Bull. Tokyo Med. Dent. Univ., 42: 67–81, 1995

HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSIS OF ADENOMATOUS HYPERPLASIA AND HEPATOCELLULAR CARCINOMA: Cellularity, Thickness of Cell Cord, and Ki-67 Proliferative Activity

BY

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ABSTRACT

To characterize adenomatous hyperplasia (AH) and hepatocellular carcinoma (HCC), and to establish their histopathological differences, morphometrical and immunohistochemical analyses, namely, cellularity, thickness of cell cord, and Ki-67 labeling index (Ki-67 LI) were done on surgically obtained hepatic lesions from patients with positive serum antibody against HCV. The hepatic lesions analyzed include chronic active hepatitis (CAH) (11 specimens), regenerative nodules of liver cirrhosis (LC) (29), AH (11), small HCC Edmondson's Grade I (GI) (19), GII (26), and GIII (14).

The results showed that AH has relatively high cellularity, and significantly greater thickness of cell cord than LC; whereas, HCC GI has significantly higher cellularity and Ki-67 LI than AH. From the data of these markers, and from the absence of conspicuous structural atypism, AH is considered to be in a different category from HCC GI. The premalignant potential of AH is supported only by its high incidence of coexistence adjacent to HCC GI or GII(6/11). Most lesions of HCC seem to develop from the liver tissue having a background of CAH or LC without passing through AH. Focal fatty changes are frequently observed within lesions of both AH and HCC GI (5/11, 8/19). When non-fatty regions of AH and HCC GI are compared, with respect to their markers, particularly Ki-67 LI, as well as the structural atypism, such as microacinus formation and pseudoglandular structure, and invasive growth into the surrounding liver parenchyma, HCC GI can be diagnosed as an early or well-differentiated malignant lesion.

Key words: Hepatocellular carcinoma, Adenomatous hyperplasia, Ki-67

INTRODUCTION

Recently developed non-invasive diagnostic methods such as ultrasonography and computed tomography have enabled us to detect liver lesions that are small in mass. Consequently, we have detected borderline type of lesions, so-called adenomatous hyperplasia (AH), in addition to small hepatocullular carcinoma (HCC), which have given us considerable information on the development of HCC (Takayama et al. [1]; Sakamoto et al. [2]; Eguchi et al. [3] Tarao et al. [4]). The criteria for making a histopathological diagnosis of AH and well-differentiated HCC corresponding to HCC GI, however, remain controversial (Kondo et al. [5]; Ohno et al. [6]; Theise et al. [7]; Ferrell et al. [8]). In particular, it is very difficult but important to be able to diagnose them from needle biopsy specimens.

The development of HCC has been considered to be intimately related to

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infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) (Tribelli et al. [9]; Tsukuma et al. [10]; Kato et al. [11]). However, most of the studies on characterization of early lesions of HCC have been performed by observing materials mixed with HBV and/or HCV infection as a background. It may be thought that the respective hepatitis virus infections contribute in different ways to the repeated liver cell injury that is followed by the development of HCC (Tsukuma et al. [10]; Kato et al. [11]; Uchida and Shikata [12]). Accordingly, it is important that characterization or observation of HCC development should be done under identical backgrounds of viral infection.

We collected surgically resected small hepatic lesions, including chronic active hepatitis (CAH), regenerative nodule of liver cirrhosis (LC), AH, and HCC, from patients with positive serum antibodies against HCV. Normal liver tissues were obtained as controls from the patients operated on for gallstones with no serum antibodies against HCV or HBV. To characterize and understand any differences among LC, AH, and well-differentiated HCC, the respective lesions in this study were analyzed morphometrically and immunohistochemically and the results summarized to establish a set of histopathological diagnostic criteria.

Materials and Methods

Specimens

Surgically resected liver tissues including HCC, AH, LC, and CAH, were selected from forty-five patients and the specimens selected were reviewed. The liver specimens were collected from Tokyo Medical and Dental University Hospital, Tokyo; Kudanzaka Hospital, Tokyo; Sanraku Hospital, Tokyo; International Medical Center of Japan, Tokyo, and Nanpuh Hospital, Kagoshima. The ages of the patients with tumors ranged from 37 to 77 years. The ages of the 8 patients with normal liver tissues, were from 56 to 74 years. The surgical specimens of hepatic lesions were classified into 6 types of lesions, according to The General Rules for the Clinical and Pathological Study of Primary Liver Cancer (Liver Cancer Study Group of Japan [13]). Because the entities of atypical adenomatous hyperplasia (AAH) and large regenerative nodule were not clearly described in this classification, these two subtypes were not used in the present study. Specimens of normal liver tissue without remarkable changes were selected from 8 patients who had been operated on for gallstones. Specimens taken from the forty-five patients with tumors included 11 CAH, 29 LC, 11 AH, 19 HCC GI (Edmondson's Classification) (Edmondson and Steiner [14]), corresponding to well-differentiated HCC, 26 HCC GII, corresponding to moderately differentiated HCC, and 14 HCC GIII, corresponding to poorly differentiated HCC. Some of the patients had more than one hepatic nodule. In some cases, different types of lesions were seen coexisting in a single nodule, e.g., HCC GI and AH in the same nodule or HCC GI and GII in the same nodule. Five lesions of AH, 10 lesions of HCC GI, 16 lesions of HCC GII, 13 lesions of HCC GIII existed soley in each nodule. The coexistence of AH and HCC GI, AH and HCC GII, HCC GI and HCC GII, and HCC GII and HCC GIII was found in 3, 3, 6, and one nodule, respectively. Except for the patients with normal liver tissues (the control group), all the patients were positive for serum antibody against HCV (C-100 antibody). Patients with positive results for serum antibody against HBsAg or HBc were omitted from this study. Tumors measuring 3 cm or less (0.6 to 3.0 cm) in the greatest diameter were selected for this study.

