

Hepatocellular Carcinomas in Native Livers From Patients Treated With Orthotopic Liver Transplantation: Biologic and Therapeutic Implications

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The gross and histopathologic characteristics of 212 non-fibrolamellar hepatocellular carcinomas (HCCs) discovered in native livers removed at the time of liver transplantation were correlated with features of invasive growth and tumor-free survival. The results show that most HCCs begin as small well-differentiated tumors that have an increased proliferation rate and induce neovascularization, compared with the surrounding liver. But at this stage, they maintain a near-normal apoptosis/mitosis ratio and uncommonly show vascular invasion. As tumors enlarge, foci of dedifferentiation appear within the neoplastic nodules, which have a higher proliferation rate and show more pleomorphism than surrounding better-differentiated areas. Vascular invasion, which is the strongest predictor of disease recurrence, correlates significantly with tumor number and size, tumor giant cells and necrosis, the predominant and worst degree of differentiation, and the apoptosis/mitosis ratio. In the absence of macroscopic or large vessel invasion, largest tumor size ($P < .006$), apoptosis/mitosis ratio ($P < .03$), and number of tumors ($P < .04$) were independent predictors of tumor-free survival and none of 24 patients with tumors having an apoptosis/mitosis ratio greater than 7.2 had recurrence. A minority of HCCs (<15%) quickly develop aggressive features (moderate or poor differentiation, low apoptosis/mitosis ratio, and vascular invasion) while still small, similar to flat carcinomas of the bladder and colon. In conclusion, hepatic carcinogenesis in humans is a multistep and multifocal process. As in experimental animal studies, aggressive biologic behavior (vascular invasion and recurrence) correlates significantly with profound alterations in the apoptosis/mitosis ratio and with architectural and cytologic alterations that suggest a progressive accumulation of multiple genetic abnormalities. (HEPATOLOGY 2001;34:502-510.)

Hepatic carcinogenesis is a multicentric process divided classically into the 3 stages of initiation, promotion, and progression.¹⁻⁷ Initiation, an irreversible step marked by DNA damage and mutations, confers upon initiated cells, a potential growth advantage over surrounding cells. In the proper promoting environment, initiated cells undergo clonal expansion because of less responsiveness to negative growth constraints, altered responses to signals for growth or terminal differentiation, and/or resistance to the cytotoxicity and mitoinhibitory effects of carcinogens. The altered foci may be capable of autonomous or clonal growth,^{4,8-10} but maintenance of a near-normal mitosis/apoptosis ratio⁶ limits expansion such that the foci may not be grossly or microscopically distinct from the surrounding liver.^{4,8-10} Tumor progression refers to accumulation of additional genetic defects that profoundly alter tumor growth, especially the relationship between mitosis and apoptosis⁶ and the capacity for invasive and metastatic growth.

Tumor progression can be divided clinically into curable and noncurable stages, which obviously are disease and treatment specific. We previously developed a statistical model allowing prognostication of hepatocellular carcinoma (HCC) recurrence after liver transplantation, based on routine gross and microscopic pathologic tumor characteristics (grade of vascular invasion, number of tumors, largest tumor size, lobar distribution).¹¹⁻¹⁴ The model allowed unambiguous identification of certain combinations of tumor characteristics that always or never led to recurrence. Unfortunately, in 30% of cases the same combination was associated with an opposite clinical outcome.¹¹⁻¹⁴

The goal of this study was to determine whether concepts derived from experimental animal hepatic carcinogenesis could be applied to the study of human HCC, and thereby enhance the predictive power of modeling. Therefore, we characterized all HCCs present in native livers removed at the time of transplantation, which provided an opportunity to study the entire spectrum of tumorogenesis, because most of the transplantations were performed for underlying cirrhosis or a combination of cirrhosis and tumor. An emphasis on features important in tumor progression enabled us to correlate gross and histopathologic tumor characteristics with vascular invasion and tumor-free survival after transplantation.

MATERIALS AND METHODS

Patient Population. The study (IRB protocols #000625 and 9507150) analyzed 212 of 358 native hepatectomy specimens containing HCC from a total of 3,432 orthotopic liver transplantation

Abbreviations: HCC, hepatocellular carcinoma; HPF, high power field.

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procedures performed between January 1981 and December 1999 at the Thomas E. Starzl Transplant Institute, University of Pittsburgh Medical Center. Fibrolamellar variants of HCC in noncirrhotic livers were excluded; and the blocks and slides from the remaining cases with HCC were unavailable for review. When more than one tumor nodule was identified, it was not always possible to distinguish independent primary tumors from intrahepatic metastasis (-es) from a single primary, on the basis of morphologic criteria alone. Any co-existent disease diagnosis was established using standard clinical and histopathologic criteria with clinicopathologic correlation. Demographic data including patient age, gender, and follow-up and other tumor information was obtained from the Electronic Data Interface for Transplantation (EDIT), which is a database maintained by the Thomas E. Starzl Transplant Institute. Tumor size and number and lobar distribution were obtained from the surgical pathology reports.

