ANTICANCER RESEARCH *25*: 3067-3074 (2005)

Hepatocellular Carcinoma in Hepatectomized Patients: Biologic and Therapeutic Implications

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Abstract. *The macroscopic and microscopic features of 60 hepatocellular carcinomas (HCC) were investigated and correlated with patients' disease-free survival. Patients and Methods: The study included 60 HCCs removed, by partial hepatectomy, from an equal number of patients. In these tumors, several macroscopic and microscopic features were assessed, graded and correlated with disease-free survival. Results: HCCs begin as small, well-differentiated tumors that have an increased proliferation rate and neovascularization. Vascular invasion, which is the strongest predictor of disease recurrence, correlated significantly with tumor number and size, the predominant and worst degree of differentiation, and the apoptosis/mitosis ratio. In the absence of macroscopic or large vessel invasion, the largest tumor size ($p=0.006$), apoptosis/mitosis ratio ($p=0.03$) and number of tumors ($p=0.04$) were independent predictors of disease-free survival. Conclusion: This study showed that, in humans, the prognosis of HCC depends on several biological factors. Aggressive biological behavior (vascular invasion and recurrence) correlated significantly with: a) alterations in the apoptosis/mitosis ratio and b) architectural and cellular alterations.*

Hepatocellular carcinoma is the predominant primary malignancy of the liver, accounting for over 80% of the total, and it ranks among the most common neoplasms in the world with an estimated annual incidence of about one million cases. Because of its prevalence and unique biological and morphological characteristics, this tumor has attracted considerable attention from several disciplines, and its clinical, molecular and pathological features have been extensively investigated and reviewed (1).

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Key Words: Hepatocellular carcinoma, hepatectomy, prognosis.

Regarding the prognosis of this tumor, the histological features yield little independent prognostic power (1). The major exception is fibrolamellar carcinoma, a distinct clinicopathological variant that is associated with a more favorable prognosis (1). Instead, the outcome is primarily governed by the stage of the tumor and clinical status of the patient, the two principal factors that determine whether curative resection can be accomplished (1). Among the morphological contributors to tumor stage is size. Small or minute carcinomas, with a maximum diameter variously defined as between 3 cm and 5 cm, manifest a better prognosis, although with larger cancers the tumor size does not directly correlate with outcome (1). Improved survival has also been associated with tumors that are encapsulated or fail to invade the surrounding hepatic parenchyma (1). Among resected tumors, an expected prognostic indicator is whether or not the surgical margin is tumor-free (1). Other histological findings are not consistently correlated with outcome, although occasional series have reported a better prognosis with clear cell carcinomas and tumors of low histological grade (1).

For a long time, dysregulation of cell growth leading to cancer was explained largely in terms of increased cell proliferation. It has now become clear that decreased cell death (apoptosis) may also contribute to the pathological cell accumulation in a neoplasm. Enhanced cell survival through inhibition of apoptosis may be one of the mechanisms through which tumor promoters exert their effect (2, 3).

Apoptosis, a term defining programmed cell death, is an active energy-requiring process that is regulated by a variety of genes. It is characterized by cytoplasmic fragmentation and nuclear condensation, and it contributes to both physiological and pathological processes (2-5).

The proliferation activity of a neoplasm can be assessed through measurement of the tumor growth fraction, by immunohistochemically identifying specific cell cycle-related antigens. A widely used marker for proliferating cells is the Ki-67 antigen, which is expressed in nuclei during all phases

of the cell cycle except G0, and the presence of which has been related to tumor recurrence, stage and grade (6). The proportion of Ki-67-labelled cells in a given cell population (Ki-67 index) provides a measure of the growth fraction (7).

Current knowledge supports that the process of hepatic carcinogenesis includes 3 stages: initiation, promotion and progression (8-13). Initiation is characterized by DNA damage and mutations, whereas promotion involves clonal expansion of initiated cells, due to reduced responsiveness to negative growth constraints. Altered responses to signals for growth or terminal differentiation are responsible for resistance to the cytotoxicity and mitoinhibitory effects of carcinogens. Previous studies have reported that the altered foci may be capable of autonomous or clonal growth (14, 15). However, maintenance of a near-normal apoptosis/mitosis ratio suppresses expansion and, given this fact, the neoplastic foci may not be grossly or microscopically distinct from the surrounding liver (12). Finally, accumulation of other genetic defects occurs, resulting in disruption of the normal apoptosis/mitosis ratio, with subsequent changes to tumor progression (12).

