intensely positive immunoreaction (++) for a score between 5 and 6.

The immunohistochemical reactions for VEGF were applied for all the cases of liver cancer included in the study. We identified the expression of the antibody both in the tumor and surrounding hepatic tissue.

Statistical analysis was performed using the Epi Info 6.04 program and consisted in counting the frequency of parameters and the percentage for qualitative variables, mean and standard deviation for quantitative variables. The comparison of percentages was made using the χ^2 (chi sqare) test, the value $p<0.05$ being considered significant.

RESULTS

a) Immunohistochemical expression of VEGF in liver cancer and surrounding tissue

Immunoreactions for VEGF protein performed in all cases of liver carcinoma highlighted a cytoplasm staining of tumor cells with a diffuse, granular pattern, in some cases VEGF immunoexpression being more intense at the margin of the tumor than in central areas. Occasionally, we observed a focal membrane immunostaining and an intense VEGF positivity in the areas of tumor invasion. All HCC and normal hepatocytes expressed VEGF with a stronger staining intensity that the negative control.

VEGF expression was graded as absent or weak (-), intermediate (+) and strong (++), according to the intensity of VEGF immunostaining in HCC and surrounding liver tissue.

Overall, VEGF protein expression was higher in HCC as compared to the surrounding liver tissue, the differences being insignificant between tumor (14 cases; 87.5%) and non-tumor (10 cases; 62.5%) areas (p=0.11). Cirrhotic nodules presented a lower VEGF expression than normal liver parenchyma.

Table 1. VEGF expression in HCC and surrounding liver tissue

	VEGF expression		P
	Negative n(%)	Positive n(%)	
HCC	2 (12.5%)	14 (87.5%)	<0.001[s]
Surrounding tissue	6 (37.5%)	10 (62.5%)	0.289[ns]

Legend: [ns] *– not significant*
       [s] *– significant*

b) The relationship between VEGF protein expression and clinical-pathological parameters of HCC patients

We observed positive VEGF immunoreaction in 14 (87.5%) of the 16 patients with HCC, more frequently than in non-tumor liver tissue (10 cases – 62.5%, p=0.11 NS). The intensity of VEGF staining was graded as follows: 4 cases VEGF+, 4 HCCs were graded VEGF++ and 2 tumors were VEGF-; in 6 cases, VEGF expression was variable, VEGF+ HCC areas alternating with focal VEGF++ areas.

We observed positive VEGF immunoexpression more frequently in males (100%), but not statistically significant different as compared to VEGF immunopositivity in women (80%) (p=0.242) (Table 2).

VEGF expression seems to be influenced by the *age* of patients: all patients ≤ 60 years old expressed VEGF, as compared to 66,67% of the patients ages > 60 years old (p=0,049).

Based on *location of the hepatic tumor*, we noted positive VEGF immunoexpression in 100% of liver cancers from the right liver lobe (RLL) and left liver lobe (LLL), while tumors located bilaterally expressed VEGF in 66.67% of cases (p=0.149).

The presence of *viral hepatic infection* was examined, being well known that it plays a role in liver carcinogenesis. From the 16 patients, 6(37.5%) were positive for hepatitis B surface antigens (AgHBs) and 2 (12.5%) presented anti-HCV antibodies. We noted a significantly higher VEGF immunoreactivity in HCCs associated with HBV infection, as compared to patients with cancer infected with HCV (p<0.001). None of the patients were infected with both viruses, while 8 (50%) did not present known viral infection. The non-tumor hepatic background was cirrhotic on 6 sections (37.5%) and non-cirrhotic on 10 sections (62.5%).

In the 6 patients with HCC associated with liver cirrhosis, the percentage of positive VEGF immunoreaction in the surrounding cirrhotic hepatic tissue was 66.67%. The positive VEGF expression was found in 14 of the 16 HCCs (87.5%) and in 4 of the 6 cases of associated hepatic cirrhosis (66.67%).