Routine Histopathologic Studies. Gross examination and histologic sectioning of the hepatectomy specimens were examined according to a previously published protocol.¹⁵ Routine hematoxylin and eosin stained sections of the tumor(s) were analyzed for predominant and worst grade of differentiation, growth pattern(s), microscopic or angiolymphatic and macroscopic or larger vessel invasion, necrosis, giant cells, and clear cell change (Fig. 1A-G). Evidence of distant metastases, lymph node metastases, resection margins, and infiltration of other organs was obtained from the clinical records and surgical pathology reports. Tumor differentiation was graded as well, moderate, and poor (Fig. 1A-G). The areas of worst differentiation were selected for counting metaphase mitotic figures and apoptotic cells (Fig. 1H-I), but areas near tumor necrosis were avoided. The apoptotic and mitotic index were determined by counting the total number of mitotic or apoptotic cells in 10 randomly selected high power fields (HPF) ($\times 400$). Vascular invasion was divided into microscopic or angiolymphatic infiltration (small thin-walled vessels) and macroscopic or larger-vessel (typical veins or arteries) invasion (Fig. 1K-L). Tumor necrosis, tumor giant cells, and clear cell change were recorded as present or absent, but not graded (Fig. 1F-J).

The surrounding liver was examined for the presence and type of cirrhosis, the activity level of chronic hepatitis, if present, and portal vein thrombosis. Chronic active hepatitis was graded as follows: 0, inactive; 1, minimally active; and 2, active; based on a combination of the severity of inflammation and interface activity.

Immunohistochemical Analyses. From the 212 cases analyzed above, 55 were randomly selected for a more detailed immunohistochemical analysis using identifiers of cell proliferation (Ki-67; Mib-1 clone; Immunotech, Westbrook, ME) and tumor angiogenesis (anti-human α smooth muscle actin; 1A4; Lot110; 1:30 dilution; DAKO, Carpinteria, CA). Staining was performed on the section containing the area of worst differentiation and on nontumorous liver using a standard indirect avidin-biotin complex technique. All Ki-67⁺ hepatocytes were counted, regardless of the intensity of the staining in 10 HPF ($\times 400$). The distribution of Ki-67 staining within the tumors and nontumorous livers was classified as homogenous and nonhomogenous.

The pattern of smooth muscle actin staining was divided into single complete vessels and branching, sinusoid-like channels (Fig. 2A-C). For scoring purposes, the number of complete vessels was counted in 10 HPF. The sinusoid-like vasculature was scored as follows: 0, no sinusoid-like pattern; +, weakly stained discontinuous and focal vessels; ++, weakly stained diffuse branching vessels/focal well-formed vessels; and +++, diffuse well-formed sinusoid-like vessels. The distribution of each type of vessels was classified as either homogenous or nonhomogenous.

Statistical Analyses. Categorical variables were compared by using the χ^2 likelihood ratio test or Fisher's exact test when appropriate. Ordinal or interval variables were compared with the Kruskal-Wallis test or Wilcoxon-Mann-Whitney U test when only 2 groups were compared. The Kaplan-Meier method was used for analysis of impact of the studied pathologic characteristics of HCC on tumor-free sur-

vival. Significance level was set up at .05. The stepwise Cox regression model with likelihood ratio test for variable selection was used to determine independent pathologic predictors of HCC recurrence.

RESULTS

Characteristics of the Patients With HCC in the Native Liver. The study patients were predominantly men (159 men, 53 women), and ranged in age from 18 to 76 years (mean \pm SD = 53.9 \pm 9.6 years). Most of the patients had at least one risk factor for HCC, as evidenced by the etiology for the underlying cirrhosis, which was present in 197 of 212 (92.8%) cases (Table 1). The causes of the underlying liver disease were hepatitis C virus-induced (n = 60) or hepatitis B virus-induced (n = 38) cirrhosis (4 had both), ethanol abuse (n = 42), cryptogenic cirrhosis (n = 22), non-A, non-B chronic hepatitis (n = 20; pre-1990), autoimmune hepatitis (n = 7), primary biliary cirrhosis (n = 5), and primary sclerosing cholangitis (n = 2). The 25 remaining cases fell into other miscellaneous categories, such as metabolic diseases and chronic hepatitis, not otherwise specified.

Gross and Microscopic Characteristics of the Liver and HCC. The pattern of cirrhosis was mixed in 113 (57.4%); the remaining livers showed a predominantly macronodular (n = 46; 23.3%) or micronodular cirrhosis (n = 38; 19.3%). The hepatitis activity level, when present, was judged as mild in 79 (37.3%) and severe in 105 (49.5%). One fourth of the cases (n = 50; 23.6%) had gross evidence of portal vein thrombosis.

A single tumor was present in 105 cases, 29 cases had 2 nodules, 7 cases had 3 nodules, and more than 3 tumor nodules were detected in 71 cases. When limited to a single lobe (n = 149), most tumors developed in the right lobe (125 right, 19 left, 4 caudate, 1 quadrate lobe). Multilobar involvement was seen in 63 cases. The median size of single and multiple tumors was 1.8 and 3.0 cm, respectively, with a standard deviation of 3.5 cm for both groups. Tumor was present in the resection margin in 13 (6.1%) cases, and 5 had lymph node metastasis.

Most HCCs appear to begin as small well-differentiated neoplasms, but eventually show heterogeneous histologic growth and differentiation patterns, consistent with the concept of tumor evolution (Fig. 3A-F). For example, most small single tumors (≤ 1.0 cm) were predominantly (23 of 28; 82%) or exclusively (13 of 28; 46%) well differentiated and did not show any evidence of vascular invasion (26 of 28; 93%). A few of these small single tumors however, showed predominantly or exclusively moderate or poor differentiation (4 of 28; 14%). Of all predominantly well-differentiated tumors, only 39 of 102 (38%) were exclusively well differentiated (i.e., the predominant grade same as the worst grade), and all but 3 of these tumors were 3 cm or less. In larger (≥ 5.0 cm) single tumors, only 3 of 16 (19%) were predominantly well differentiated and none were exclusively well differentiated. Eight of 10 (80%) exclusively poorly differentiated tumors were 3 cm or greater.