The purpose of this study was to evaluate several macroscopic and microscopic features of hepatocellular carcinomas removed at the time of hepatectomy and to correlate them with patients' disease-free survival.

Patients and Methods

The study included 60 consecutive hepatectomy specimens of primary hepatocellular carcinomas, confirmed by previous biopsy, resected from an equal number of patients, at Patras University Hospital, Greece, during an 11-year time-period (1994-2004). Archival tissues and data derived from the pathology records, as well as clinical follow-up, were readily available for all patients. There were 36 men and 24 women, aged between 43 and 59 years. All the patients underwent hepatectomy for therapeutic reasons. All the demographic and clinicoepidemiological features pertaining to the patients were retrieved from their medical records. The slides and the pathology report for each patient were drawn from the files of the Pathology Department and were reviewed in order to confirm the pathological grade and stage.

Macroscopic examination of the specimens. Macroscopic evaluation of the hepatectomy specimens was based on a standard protocol (16). Information regarding the major diameter of the tumor(s), the number of tumor nodules and their distribution within liver lobes, were obtained from the pathology reports.

Microscopic evaluation of the specimens. Routine histopathological examination: Paraffin sections (4 μm thick) were stained with hematoxylin and eosin (H&E) and various features were evaluated, including: the predominant and worst tumor grade (according to Edmondson criteria) (17), the pattern of the tumor (microtrabecular, pseudoglandular, solid), vascular invasion (macroscopic or large vessel and microscopic or angiolymphatic), the presence or absence of necrosis within the tumor, the presence or absence of giant tumor cells and tumor clear cell changes.

Table I. *HCC and underlying disease (N=60).*

Hepatitis B	27
Hepatitis C	5
Hepatitis B+C	3
Alcohol	12
Unknown cause	13
Total	60
Co-existent cirrhosis 32	
Macronodular	10
Micronodular	5
Mixed	17
Total	60

Immunohistochemical and in situ hybridization (TUNEL) methods. Immunohistochemistry was performed on sections from one selected paraffin block of each case. Histological sections 4 μm thick, mounted on gelatin-coated glass slides, were dewaxed in xylene and hydrated through a series of graded concentrations of alcohol. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide for 15 min. For antigen retrieval, the slide sections were then processed in a microwave oven twice for 5 min each time at high power. Subsequently, a standard streptavidin-biotin-peroxidase techique (Multilink kit, Biogenex, San Ramon, CA, USA) was applied to detect the antigens. Sections were incubated for 30 min with the primary antibodies. These included mouse monoclonal antibodies to: Hepatocyte (Dako USA) at a 1:50 dilution, Ki-67 (Dako), at 1:40 dilution and CD34 antigen (Biogenex, USA) at 1:20 dilution. The anti-Hepatocyte antibody was used in order to confirm the hepatocytic origin of the tumors. The anti-Ki-67 and anti-CD34 antibodies were used to assess the degrees of proliferation and neovascularization, respectively. Diaminobenzidine (Sigma Fast DAB tablets, D-4293, St. Louis, MO, USA) was used as a chromogen. Sections were counterstained with Harris hematoxylin. All procedures were performed at room temperature. Between steps, the sections were washed in TBS. For positive control purposes, paraffin sections from human tonsils were used. For each antibody-negative control, studies were performed substituting the primary antibodies with normal rabbit serum. Nuclear staining for Ki-67 and cytoplasmic staining for CD34 were considered as positive.

To assess the presence of apoptosis, a standard TUNEL method was used. In paraffin sections, fragmented nuclear DNA associated with apoptosis was labelled *in situ* with digoxigenin-deoxyuridine (dUTP) and was introduced by terminal deoxynucleotidyl-transferase (TdT), according to a standard method using the ApopTag Plus peroxidase kit (Roche). In order to avoid an overestimation of the TUNEL-positive cells, only cells that exhibited both morphological features of apoptosis on light microscopy, not associated with inflammation, and positive TUNEL staining for fragmented DNA were considered as positive. For negative control purposes, some slides were incubated with a labelling solution that did not contain Tdt.