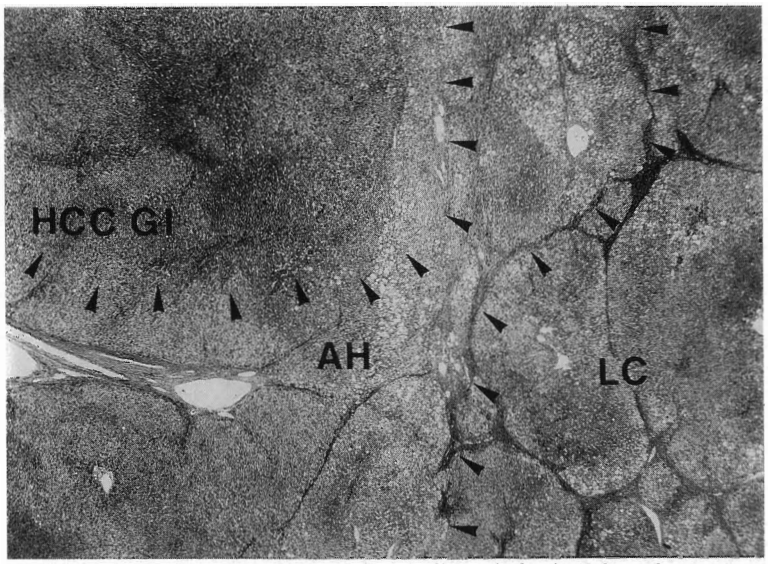

Fig. 1. A representative nodular lesion in liver cirrhosis (LC). Adenomatous hyperplasia (AH) coexists adjacent to hepatocellular carcinoma (HCC GI) in LC. The boundary between each lesion is indicated by arrowheads (H & E staining, X 20).

Representative lesions of LC, AH, and HCC GI are shown in Figs. 1 through 4.

Histological Analysis

Histological observations were performed on 4 μm-thick sections of 10% formalin-fixed and paraffin-embedded tissues. Histopathological diagnosis was principally obtained by observing the histologic sections stained with hematoxylin and eosin (H & E). Histological analysis was done in several foci of each lesion. When one hepatic nodule included different types of lesions, analysis was done separately for each type of lesion.

Cellularity (/0.0625 mm^2) was determined by counting the nuclei of hepatocytes (epithelial cells) in 10 to 20 foci of 0.0625 mm^2 in respective lesions under the light microscope. Non-parenchymal cells were not counted. For both cellularity and Ki-67 labeling index (Ki-67 LI), even if a hepatocyte contained more than one nucleus, it was counted as one to represent the existence of a single cell. Thickness of the cell cord (in μm) of the respective lesions was determined by measuring 10 epithelial cell cords on 8 μm-thick sections stained with silver impregnation.

Immunohistochemistry

To detect the presence of Ki-67 positive cells in the specimens examined, they were pretreated by autoclave-processing in 0.01M citrate buffer (pH 6.0) at 121°C for 10 min. The conventional Streptavidin Biotin (SAB) method was used to detect Ki-67 positive cells in 4 μm-thick sections of formalin-fixed, paraffin-embedded tissues. Briefly, after the paraffin was removed and endogenous peroxidase blocked with 0.3% hydrogen peroxide (H_2O_2) in methanol for 30 min at room temperature, the sections were incubated for 60 min at room temperature with monoclonal mouse antibody against human Ki-67, MIB-1 (x100 dilution, IMMUNOTECH, Marseille Cedex, France). After washing with phosphate-buffered saline (PBS), pH 7.4, the SAB method with peroxidase

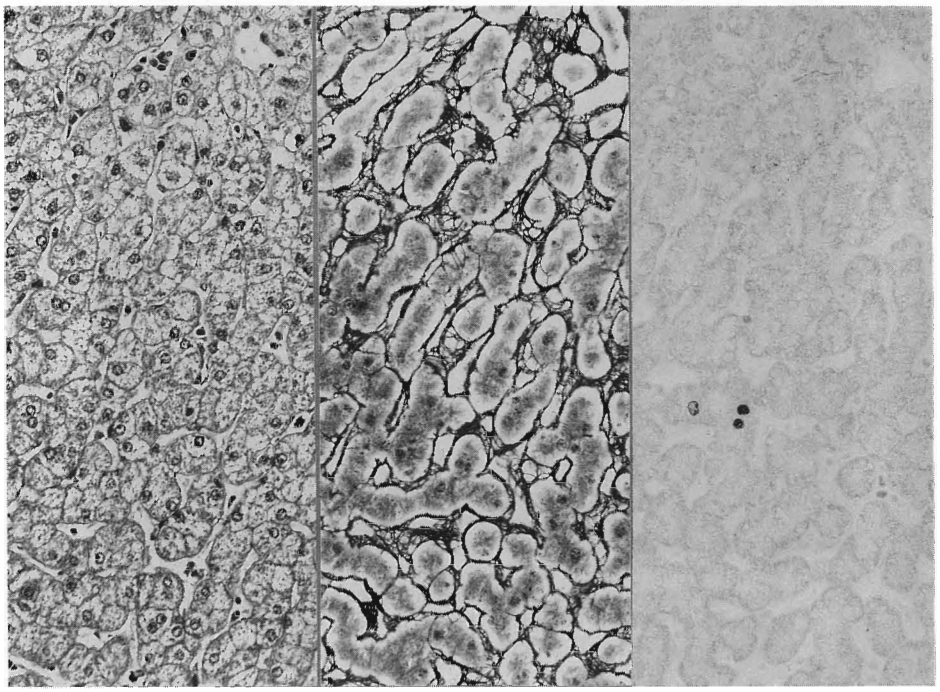

Fig. 2. A representative focus of a regenerative nodule in liver cirrhosis (LC). The left, middle and right portions are stained with H & E stain, silver staining, and anti-Ki-67 immunostaining, respectively (X 200).