A trabecular pattern predominated in 174 cases and was the secondary pattern in 21 cases; a pseudo-acinar pattern was observed in 66 cases and predominated in 29 cases. Clear cell change was present in 93 (43.9%) and giant tumor cells in 82 (38.7%) cases. Slightly more than one third (n = 78; 36.8%) of the tumors showed necrosis. One half of the tumors (n = 102; 48.1%) showed microscopic or angiolymphatic invasion and a subset of these (n = 40; 18.9%) also showed macroscopic or larger vessel involvement. The mitotic figure count in the

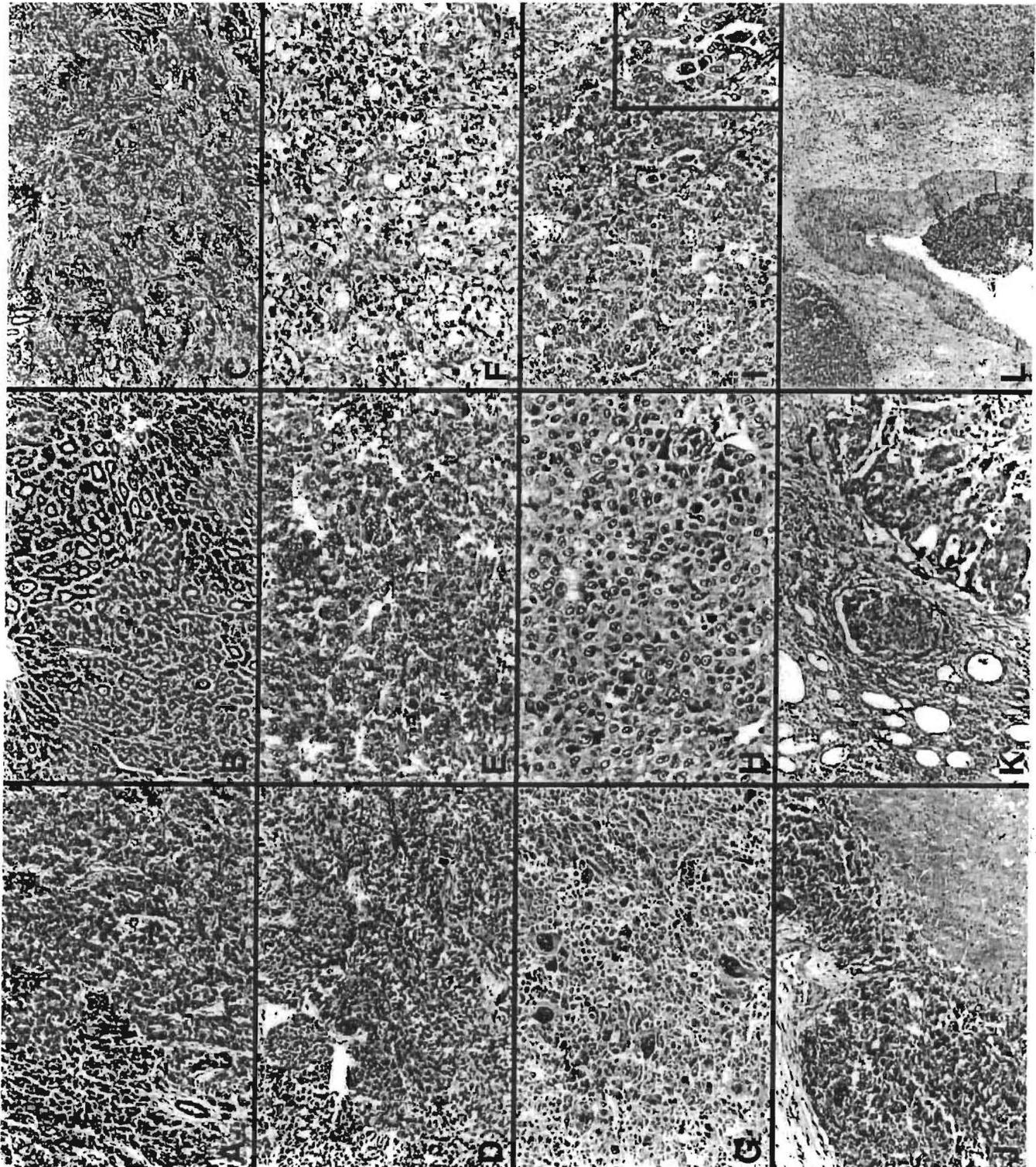


FIG. 1. Illustration of the various routine histopathologic parameters evaluated in the HCC present in the native livers removed at the time of transplantation. (A) Well-differentiated HCC; (B) pseudo-acinar growth pattern; (C) fibrolamellar-like growth pattern; (D) moderately differentiated HCC; (E) poorly differentiated HCC; (F) clear cell change; (G) multi- and mononuclear tumor giant cells; (H) mitoses; (I) apoptosis (*inset* showing greater detail); (J) tumoral necrosis (*bottom right*); (K) microscopic or angiolymphatic invasion; (L) macroscopic or arterial invasion.

areas of worst differentiation ranged from 1 to 42 per 10 HPF ($\times 400$) (mean = 10.9 ± 8.9 , median = 9). In the same areas, apoptosis counts ranged from 4 to 69 per 10 HPF (mean = 32.2 ± 15.2 , median = 31). The ratio of apoptosis to mitosis

in the same area ranged from 1.09 to 31 (mean = 4.74 ± 4.12 , median = 3.63).