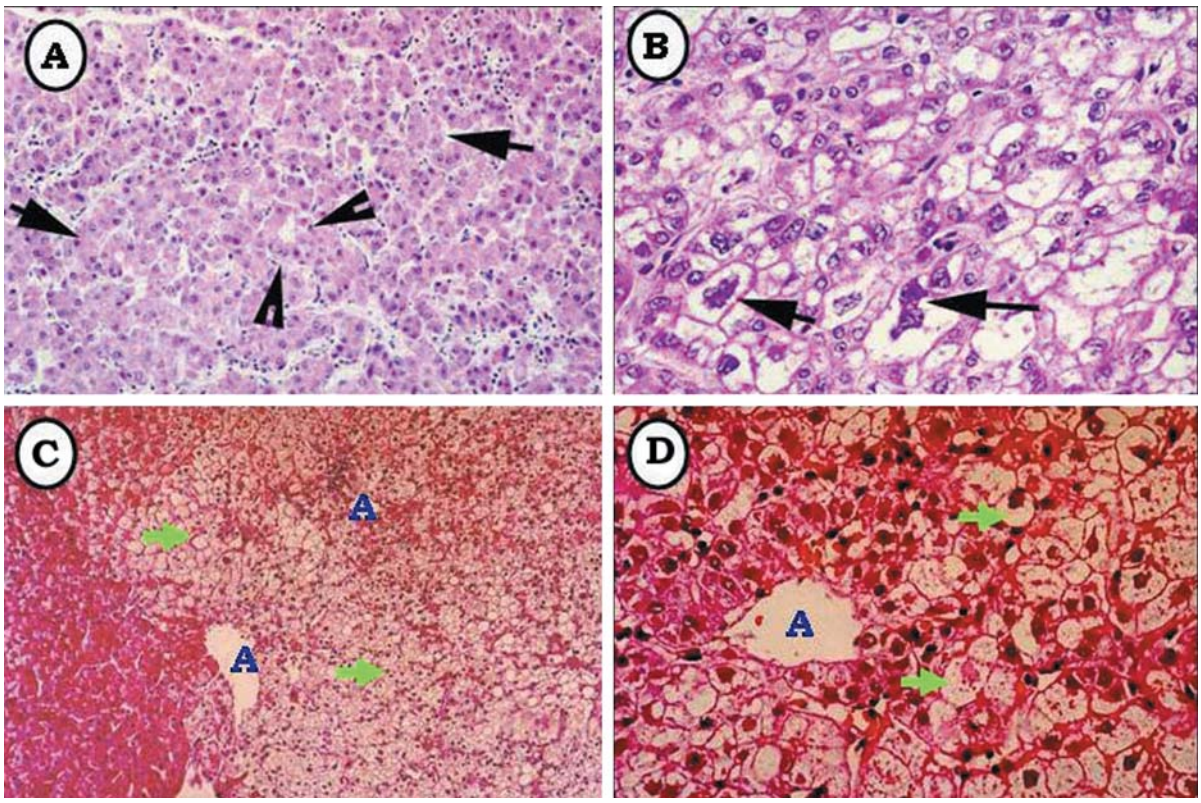

Figure 1. *A. HCC Edmondson grade I. The neoplastic cells form rosettes (arrowheads) and pseudoglands (arrows) (H&E x 250). B. HCC Edmondson grade III. The tumor harbors cellular atypia and pleomorphism (arrows) (H&E x 400). C, D. Clear cell changes (arrows). A: Abnormal feeding arteries. (C: H&E x 100, D: H&E x400).*

Morphometric analysis. All H&E, immunohistochemical and *in situ* hybridization slides were analyzed and scored in a blind fashion without knowledge of the clinicopathological data. Cell counts were performed manually on tumor and non-neoplastic surrounding parenchyma at x400 magnification using a 10x10 microscope grid (approximately 500 cells are included in such a grid). All counts were estimated by visual inspection of 5 different fields per section. For each field, the number of mitoses (in H&E stains-mitotic index-M), the percentage immunoreactivity for Ki-67 and CD34 and the presence of the apoptotic bodies (apoptosis-A) was obtained. The values in the same field did not differ by more than 10%. The average scores were then calculated. Finally, the apoptosis/mitosis (A/M) ratio was obtained by dividing the apoptotic index by the mitotic index in each case.