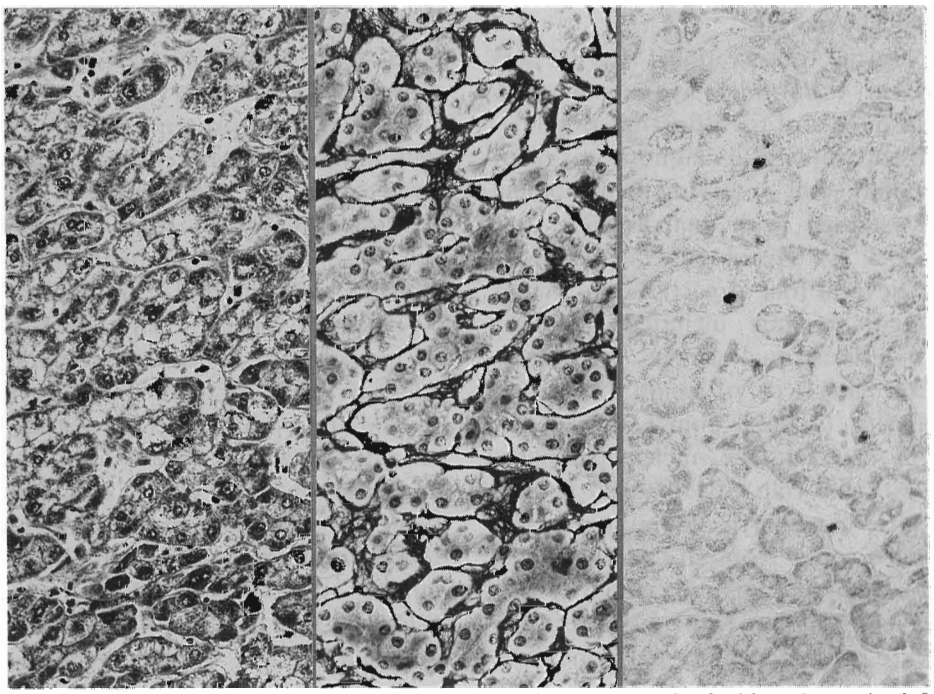

Fig. 3. A representative focus of adenomatous hyperplasia (AH), stained with H & E stain (left), silver staining (middle), and anti-Ki-67 immunostaining (right) (X 200).

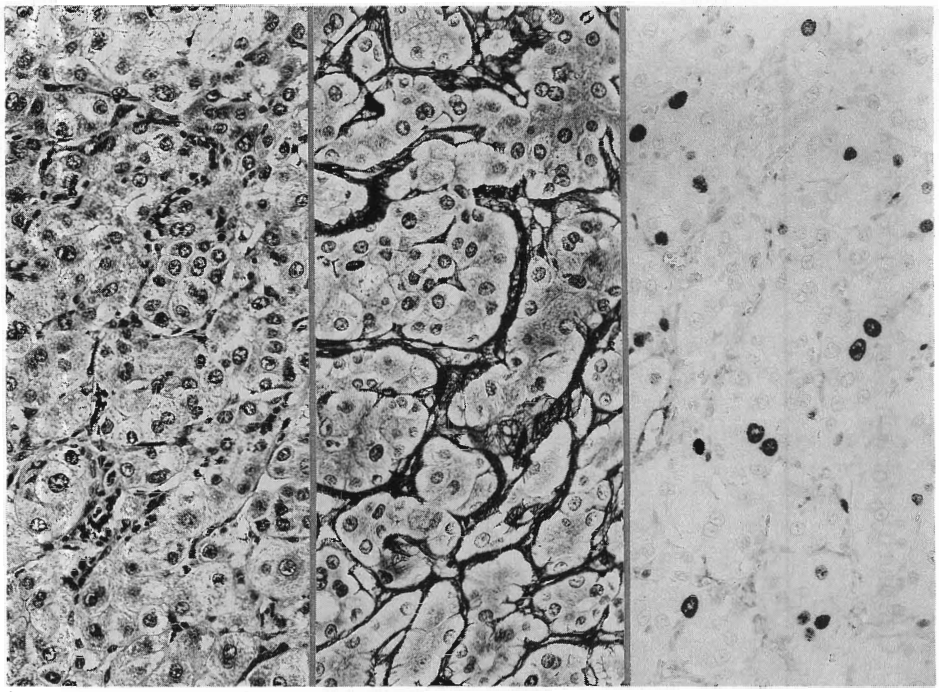

Fig. 4. A representative focus of hepatocellular carcinoma GI (HCC GI), stained with H & E stain (left), silver staining (middle), and anti-Ki-67 immunostaining (right) (X 200).

(DAKO LSAB kit, DAKO, Copenhagen, Denmark) was used. Pale counterstaining for nuclei was achieved with Meyer's hematoxylin solution. The Ki-67 labeling index (Ki-67 LI) is expressed in terms of the number of Ki-67 antigen positive hepatocytes out of 1,000 hepatocytes. In general, more than 1,000 hepatocytes were randomly counted in each lesion. However, in a few lesions where the distribution of positive cells was markedly uneven, more than 3,000 hepatocytes were counted.

Identification of Focal Fatty Change in Epithelial Cells

Fatty changes were identified in the lesions in which more than 50% of the epithelial cells in one focal area were occupied by fat droplets.