Correlation Between Different Tumor Characteristics. Recognition of vascular invasion is a direct indication of tumor aggres-

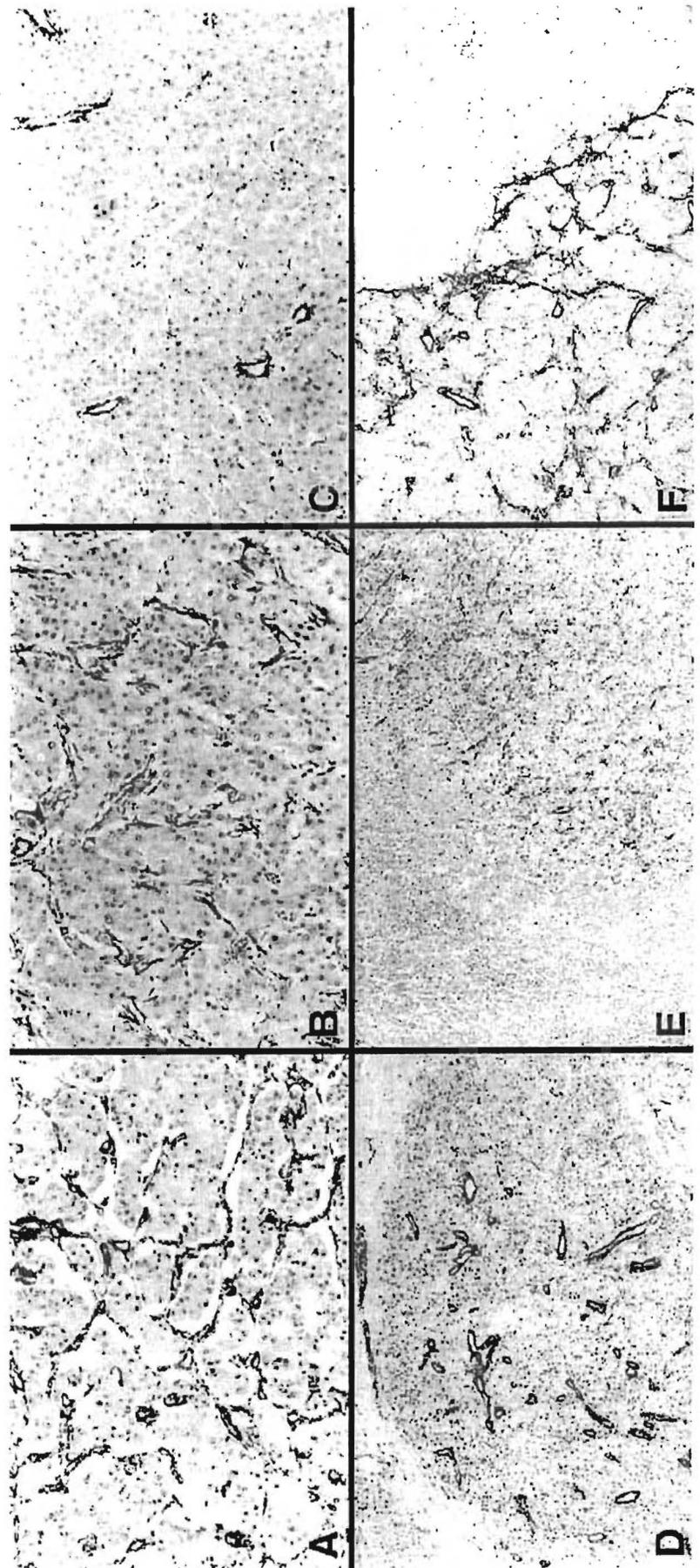


FIG. 2. Grading of tumor angiogenesis. (A) Diffuse prominent sinusoidal-like vessels (+++); (B) weakly stained, diffuse sinusoidal-like vessels (++); (C) weakly stained, discontinuous sinusoidal-like vessels; and (D) countable complete vessel pattern. Recognition of tumor angiogenesis. (E) Note the development of the sinusoidal-like tumor angiogenesis within the tumor (right), in contrast to the surrounding non-neoplastic liver (left). (F) Tumor angiogenesis also varied in different areas of an evolving tumor. Note the combination of the sinusoidal-like vessels and single complete vessels in the moderately differentiated area of the HCC (left), which evolved from a well-differentiated tumor that shows only a weak sinusoidal pattern (right).