Of the 14 HCCs with sizes ≥ 5cm, in 12 cases we noted positive VEGF expression (85.71%), while 2 cases (14.29%) were VEGF negative (p=0.568).

Table 2. Correlation between VEGF expression and clinical-morphological parameters of patients with HCC

Clinical-morphological parameters		Cases n(%)	VEGF expression		p
			Positive n (+ → ++)	Negative n (-)	
Sex	males	6 (37.5%)	6 (100%)	0 (0%)	0.242[ns]
	women	10 (62.5%)	8 (80%)	2 (20%)	
Age	≤60	10 (62.5%)	10 (100%)	0 (0%)	0.049[s]
	>60	6 (37.5%)	4 (66.67%)	2 (33.33%)	
Localization	RLL	6 (37.5%)	6 (100%)	0 (0%)	0.149[ns]
	LLL	4 (25%)	4 (100%)	0 (0%)	
	bilateral	6 (37.5%)	4 (66.67%)	2 (33.33%)	
Viral infection	HBV	6 (37.5%)	6 (100%)	0 (0%)	<0.001[s]
	HCV	2 (12.5%)	0 (0%)	2 (100%)	
	not confirmed	8 (50%)	8 (100%)	0 (0%)	
Associated pathology	cirrhosis	6 (37.5%)	4 (66.67%)	2 (33.33%)	0.149[ns]
	hepatitis	4 (25%)	4 (100%)	0 (0%)	
	other pathology	6 (37.5%)	6 (100%)	0 (0%)	
Size of the tumor	<5cm	2 (12.5%)	2 (100%)	0 (0%)	0.568[ns]
	>5cm	14 (87.5%)	12 (85.71%)	2 (14.29%)	
Differentiation of the tumor	well (G1)	3 (18.75%)	2 (66.67%)	1 (33.33%)	0.71[ns]
	moderately (G2)	12 (75%)	10 (83.33%)	2 (16.67%)	
	poorly (G3)	1 (12.5%)	1 (100%)	0 (0%)	
Capsular formation	present	10 (62.5%)	8 (80%)	2 (20%)	0.242[ns]
	absent	6 (37.5%)	6 (100%)	0 (0%)	
Capsular infiltration	present	10 (62.5%)	8 (80%)	2 (20%)	0.242[ns]
	absent	6 (37.5%)	6 (100%)	0 (0%)	
Vascular invasion	present	12 (75%)	10 (83.33%)	2 (16.67%)	0.383[ns]
	absent	4 (25%)	4 (100%)	0 (0%)	
Intrahepatic metastases	present	8 (50%)	6 (75%)	2 (25%)	0.13[ns]
	absent	8 (50%)	8 (100%)	0 (0%)	

Legend: [ns] – not significant; [s] – significant; RLL – right liver lobe; LLL – left liver lobe; HBV – hepatitis B virus; HCV – hepatitis C virus;

Although the *histological type* of HCC did not influence the expression of VEGF, immunoreactions for VEGF protein were positive in 87.5% of trabecular/sinusoidal type (Figs. 1, 2 and 3), acinar (Figs. 3 and 4) and pelioid type (Fig. 5), significantly more frequently than in carcinomas with solid pattern, clear cells (Fig. 6), with fat deposition and bile secretion (Fig. 7).

In multinodular HCC, hepatic cells from internodular fibrous stroma presented a positive VEGF expression significantly higher than tumor cells from HCC nodules (Fig. 8).

According to the presented differentiation grade, tumors were classified as well, moderately or poorly differentiated in 3 (18.75%), 12 (75%) and 1 case (12.6%), respectively. The patient with poor HCC differentiation was older than the other patients (>60 years old) and presented a higher level of serum α-FP. We obtained positive VEGF reaction in 66.67% of well differentiated HCC; moderately differentiated carcinomas were positive in 83.33% of cases, while in the poorly differentiated carcinoma we noted a weakly positive VEGF expression. The results obtained point out a relationship between *the degree of tumor differentiation* and VEGF expression, but without reaching the point of statistical significance.