Identification of Capsule Formation or Invasive Growth of the Tumor Mass

When partial or total absence of a capsule surrounding the tumor mass was found histologically, capsule formation of the lesion was determined as negative. Invasive growth was determined by finding the irregular infiltration or replacement of the surrounding non-tumorous liver tissue by the tumor cells.

Statistical Analysis

All data are expressed as mean±standard deviation (SD). Statistical significance of any difference between two groups was determined by the Student's t-test. The data were statistically analyzed for incidence, by the Chi-square test.

Results

Morphometrical Analysis

Cellularity. The cellularity was significantly lower in LC than in normal liver tissue ($p<0.05$) and relatively lower in CAH and AH than in normal liver tissue (Fig. 5). But it was relatively higher in

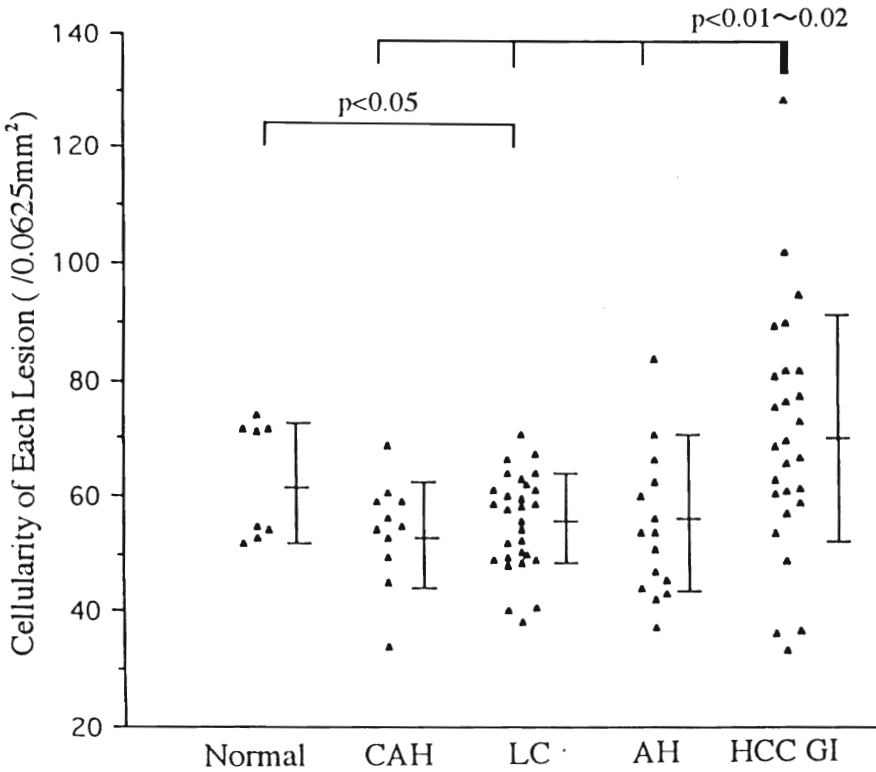

Fig. 5. Cellularity of various liver lesions. The cellularity (/0.0625 mm^2) was determined by counting epithelial nuclei under the light microscope.

HCC GI than in normal liver tissues. Further, it was significantly higher ($p< 0.01–0.02$) in HCC GI than in CAH, LC, and AH. HCC GII and GIII showed a higher cellularity than HCC GI (Table 1).

Thickness of liver cell cord. The thickness of the liver cell cord showed a gradual increase from normal liver tissues to HCC GIII (Fig. 6, Table 1). Particularly, it was significantly thicker in CAH and LC than in normal liver tissue. AH showed a significant increase of thickness of cell cord compared with that of LC. Further, it was relatively thicker in HCC GI than in AH.

Ki-67 LI. Ki-67 LI was significantly higher in CAH, LC, AH, and HCC GI than in normal liver tissue (Fig. 7). Further, significant increase of Ki-67 LI was revealed in HCC GI compared with that in AH. Also, a significant increase of Ki-67 LI was clearly shown in accordance with the severity of the grade of HCC (Table 1).

Comparison between AH and HCC

To characterize respective lesions, histopathological findings concerning capsule formation, growth pattern (Fig. 8), microacinus formation (Fig. 9, left), pseudoglandular structure (Fig. 9, right), focal fatty change (Fig. 10), and residual portal triads (Fig. 11) within the tumor mass are summarized in Table 2. Invasive growth and absence of capsule formation were often seen in HCC GI (6/19, 12/19) in contrast to no invasive growth in AH (0/11). As the grade of HCC increased, the invasive growth became more apparent. Microacinus formation, a marker of structural atypism, was found in 18 out of