TABLE 1. Etiology of Underlying Cirrhosis, Which Was Present in 197 of 212 Cases With Coexistent HCC in the Native Liver Removed at Transplantation

Etiology of Cirrhosis	Number of Cases*
Hepatitis C virus	60
Hepatitis B virus	38
Ethanol abuse	42
Cryptogenic cirrhosis	22
Non-A, non-B chronic hepatitis (pre-1990)	20
Autoimmune hepatitis	7
Primary biliary cirrhosis	6
Primary sclerosing cholangitis	2
Other	25

*Some of the patients have more than one disease and are listed under each.

siveness and is the single most significant predictor of tumor recurrence after transplantation.^{13,16} Microscopic or angiolymphatic invasion correlates significantly with tumor nodule number and tumor size as well as the presence of giant cells and necrosis ($P < .01$; Table 2). There was also a significant correlation between angiolymphatic invasion and the predominant and worst degree of differentiation ($P < .01$; Table 3). The predominant differentiation pattern is more predictable of angiolymphatic invasion for the poorly differentiated cases, but the worst degree of differentiation better predicted angiolymphatic invasion in the well-differentiated cases. If the predominant differentiation was poor, all of the cases (10 of 10; 100%) had some level of vascular invasion. Conversely, if the worst area of the tumor was still well differentiated, then 35 of 39 (89.7%) cases did not have any level of vascular invasion (Table 3). There were no significant associations among the presence of clear cell change, histologic growth pattern, hepatitis activity, etiology of the primary liver disease, and any level of vascular invasion (Table 2).

There were also significant correlations between the apoptosis/mitosis ratio and other pathologic and clinical (see below) tumor characteristics. For example, both predominant and worst degree of differentiation were strongly correlated with apoptosis, mitosis, and the apoptosis/mitosis ratio ($P < .01$): the ratio decreases with dedifferentiation. There was a very strong correlation ($P < .0001$) between the apoptosis/mitosis 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. The apoptosis/mitosis ratio was also significantly lower in tumors with clear cell change ($P < .05$), giant cells ($P < .01$) and tumor necrosis ($P = .001$). We failed to establish a significant correlation between tumor number and those parameters. The mean with 95% CI boundaries for the apoptosis/mitosis ratio for the predominant grade of differentiation is shown in Fig. 4.

Immunohistochemical Findings. In all the cases studied, the number of Ki-67⁺ hepatocytes per 10 HPF was significantly higher in the tumor than in the surrounding liver ($P < .0001$). However, in most of the cases (49 of 55; 89%), the apparent non-neoplastic hepatocytes in the surrounding liver also showed increased Ki-67 labeling (mean 4.81 ± 4.77 ; range, 0 to 14; median, 2.5) compared with historical controls of normal human liver.¹⁷ In general, cases with relatively high Ki-67 labeling in the non-neoplastic hepatocytes also showed high Ki-67 labeling in the tumor and/or had active hepatitis. There

were also significant correlations between apoptosis, mitosis, and Ki-67 labeling, as expected, and seen in previous studies ($P < .01$). When the apoptosis/mitosis ratio and Ki-67 labeling were compared, again a strong correlation was found, but it was slightly lower ($P < .05$) than for the correlation between mitosis and apoptosis (see above).

Almost all of the tumors (50 of 56; 89.3%) showed a diffuse sinusoid-like staining pattern with smooth muscle actin, whereas this pattern was not seen in the surrounding non-neoplastic liver (Fig. 2). Within the tumors, the number of vessels (range 3-39; mean 10.2 ± 6.8 , median 8) was significantly higher ($P < .01$) than in the surrounding non-neoplastic nodules, which showed no vessels in 37 cases, 1 vessel in 6 cases, and 2 vessels in 13 cases. Although angiogenesis significantly correlated with neoplastic transformation, there was no statistical correlation between angiogenesis and mitosis, Ki-67, differentiation, vascular invasion, tumor size, or tumor number. If the number of complete vessels within a tumor was more than 5, the apoptotic cell number decreased and the correlation between the vessel number and apoptosis was statistically significant ($P < .01$).

Tumor vascularity (19 of 56; 34%) and Ki-67 labeling (25 of 56; 45%) were judged to be heterogeneous within the tumors, and the heterogeneity was often related to obvious differences in tumor morphology (i.e., differentiation or histologic pattern). However, in other cases regional differences in Ki-67 labeling within the tumors had no obvious morphologic correlate, except that in 5 cases, staining was increased at the periphery of the tumor. There was no significant correlation between the tumor growth pattern or clear cell change and Ki-67 and/or vessel number or heterogeneity.

Correlation Between Tumor Characteristics and Tumor-Free Survival After Transplantation. Because patients with macroscopic or larger vessel invasion invariably suffer HCC recurrence,¹⁶ pathologic examination of native liver becomes especially valuable in terms of disease prognosis for patients with no or microscopic (angiolymphatic) invasion. To investigate the potential role of tumor characteristics for postoperative HCC progression in patients without large vessel invasion, the Cox proportional hazard model was used. Largest tumor size ($P < .006$), apoptosis/mitosis ratio ($P < .03$), and number of tumors ($P < .04$) were found to be independent predictors of postoperative tumor-free survival. The hazard of recurrence increases 3.1-fold (95% CI: 1.1-8.8) for the apoptosis/mitosis ratio below 3.5 compared with the respective value above 3.5.

The apoptosis/mitosis ratio alone can also serve as a predictor of disease progression. The patients with tumors showing a ratio above 3.5 had significantly superior ($P < .002$) post-transplantation tumor-free survival compared with patients who had tumors with a lesser ratio (Fig. 5). This finding is not surprising because the extent of vascular invasion, which is the most important predictor of disease progression, correlates well with the apoptosis/mitosis ratio ($P < .0001$). Moreover, in the absence of macroscopic or large vessel invasion, none of 24 patients with the apoptosis/mitosis ratio of above 7.2 had HCC recurrence during the follow-up period (3.4 ± 0.5 years).