Statistical analysis. The results are expressed as mean±SD values. Intergroup comparisons, regarding correlation of pathological parameters with staining results, were performed using one-way analysis of variants (ANOVA). Whenever the equal variance test or normality tests failed, the Kruskal-Wallis nonparametric test was applied. In order to address the problem of multiple comparisons, the ANOVA and Kruskal-Wallis tests were followed by a *post hoc* Bonferroni test. The Spearman rank correlation test was used to investigate any possible correlations between the A/M ratio and other pathological factors. Finally, the Cox proportional hazards model was employed to reveal the effects of other prognostic factors (predominant and worst tumor grade, pattern of the tumor vascular invasion, presence or absence of tumor necrosis, presence or absence of giant tumor cells and tumor clear cell changes) on survival. The Kaplan-Meier procedure was also used to compare the survival curves. Data were analyzed using the SPSS statistical package (SPSS®, Release 10.0.1). Any $p<0.05$ was considered significant.

Table II. *Correlation between vascular invasion level and grade.*

	Vascular invasion negative	Vascular invasion positive	Macroscopic and microscopic	Total
Predominant grade				
I	23	11	0	34
II	9	3	7	19
III	0	3	4	7
Worst grade				
I	7	5	0	12
II	25	9	7	41
III	0	3	4	7
Total	32	17	11	60

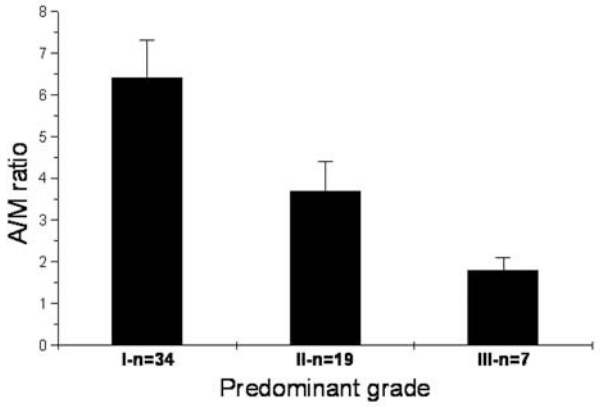

Figure 2. *Correlation between A/M (apoptosis/mitosis) ratio and predominant grade.*

Results

Routine histopathological features. All tumors exhibited positive immunostain for the Hepatocyte antigen. Table I lists all the causes for the underlying diseases. It should be emphasized that, in 13 cases, no evident underlying cause could be established. In 32 cases, hepatocellular carcinoma co-existed with cirrhosis. Forty-three tumors showed a microtrabecular/microacinar growth pattern and 17 tumors a solid growth pattern. Thirty-four tumors had predominant grade I (Figure 1A), 19 grade II and 7 grade III (Figure 1B). Table II shows an obvious significant correlation between angiolymphatic invasion and the predominant and worst degree of differentiation. In addition, as shown in Table III, vascular invasion at any level (macroscopic and microscopic) was more likely to be present: a) in tumors with a solid growth pattern compared to a microacinar pattern (Figure 1A) ($p=0.032$); b) when giant cells and clear cells (Figures 1C and 1D) were observed within the tumor ($p=0.003$ and $p=0.031$, respectively); and c) when tumor necrosis was present ($p=0.012$). This study failed to demonstrate any significant correlation between the number of neoplastic nodules and the presence of vascular invasion.