Capsular formation (p=0.242), *capsular infiltration* (p=0.242), *vascular invasion* (p=0.383) and *intrahepatic metastases* (p=0.13) were observed more frequently in patients with positive VEGF expression (80%, 80%, 83,33% and 75%, respectively) than in those with negative VEGF expression (20%, 20%, 16,67% and 25%, respectively) (Table 2).

Based on the intensity of VEGF expression in HCC and surrounding liver tissue, the tumors were classified into 2 groups: HCCs with tumor VEGF expression higher than the one in surrounding non-tumor tissues (T>N) – 8 cases, and HCCs with VEGF expression ≤ than that in the surrounding liver tissue – 8 cases (T≤N).

We could not remark a significant correlation between the VEGF expression and the level of tumor invasion, the tumors associated with invasion of liver pedicule, gallbladder and retroperitoneal extension expressed VEGF with a variable intensity (associating weakly colored and intensely colored areas).

Associations were found between VEGF overexpression and poor prognosis factors, such as young age (p=0.049), male gender (p=0.242) and the presence of AgHBs (p<0,001) (table 2).

DISCUSSIONS

VEGF – a potential tumor angiogenesis factor induced by hypoxia was extensively described in the last years (Kim KR, 2002; Cejudo-Martin P, 2002).

VEGF is the most investigated angiogenic factor in HCC. Its expression increases gradually from low-grade dysplastic nodules, to high-grade dysplastic nodules and early HCC. Small HCC show an increased expression of neoangiogenesis and cell proliferation activity, as compared to advanced HCC (Park YN, 2000). Tumor expression of VEGF (mRNA and protein expression) is correlated significantly with serum level of VEGF in patients with HCC, providing the basis for using serum VEGF as prognostic marker (Poon RT-P, 2003). Serum concentration of VEGF increases with the stage of HCC, patients with metastases presenting the highest levels (Jinno K, 1998).

In 1993, Kim et al. (Kim KJ, 1993) demonstrated that blocking the action of a paracrine mediator VEGF that acts on the vascularization, can have a significant inhibitory effect on tumor growth, the authors highlighting the significance of VEGF as an important mediator of tumor angiogenesis.

In the study of Deli G. (2005), VEGF positive expression was found in 69.1% of the 105 HCC investigated and in 79.4% of the surrounding cirrhotic liver tissue, these data giving evidence that positive VEGF expression is significantly higher in surrounding cirrhotic liver than in tumor tissues. VEGF expression significantly correlated with capsular infiltration, vascular invasion, intrahepatic metastases and lower survival rate, these results suggesting the important role of VEGF in angiogenesis and prognosis of HCC.

El-Assal et al. (1998) remarked a significantly higher VEGF expression in cirrhotic liver than in non-cirrhotic tissues, and Shimoda K. (1999) and then Feng DY. (2000) found a VEGF positivity rate significantly lower in HCC than in the surrounding cirrhotic liver tissue (66,7% vs. 85,4%).

It is possible that hepatocytes, in cirrhotic liver, are in a sustained reduced blood flow and that the low pressure of oxygen elevates VEGF transcription and protein synthesis El-Assal ON, 1998). The excessive produced VEGF and secreted by hepatocytes and HCC cells can subsequently act on endothelial cells, resulting in capillarization of sinusoidal endothelial cells and appearance of new blood vessels (Jeng KS, 2004).

Positive VEGF expression is higher in marginal than in central areas of HCC (An FQ, 2000). Tumor cells that express VEGF can proliferate more rapidly than those that do not express VEGF. Rapid cell proliferation in the center of the tumor can lead to increased interstitial fluid, with closure by compression of capillaries and consecutive tumor necrosis (Plate KH, 1992), areas with central necrosis causing suppression of VEGF protein synthesis (Lang KJ, 2002).