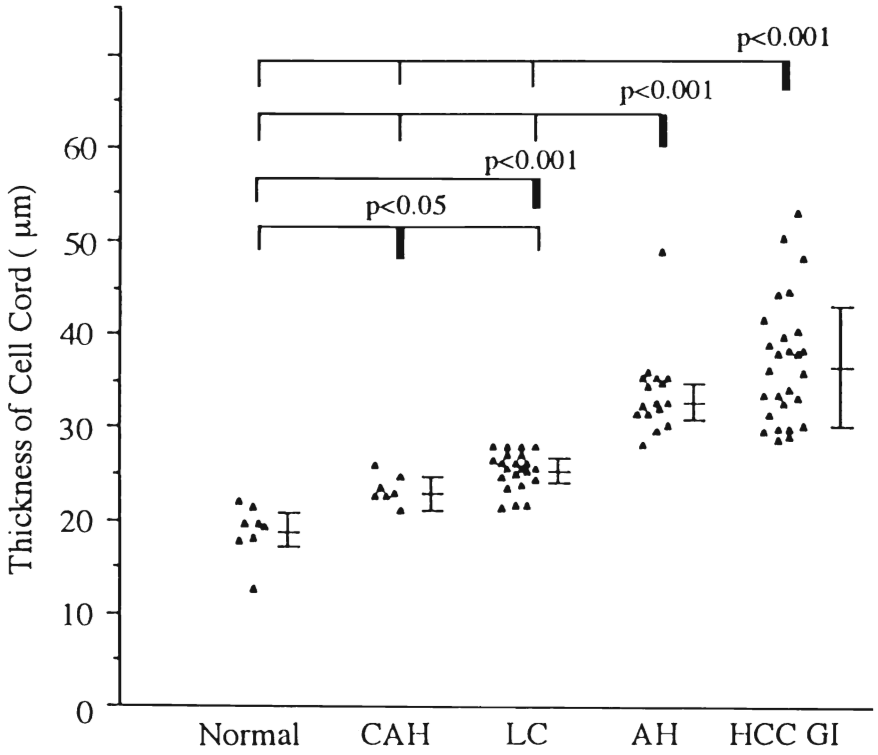

Fig. 6. Thickness of cell cord in various liver lesions.

Table 1. Quantitative Analysis of Cellularity, Thickness of Cell Cord and Ki-67 Labeling Index of Respective Hepatic Lesions

Type of lesions	Cellularity (/0.0625 mm^2)	Thickness (μm)	Ki-67 LI (positive/1000)
Normal (n=8)	62.7±10.1	7.5±1.1	3.4±3.8
CAH (n=11)	54.0±9.0	9.3±0.7	34.3±26.1
LC (n=29)	55.5±8.2	10.2±0.8	32.8±29.2
Untreated (n=7)	57.9±6.3	10.2±0.8	20.7±19.5
Treated (n=8)	56.1±5.1	10.1±0.6	15.7±10.4
AH (n=11)	56.5±14.8	13.5±1.2	36.1±18.6
Fatty change (n=5)	43.3±3.6	15.1±2.6	46.7±28.6
Non-fatty (n=11)	62.3±13.6*	13.0±0.9*	31.1±20.4**
HCC GI (n=19)	73.0±18.7	14.6±2.3	82.4±61.6
Fatty change (n=8)	52.0±18.1	16.0±2.4	51.5±23.7
Non-fatty (n=15)	76.8±18.4	14.7±2.7	89.0±65.4
HCC GII (n=26)	85.3±20.5	19.4±7.0	123.6±105.2
HCC GIII (n=14)	94.7±25.6	38.6±14.0	256.3±125.2

Each data represents mean value±standard deviation. Ki-67 LI: Ki-67 labeling index. CAH: chronic active hepatitis. LC: regenerative nodule in liver cirrhosis. Treated: lipiodol and/or transarterial embolization. AH: adenomatous hyperplasia. HCC: hepatocellular carcinoma. I, II, III: Edmondson's classification. *: Significantly different ($p<0.05$) from non-fatty parts of HCC GI. **: Significantly different ($p<0.01$) from non-fatty parts of HCC GI.

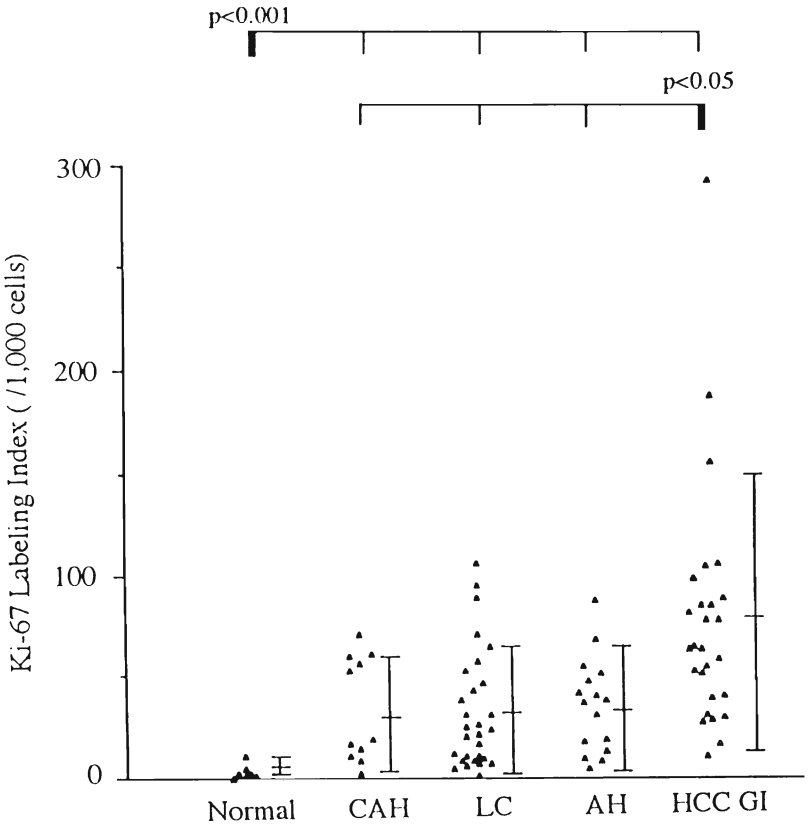

Fig. 7. Ki-67 labeling indices (LI) in various liver lesions.