Figure 2 displays the mean±SD values for the A/M ratio in relation to the predominant tumor grade. It is clear that this ratio is significantly decreased towards tumors of grade III (6.2±2.3 in grade I tumors *vs.* 1.8±0.9 in grade III tumors-$p<0.001$). There were also significant correlations between the A/M ratio and the extent of vascular invasion (distribution of those with none, microscopic or angiolymphatic, and macroscopic or larger vessel invasion): a lower ratio in those with increasing extent of vascular invasion ($p<0.001$). The A/M ratio was also significantly lower in tumors with clear cell change ($p<0.01$), giant cells ($p<0.001$) and tumor necrosis ($p<0.001$) (data not shown).

Immunohistochemical and in situ hybridization results. In all 60 cases, the Ki-67 index (proliferation index) (Figures 3B, 3C and 3D) was higher in the tumor than in the surrounding liver (data not shown – $p<0.001$). Apoptotic bodies were more frequently present in the well-differentiated neoplasms (Figure 3A). In addition, significant correlations between apoptosis, mitosis and Ki-67 values were recorded ($p<0.01$).

All the 60 tumors showed a diffuse staining pattern with the CD34 antigen (Figures 4A and 4B). Within the tumors, the mean±SD values of the vessels were 12.3±3.4 compared to 5.1±1.2 of the surrounding non-neoplastic parenchyma ($p<0.01$). When the number of vessels within a tumor increased, the apoptotic cell number decreased ($p<0.01$), suggesting that angiogenesis might contribute to tumor progression by altering the A/M ratio.

Statistical analysis regarding disease-free survival and tumor recurrence. Cox regression analysis revealed that the presence of vascular invasion is an independent prognostic factor of recurrence (95%CI: 1.42-16-$p<0.001$). Vascular invasion was significantly correlated with the worst grade and the A/M ratio. In the absence of vascular invasion, the size of the largest tumor nodule ($p=0.006$), the A/M ratio ($p=0.03$) and the number of tumor nodules ($p=0.04$) were independent prognostic factors and were significantly correlated with disease-free survival. More specifically, tumors with A/M>3 displayed higher disease-free survival compared to those with A/M<3 ($p<0.01$) (Figure 5). From the 8 cases with A/M>6, none developed recurrence 5 years after hepatectomy. Lastly, patients with co-existent cirrhosis had significantly shorter disease-free survival compared to those without cirrhosis ($p<0.01$ – data not shown). These results are in agreement with the data of the literature (1).

Discussion

This study showed that hepatocarcinogenesis is a multistep and multifocal process (8, 12, 13).

The majority of HCCs in this study appeared to begin as small well-differentiated neoplasms without vascular invasion, which is similar to other studies (11). The distinction between dysplasia and carcinoma in such cases is currently based on the subjective opinion of the pathologist, who relies on a combination of architectural and cellular alterations (10, 11, 18, 19). The validity of this distinction is substantiated by significantly increased Ki-67 labelling within the tumor cells (20, 21) compared with the surrounding liver and the induction of tumor angiogenesis, confirmed by CD34 antigen staining (22-24). The fact is that the small, well-differentiated HCCs show increased cellular proliferation, but the differentiation, and the ratio of cell

proliferation to apoptosis, remains near normal such that complete excision is curative.

Previous studies have reported that the development of "tumor(s) within a tumor" was recognized histologically by both a lesser degree of differentiation pleomorphism and a higher Ki-67 index (11, 18, 19, 25, 26). In such cases, less differentiated foci are always surrounded by better differentiated neoplastic hepatocytes, similar to previous studies (11, 18, 19, 26). Eventually, less-differentiated foci are capable of developing vascular invasion and metastasize, as evidenced by the correlation between vascular invasion and recurrence of disease (11, 27). The acquisition of these aggressive features may be due to abnormalities that lead to alterations in growth (A/M ratio) (12, 13, 28). In fact, the increased proliferation observed in the small, well-differentiated lesion may promote genetic instability and tumor progression (13). Thus, routine histopathological findings such as vascular invasion, tumor giant cells, and the A/M ratio may be helpful markers in the attempt to predict the biological behavior of the tumor. It should be mentioned that the mitotic and apoptotic processes of a tumor are regulated and controlled by several cell cycle proteins (25, 29-32).