In the cirrhotic liver tissues surrounding HCC, VEGF expression is modulated by inflammatory cytokines released from inflammatory infiltrate cells (basic fibroblast growth factor, transforming growth factor α and β, epidermal growth factor and platelet-derived growth factor) that act on VEGF expression, suggesting its role in the development of liver cirrhosis

(Yamaguchi R, 1998; Steinbrech DS, 1999; Mandriota SJ, 1997).

In our study we observed positive VEGF expression in 87,5% of the studied HCC and we found a positive relationship between the overexpression of VEGF and the factors of poor prognosis, like age of patients ≤60 years (p=0.049), male sex (p=0.242), presence of AgHBs (<0,001); capsular infiltration (p=0.242), vascular invasion (p=0.383) and intrahepatic metastases (p-0.13) were observed more frequently in patients with positive VEGF expression than in those with negative immunoreaction (80%, 83.33% and 75%, respectively vs. 20%, 16.67% and 25%, respectively).

Angiogenesis is an important process involved in the development, prognossis, growth and metastasis of malignant tumors (Gullino PM, 1990). The role of VEGF in the development of HCC was investigated especially regarding its proangiogenic potential. Studies on the mRNA and protein expression of VEGF (Mise M, 1996) reported a higher VEGF mRNA expression in tumor tissues than in surrounding liver tissues in 60% of HCC cases (Miura H, 1997). Yao et al. (2005) observed a VEGF mRNA expression level significantly higher in HCC than in surrounding tissue.

The VEGF protein was reported to be intensely localized in HCC cells; however, these studies did not compare tumor cell with surrounding liver tissue localization. In the study of Tseng P-L (2008), 35 patients (31%) exhibited stronger VEGF expression in the surrounding liver tissue than in HCC, the results being similar to those obtained by El-Assal et al. (1998); similar results were also discussed in the study of Yamaguchi R. (1998), who did not emphasize their clinical significance. Although these results were explained by the possibility that VEGF expressed in the liver may be secreted by normal hepatocytes, this hypothesis seems to be contrary to previous studies on mRNA expression (Yao DF, 2005). Another possible explanation for the results mentioned above is that VEGF expressed in the liver is released by HCC cells, the end product (the protein) being stored in the surrounding liver tissue, where it has a paracrine effect – this theory seems more probable because it is consistent with previous studies on VEGF mRNA expression and with present IHC studies. Some previous studies observed a significantly higher VEGF expression in the cirrhotic tissue surrounding HCC than in the non-cirrhotic liver tissue (Deli G, 2005; Shimoda K, 1999), underlining the hypothesis that a substantial reduction in the blood flow and a decrease in oxygen pressure regulate VEGF transcription and protein synthesis in cirrhotic liver tissues.

The association between hepatitis B or C viral load and a poor prognosis for HCC is well documented (Ohkubo K, 2002). In the study of Tseng P (2008), the presence of viremia (either HBV or HCV) was associated with VEGF overexpression in patients with HCC, being a poor prognosis factor in these patients, Helaly GF (2006) discussing the existence of a weak correlation between the level of hepatitis C viremia and VEGF. It is possible that VEGF overexpression observed in patients with viremia may be induced by the ongoing regeneration and the active inflammation in the liver. After independently analyzing survival, HBV viremia was considered a better predictive factor than HCV viremia for the prognosis of HCC.

CONCLUSIONS

Positive expression of VEGF in HCC is significantly correlated with capsular infiltration, vascular invasion and intrahepatic metastases.

VEGF expression was higher in HCC than in cirrhotic nodules; our results suggest that VEGF can play an important role in the angiogenesis and prognosis of HCC, as well as in the angiogenesis of liver cirrhosis.