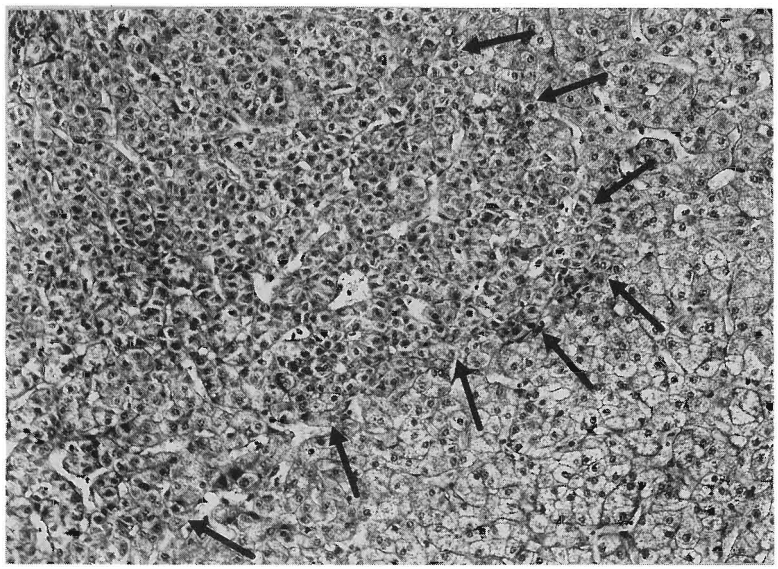

Fig. 8. Invasive growth of HCC. Invasive growth was determined by finding the irregular infiltration or replacement of the surrounding non-tumorous liver tissue by the tumor cells. The boundary between HCC and the surrounding non-tumorous liver tissue is indicated by arrows (H & E staining, X 100).

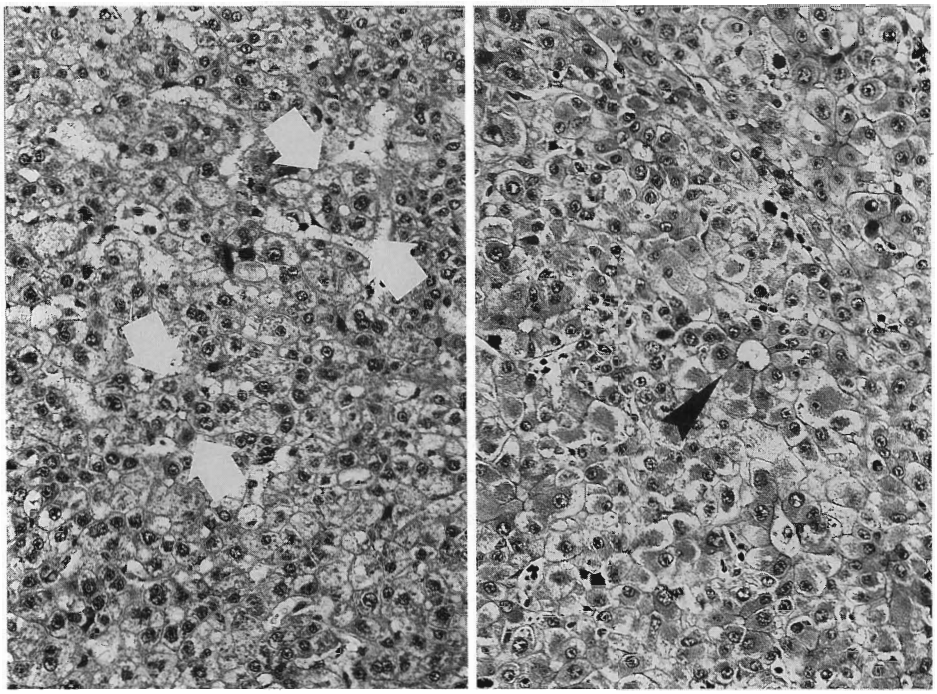

Fig. 9. Microacinus formation in HCC GI as a marker of structural atypism. Structural atypism was determined by microacinus formation (arrows) (left) and pseudoglandular structure (arrowhead) (right) (H & E staining, X 200).

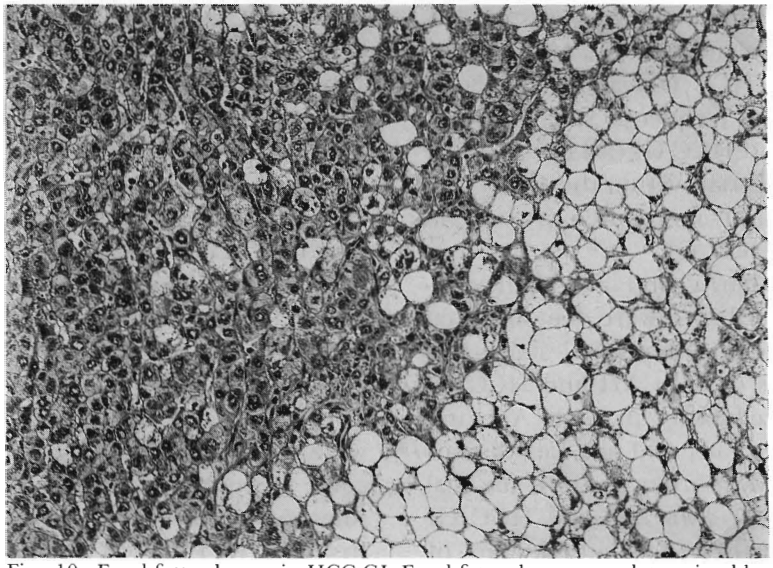

Fig. 10. Focal fatty change in HCC GI. Focal fatty change was determined by the presence of lipid droplets in more than 50% of epithelial cells. In this figure, focal fatty change mainly occupies the lower right corner, extending to the upper right corner (H & E staining, X 100).