The apoptosis/mitosis may help distinguish between multiple versus single origin of multiple tumors (stage T4 vs. T2 or 2 T1 tumors). However, tumor genetic analysis is required to evaluate this hypothesis.

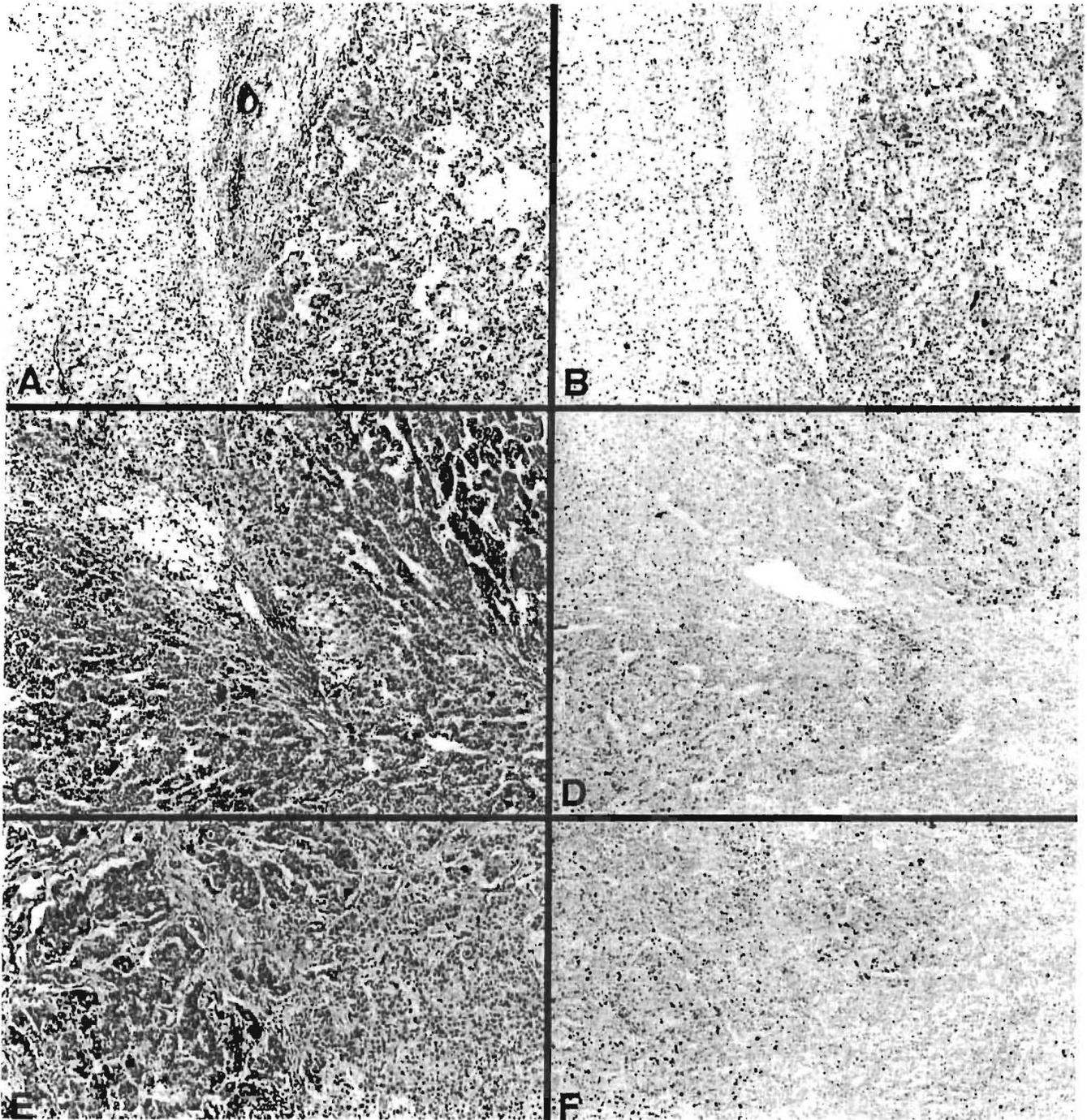


FIG. 3. Most of the HCCs showed heterogeneous histologic growth and differentiation patterns, consistent with the concept of tumor progression. More poorly differentiated areas showing a higher rate of proliferation always developed within a better-differentiated area at the periphery. The same tumor is illustrated in (A) smooth muscle actin staining and (B) Ki-67 nuclear labeling. Note that the well-differentiated area is on the left, shows clear cell change, less neoangiogenesis, and a lower Ki-67 labeling index, compared with the moderately differentiated area on the right. In an effort to capture tumor heterogeneity for data analysis, both the predominant and worst grade of differentiation were recorded. The same concept is illustrated in C and D (same regions of tumor) and E and F (same regions of tumor). Note that the areas of increased cellularity and decreased differentiation show higher Ki-67-labeling indexes than the surrounding better-differentiated neoplastic cells.