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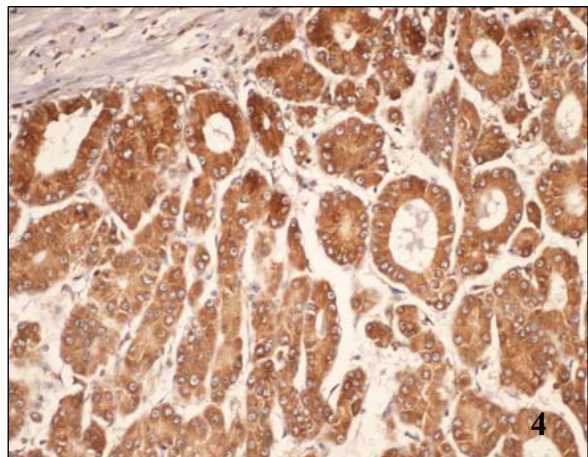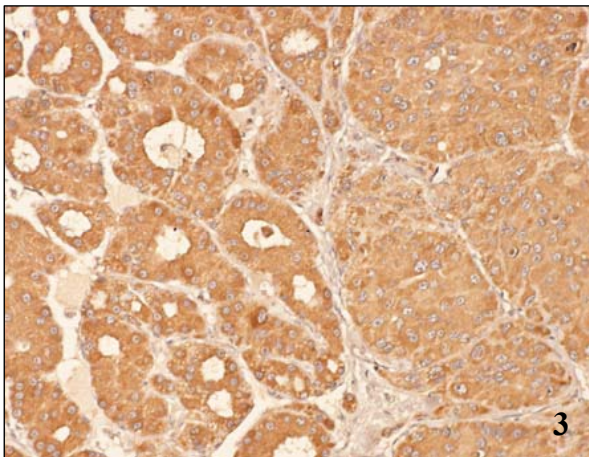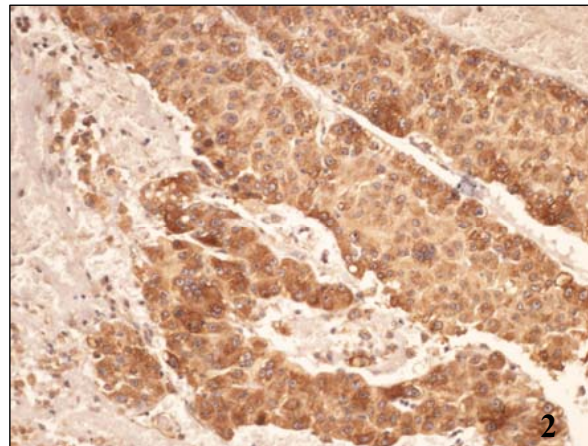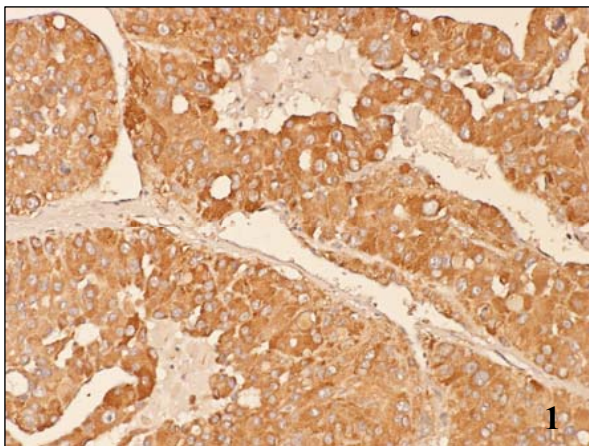
Fig. 1. Positive VEGF expression in trabecular HCC cells (LSAB+, DAB x 200)
Fig. 2. Positive VEGF expression in trabecular HCC cells (LSAB+, DAB x 200)
Fig. 3. Sinusoidal and acinar HCC, VEGF ++ (LSAB+, DAB x 200)