Another interesting aspect of hepatic carcinogenesis and tumor progression is the fact that, although most hepatocellular carcinomas begin as well-differentiated neoplasms that slowly enlarge and evolve, as discussed above, a small subset (roughly 10% as reported in the literature) quickly display aggressive characteristics, similar to flat ulcerative carcinomas of the bladder and colon (33, 34). In our study, we did not observe any such cases. However, with this information in mind, we were tempted to speculate that tumor size alone may not be adequate to predict the biological behavior of the tumor. More research in this field, in order to clarify those genetic abnormalities that contribute to the early aggressiveness of such tumors, is warranted.

Finally, angiogenesis is an obviously important aspect of hepatic carcinogenesis. The results of this study showed a correlation between microvascular density and tumor aggressiveness, size, differentiation, mitosis, or any level of vascular invasion. These are in agreement with previous studies (23, 35, 36). When the number of vessels within a tumor increased, the apoptotic cell number decreased, suggesting that angiogenesis might contribute to tumor progression by altering the A/M ratio.

In conclusion, this study demonstrated that liver carcinogenesis in humans is a multistep and multifocal process. Aggressive biological behavior (vascular invasion and recurrence) correlates significantly with alterations in the A/M ratio and with architectural and cytological alterations, that suggest a progressive accumulation of multiple genetic abnormalities.

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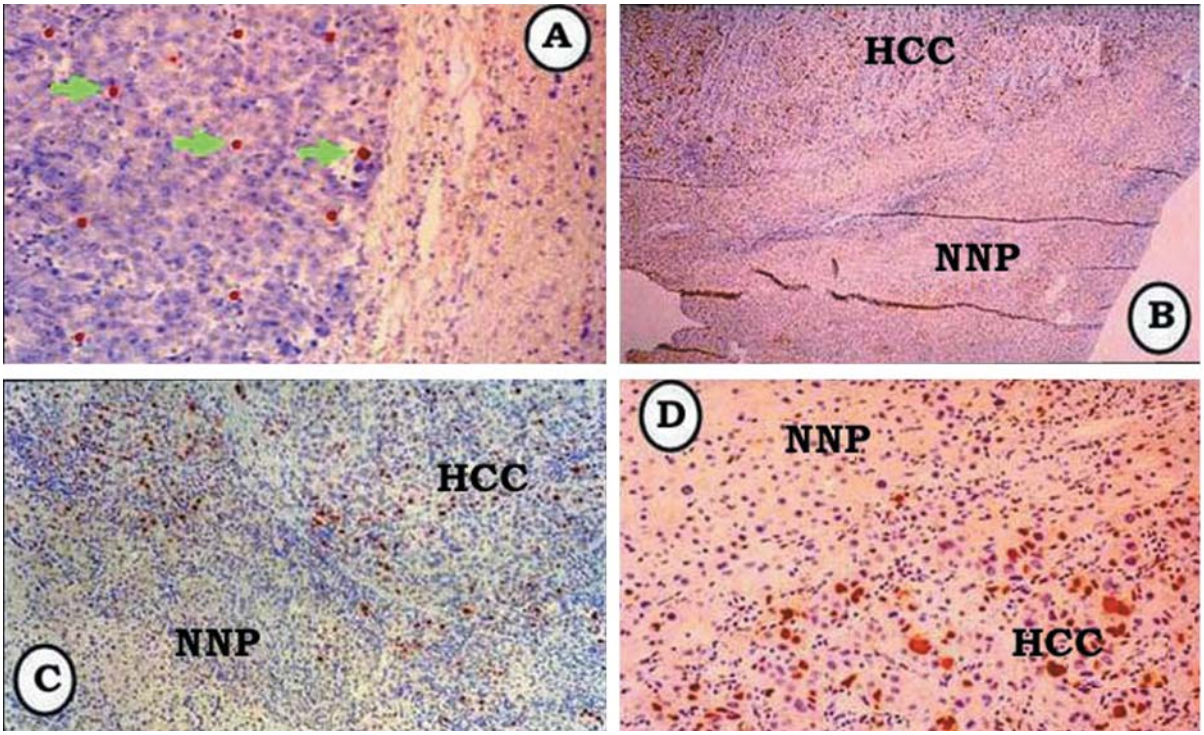