DISCUSSION

This study shows that the multistep and multifocal concept of hepatic carcinogenesis elucidated largely in experimental animal models, can be used to recognize the various stages of HCC development in humans and guide clinical manage-

men.^{1,6,7} It also shows that hepatic tumor biology is similar to the sequential evolution of colon¹⁸ and bladder cancer.¹⁹⁻²²

The majority of HCCs in this study appear to begin as small well-differentiated neoplasms without vascular invasion, which is similar to other studies.⁵ The distinction between dysplasia

TABLE 2. Correlation Between Any Level of Vascular Invasion and Various Gross and Histopathologic Tumor Parameters

	Negative for Vascular Invasion	Positive for Any Level of Vascular Invasion	P
Pattern			
Trabecular	92 (83.6%)	86 (84.3%)	.329
Acinar	12 (10.9%)	13 (12.7%)	
Fibrolamellar	6 (5.5%)	3 (3%)	
Giant cell			
(-)	81 (73.6%)	49 (48%)	<.01
(+)	29 (26.4%)	53 (52%)	
Clear cell change			
(-)	68 (61.8%)	51 (50%)	.124
(+)	42 (38.2%)	51 (50%)	
Tumor necrosis			
(-)	90 (81.8%)	44 (43.1%)	<.01
(+)	20 (18.2%)	58 (56.9%)	
Hepatitis activity			
(-)	12 (10.9%)	15 (14.7%)	.492
+	43 (39.1%)	36 (35.3%)	
++	55 (50%)	51 (50%)	
Tumor number			
1	78 (70.9%)	27 (26.5%)	<.01
2	19 (17.3%)	10 (9.8%)	
3	—	7 (6.8%)	
Multiple	13 (11.8%)	58 (56.9%)	
Total (n = 212)	110	102	

and carcinoma in such cases is currently based on the subjective opinion of the pathologist who relies on a combination of architectural and cytological alterations.^{3,5,23,24} The validity of this distinction is substantiated by significantly increased Ki-67 labeling within the tumor cells^{25,26} compared with the surrounding liver and the induction of tumor angiogenesis (diffuse sinusoid-like and/or complete vessels) recognized on smooth muscle actin staining. Factor VIII or CD34 staining can also be used to highlight endothelial cells of tumor angiogenesis.²⁷⁻²⁹ As in bladder carcinogenesis,²⁰ these small well-differentiated neoplasms show increased cellular proliferation, but "social" interactions, differentiation, and the ratio of cell proliferation to apoptosis remains near normal such that complete removal brings about a cure.

An overriding conclusion of this and other studies in experimental animals and humans is that HCCs progress.^{5,6,30-32} As the tumors enlarge, foci within the HCC become less well differentiated: only 18% of all tumors remained exclusively

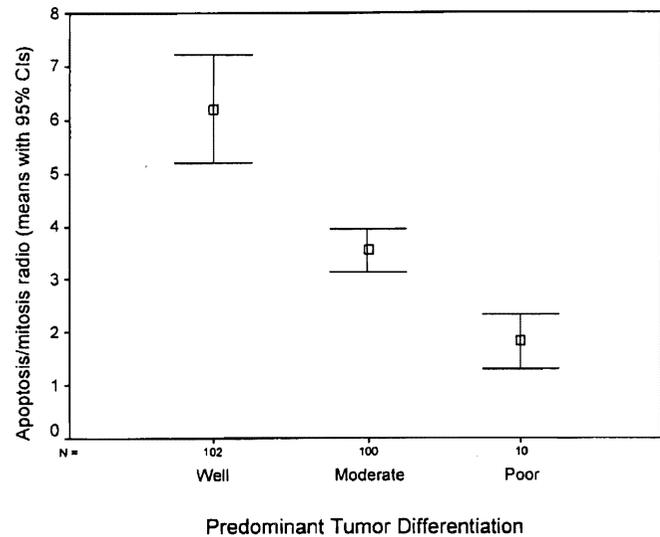


FIG. 4. Correlation between predominant grade of tumor differentiation and apoptosis/mitosis ratio.

well differentiated, and all but 3 of these were smaller than 3.0 cm. As in other studies,^{5,23,24,32,33} the development of "tumor(s) within a tumor" was recognized histologically by both a lesser degree of differentiation or growth pattern, pleomorphism, and higher Ki-67 labeling index. In such cases, less differentiated foci are always surrounded by better differentiated neoplastic hepatocytes, similar to previous studies.^{5,23,24,33}

Eventually dedifferentiated foci acquire an ability to invade the vasculature and metastasize, as evidenced by the correlation between vascular invasion and recurrence after transplantation.^{5,16,34} Acquisition of these aggressive characteristics is likely attributable to further genetic instability and abnormalities, as evidenced by the appearance of tumor giant cells and poor differentiation, which lead to profound alterations in growth (apoptosis/mitosis ratio), as in experimental animal studies^{6,7,35} and in human breast cancer.³⁶ In fact, the increased proliferation observed in the small, well-differentiated lesion may promote genetic instability and tumor progression.⁷ Thus, routine histopathologic findings such as vascular invasion, tumor giant cells, and the apoptosis/mitosis ratio are likely to be surrogate markers of genetic instability and abnormalities, marking clinically important events in tumor progression.