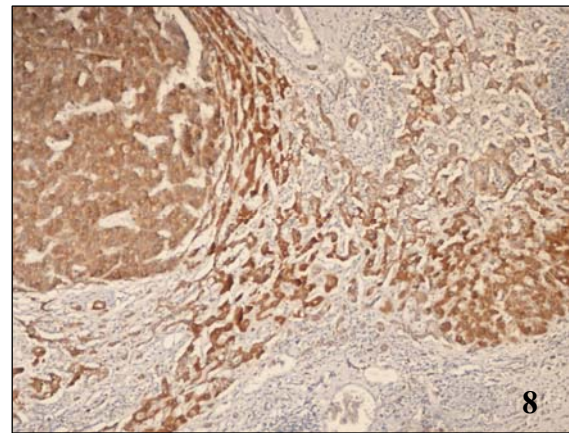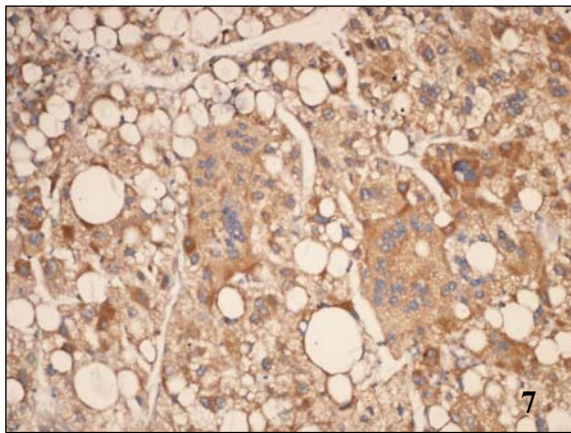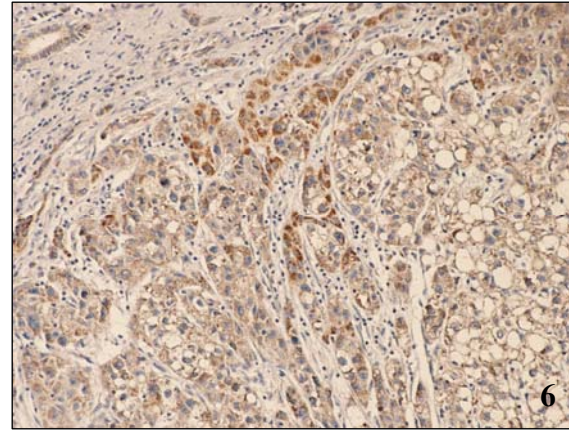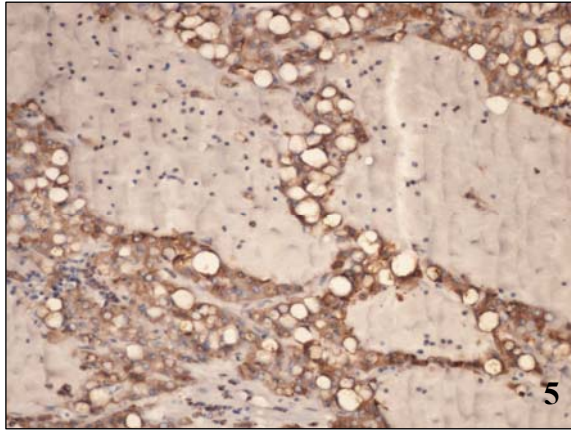Fig. 4. Acinar HCC with VEGF ++ expression (LSAB+, DAB x 200)

Fig. 5. Pelioid HCC, VEGF + (LSAB+, DAB x 200)
Fig. 6. Clear cell HCC, VEGF weakly positive (LSAB+, DAB x 200)
Fig. 7. HCC with fat deposition and bile secretion VEGF+ (LSAB+, DAB x 200)
Fig. 8. Sinusoidal HCC. VEGF + expression in tumor nodules and VEGF++ expression in surrounding normal hepatocytes (LSAB+, DAB x 200)