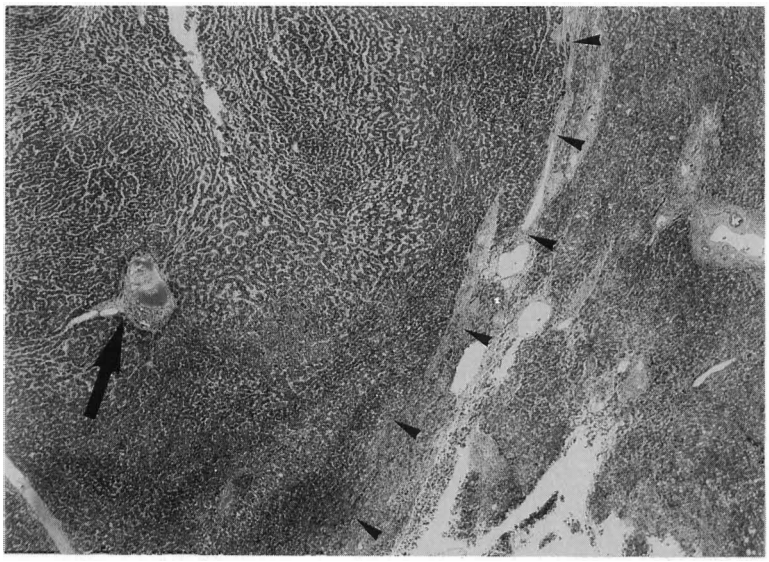

Fig. 11. A residual portal triad (arrow) within HCC GI. Original portal triads are left within HCC lesion. Non-tumorous liver tissue is seen on the right side of HCC GI. The boundary between HCC and non-tumorous liver tissue is indicated by arrowheads (H & E staining, X 20).

19 HCC GI lesions, whereas it was not observed in any AH lesions (0/11). Invasive growth was found in one HCC GI lesion where microacinus formation was not detected. Pseudoglandular structure also was often seen in HCC GII and GIII. Focal fatty change was frequently seen in lesions of both AH and HCC GI (5/11, 8/19). The residual portal triad within the tumor was found significantly more often ($p<0.05$) in AH than in HCC GI.

Higher cellularity and a tendency toward thinner cell cord were demonstrated more frequently in non-fatty lesions than in fatty lesions of both AH and HCC GI ($P<0.05$ on cellularity in both AH and HCC GI), when fatty and non-fatty lesions were compared in order to know the real characteristics of both AH and HCC GI (Table 1). Further, non-fatty lesions of HCC GI showed a relatively higher Ki-67 LI than their respective fatty lesions within HCC GI. All of the markers, including cellularity, thickness of cell cord, and Ki-67 LI, were significantly higher ($p<0.01$–0.05) in HCC GI than in AH, when non-fatty lesions were compared. Six out of 11 AH lesions were located in direct connection with HCC GI or GII lesions. No significant differences in cellularity, thickness of cell cord, and Ki-67 LI were observed between solitary AH (lesions of AH alone) and AH adjacent to HCC (Data are not shown). The ratios of cellularity, thickness of cell cord, and Ki-67 LI of AH and HCC GI compared with those of their respective non-tumorous liver tissues, including CAH and LC, are shown for each individual case (Fig. 12). In this analysis, non-fatty parts of both AH and HCC GI were selected. The ratio of thickness of cell cord of tumor cells to that of non-tumorous cells was significantly greater ($p<0.05$) in HCC GI than in AH.

Difference of Ki-67 LI between Sections of Liver Cirrhosis with and without Anticancer Treatment of HCC

Treatment for HCC includes trans-

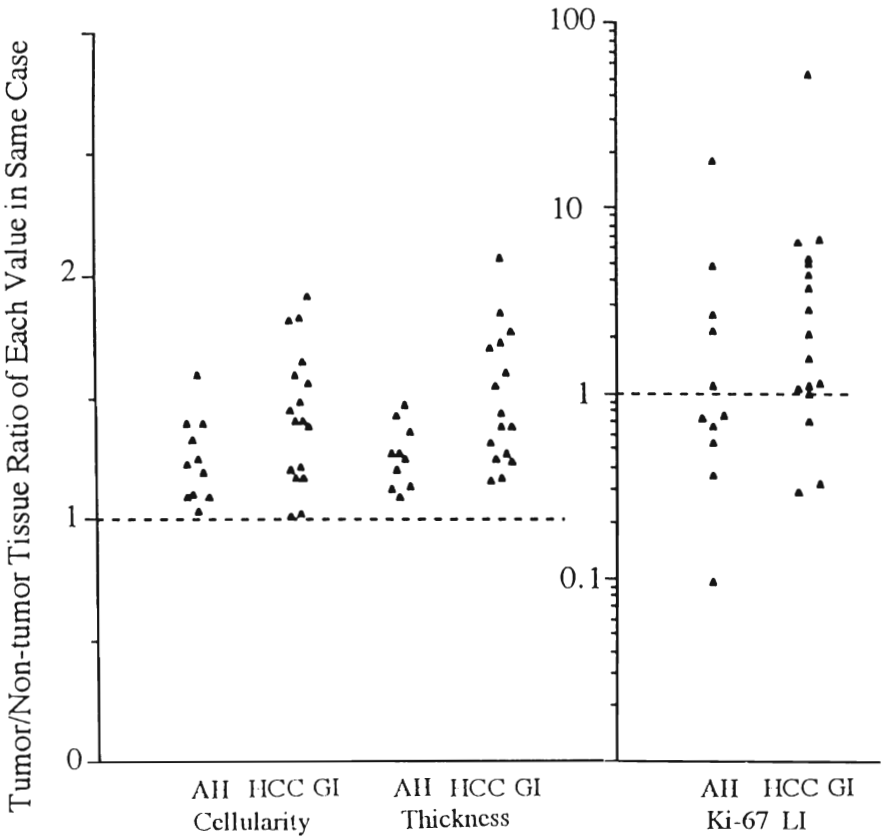

Fig. 12. Comparison of ratios of markers of each individual AH and HCC GI to those of their respective non-tumorous liver tissues. Non-fatty parts of both AH and HCC GI lesions were selected for this comparison. Only the thickness of cell cord was significantly different ($p<0.02$) between AH and HCC (1.26±0.13, 1.50±0.27, respectively).