Figure 3. *A. Numerous apoptotic bodies within an HCC Edmondson grade I (arrows-TUNEL peroxidase x250). B, C, D. Different expression of Ki-67 antigen (proliferation index): High Ki-67 expression in tumor cells (HCC) and lower Ki-67 expression in the surrounding non-neoplastic parenchyma (NNP) (streptavidin-biotin peroxidase x25(B), x100(C), x250(D).*

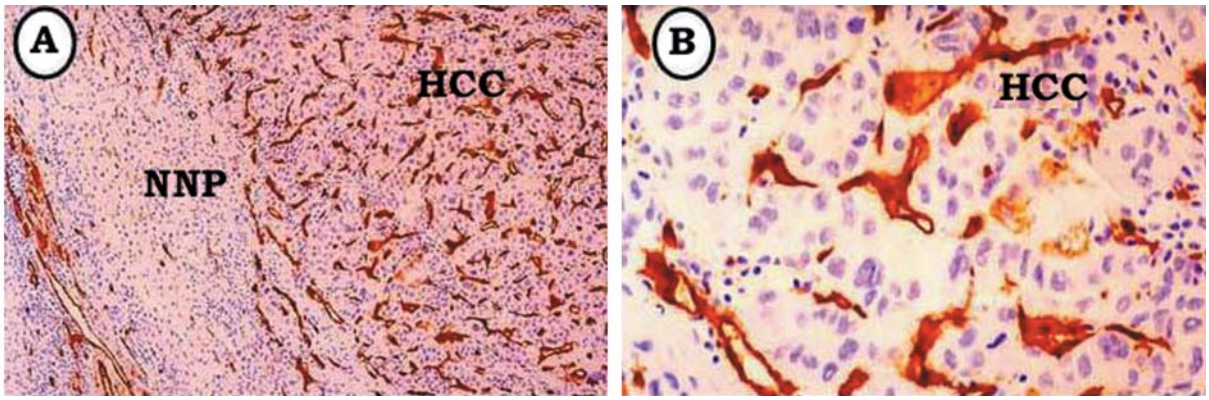

Figure 4. *A, B. Differences in neo-vascularization: high degree of neovascularization (expression of CD34 antigen) in tumor areas (HCC) and lower degree in the surrounding non-neoplastic parenchyma (NNP) (streptavidin-biotin peroxidase x100(A), x400(B).*

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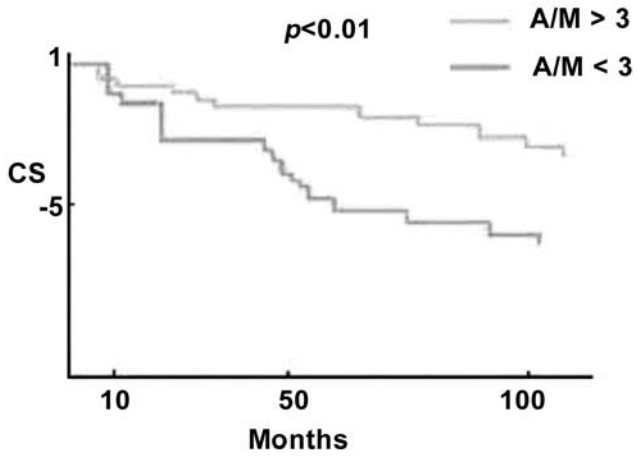

Figure 5. *Correlation between disease-free survival and A/M (A/M: apoptosis/mitosis ratio).*

Table III. *Correlation between vascular invasion (macroscopic and microscopic) and gross and histopathological features of the tumors.*

	Vascular invasion absent	Vascular invasion present	*p*
Growth pattern			
Microtrabecular/microacinar	29	14	0.032
Solid	3	14	
Giant cells			
No	29	11	0.003
Yes	3	17	
Clear cells			
No	26	11	0.031
Yes	6	17	
Tumor necrosis			
No	29	13	0.012
Yes	3	15	
Number of tumors			
1	30	22	0.192
2	2	3	
3	0	3	
Total	32	28	

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Received May 4, 2005
Accepted May 18, 2005