TABLE 3. Correlation Between the Level of Vascular Invasion and the Predominant and Worst Areas of Tumor Differentiation

	Negative for Vascular Invasion	Positive for Microscopic or Angiolymphatic Invasion	Positive for Microscopic and Macroscopic Vascular Invasion	Total
Predominant differentiation				
Well	72 (70.6%)	18 (17.6%)	12 (11.8%)	102 (48.1%)
Moderate	37 (37%)	42 (42%)	21 (21%)	100 (47.2%)
Poor	0 (0%)	3 (30%)	7 (70%)	10 (4.7%)
Worst differentiation				
Well	35 (89.7%)	3 (7.7%)	1 (2.6%)	39 (18.4%)
Moderate	60 (55%)	31 (28.4%)	18 (16.5%)	109 (51.4%)
Poor	14 (21.9%)	18 (16.5%)	21 (32.8%)	64 (30.2%)
Total	109 (51.4%)	63 (29.7%)	40 (18.9%)	212 (100%)

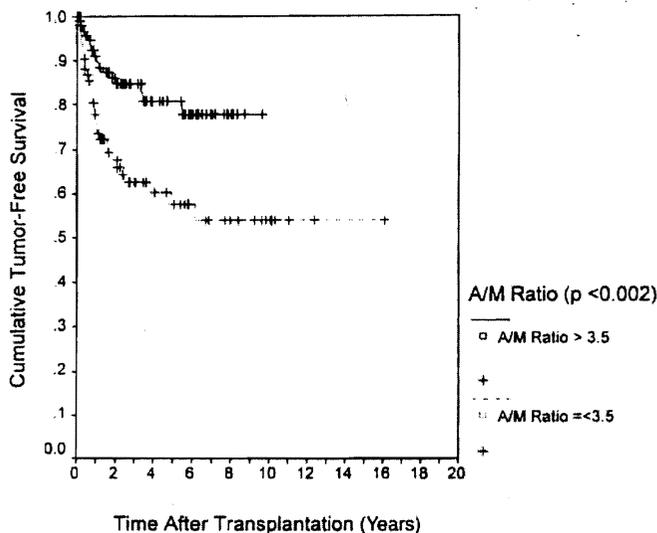


FIG. 5. Correlation between apoptosis/mitosis ratio and the cumulative tumor-free survival after transplantation.

Appreciation of tumor heterogeneity is also a consistent conclusion in molecular and genetic analyses of HCC,^{23,30,37,38} which show that both genetic and epigenetic alterations contribute to tumor phenotype.^{30,31,37-40} Genes with reported somatic mutations include the tumor suppressor genes such as p53, mannose-6-phosphate/insulin-like factor 2 receptor, β -catenin, p16, and retinoblastoma, which are either directly or indirectly involved with cell cycle progression or invasive growth. Germ line mutations of p16, adenomatous polyposis coli, and breast cancer gene 2 have also been reported,³⁸ as have oncogenic activation of several cellular genes such as cyclin D and cyclin A.³⁸

Regulation of mitosis and apoptosis through the cell cycle-related proteins p16, p21, p27, and cyclin D is an intriguing aspect of liver carcinogenesis.^{4,32,38,41-43} For example, inactivation of p16 by posttranscriptional regulation appears to participate in both the early developmental stage and in tumor progression,³⁸ whereas reduced p21 expression caused by p53 mutations seems to occur at a later stage. Consistent with our observation of an altered apoptosis/mitosis ratio and aggressive biologic behavior, poorly differentiated tumors and the inner nodules of heterogeneous tumors more often show p53 mutations,^{32,38} and Rb dysfunction is associated with malignant progression and metastasis of HCC.³⁰ Given the results of this study, it will be important in future studies to carefully correlate genetic alterations with tumor morphology and growth characteristics using microdissection.

Although most HCCs begin as well-differentiated neoplasms that slowly enlarge and evolve, as discussed above, a small subset (roughly <20%) quickly acquire aggressive characteristics similar to flat carcinomas of the bladder^{21,22} and colon.¹⁸ Because of this biology, prognostication of tumor behavior based on size alone will be inaccurate for a minority of cases. It will be interesting to determine whether these early aggressive lesions follow molecular pathways distinct from more conventional tumors (or share some pathways but not others), similar to the spectrum of genetic alterations uncovered in flat and polypoid bladder²⁰ and colon¹⁸ cancers.

Angiogenesis is an obviously important aspect of hepatic carcinogenesis, which already is used for tumor treatment.

Unfortunately, this study failed to show a correlation between microvascular density and tumor aggressiveness, size, differentiation, mitosis, or any level of vascular invasion, which is consistent with some previous studies^{28,44,45} but in contrast with others.^{27,28} However, if the number of complete vessels within a tumor was more than 5, the apoptotic cell number decreased, suggesting that angiogenesis might contribute to tumor progression by altering the apoptosis/mitosis ratio. In our opinion, the lack of consensus between HCC angiogenesis and tumor biology is at least partially attributable to lack of standardized quantification methods.^{27,28,44,45} In addition, the development of large complete vessels in HCC angiogenesis is significantly different than other neoplasms^{46,47} and current methods do not consider the caliber of any complete vessels. More work is needed in this area.

Lastly, it is likely that the TNM system will require modification to better predict prognosis, as we previously suggested.¹⁴ Although some aspects of tumor progression are captured with the current system, the variable significance of multifocality and other aspects of tumor progression, such as microscopic vascular invasion and the apoptosis/mitosis ratio, are ignored. For example, this study showed that if 2 separate HCCs maintain a relatively high apoptosis/mitosis ratio, they probably arose as 2 separate T1 primaries and do not represent a multifocal aggressive neoplasm arising from intrahepatic metastasis.

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