Table 2. Summary of the Histopathological Findings in Hepatic Nodules

Type of lesions (number)	Capsule formation*	Growth pattern	Micro-acinus	Pseudo-gland	Fatty change	Portal triad
AH (n=11)	2	E:11 I: 0	0	0	5	11
HCC GI (n=19)	7	E: 13 I: 6	18	6	8	11
HCC GII (n=26)	15	E: 15 I: 11	18	18	5	4
HCC GII (n=14)	9	E: 1 I: 13	7	13	1	2

HCC: hepatocellular carcinoma. I, II, III: Edmondson's classification
*: completely encapsulated case. E: expansive growth, I: invasive growth.

arterial embolization (TAE) and/or selective injection of Lipiodol combined with anticancer drugs (LIP). To find out whether such treatments given for HCC have any influence on the surrounding liver tissue, Ki-67 LI was compared between two groups—LC of treated and untreated patients. No significant difference was found between the two groups (Table 1). Furthermore, there was no

significant difference in the Ki-67 LI between the patients treated with LIP and TAE (Data are not shown).

Discussion

To characterize and establish histopathological features of AH and well-differentiated HCC, we carried out morphometrical and immunohistochemical analyses on various liver lesions, including normal liver tissue, CAH, LC, AH, HCC GI, GII, and GIII. Recently, the assay system for serum antibody against HCV has been introduced. Accordingly, all hepatic lesions, except for the control group consisting of normal liver tissues, were collected in a background of positive serum antibody against HCV, to exclude any influence by the HBV infection (Tsukuma et al. [10]; Kato et al. [11]).

Of the available morphometric markers, cellularity was selected to represent the density of the liver cells. Thickness of cell cord, microacinus formation, and pseudoglandular structure were chosen for determination of structural atypism. It is supposed that the thickness of cell cord reflects not only the size of liver cells but also their cellular arrangement. Microacinus formation or pseudoglandular structure can be identified as a marker of dedifferentiation of liver cells. Further, focal fatty changes within the tumor mass were observed as an indicator of the abnormal metabolism or metabolic differentiation of the liver cells. Cellular atypism, such as the nucleo-cytoplasmic ratio was not checked in this study, because it is not considered as a reliable means to distinguish very well-differentiated HCC (HCC GI) from LC (Motohashi et al. [15]). Ki-67 LI was calculated in respective lesions as an indirect marker of cellular proliferative activity in this work. The Ki-67 antigen is closely related to the cell cycle, and is usually expressed in late G1, S, G2, and M phases (Mcgurrin et al. [16]; Shepherd et al. [17]; Weidner et al. [18]). Further, immunohistochemical staining for the Ki-67 antigen is much more stable than that for the proliferating cell nuclear antigen (PCNA), which has been often used for indicating cellular proliferative activity in hepatic lesions (Terada and Nakanuma [19]; Kitamoto et al. [20]; Adachi et al. [21]; Ojanguren et al. [22]). Ki-67 LI in cancer tissue has been shown to have a relative correlation with the grade of malignancy or the prognosis of cancer patients (Sphepherd et al. [17]).

As a result, regarding the thickness of cell cord, AH is located between non-tumorous lesions (CAH and LC) and HCC GI, which is considered to be a malignant lesion. However, when the cellularity and Ki-67 LI of AH and LC were compared, the differences were not significant. These findings with respect to cell proliferative activity support the conclusion that it is reasonable to call AH a macroregenerative nodule as it has been diagnosed in the U.S.A. (Theise et al. [7]; Ferrell et al. [8]). When microacinus formation and/or pseudoglandular structure are selected as markers of definite structural atypism, we can gain a clear distinction between AH and HCC GI by observing whether or not these structures can be seen within the lesions. Additionally, the irregular invasive growth of tumor cells into the adjacent liver parenchyma is also a helpful finding for the identification of malignancy. Further, focal fatty change as an indication of abnormal metabolism or metabolic differentiation was frequently found within the lesions of both AH and HCC GI. All of the markers, including cellularity, thickness of cell cord, and Ki-67 LI, showed a significantly clear difference ($p<0.01$–0.05) between the non-fatty lesions of AH and those of HCC GI (Table 1). Consequently, AH can be diagnosed separately

from HCC GI by observing non-fatty lesions with respect to several markers, including cellularity, thickness of cell cord, and Ki-67 LI of tumor cells, as well as the absence of conspicuous structural atypism, including microacinus formation and pseudoglandular structure, even though AH and HCC GI are similar with regard to the frequent coexistence of focal fatty change. The non-fatty parts of AH showed a relatively high cellularity in comparison with those of LC. However, there were relatively high values of Ki-67 LI in the fatty lesions of AH, compared to the values in non-fatty lesions. This might be because the area of fatty change was relatively small compared with that of HCC GI, and also because of the relatively small number of AH with such lesions that we could observe in our limited materials. Six out of 11 AH lesions coexisted adjacently to HCC GI or GII. These findings suggest that HCC GI developed within AH, after further transformation. Accordingly, this result can support the proposed multi-step carcinogenesis of HCC in which AH being suggested as a premalignant lesion ([1–3, 19], Terada et al. [23]). Inversely, a different mode of carcinogenesis without passing through AH, can also be introduced for HCC, because the remaining five AH and 10 HCC GI lesions were detected solitarily in this study (Grigioni et al. [24]).

In HCC lesions, the values of all three markers of cellularity, thickness of cell cord, and Ki-67 LI correlated with the grade of HCC. Consequently, these selected markers can be considered suitable for this work. There was no difference in Ki-67 LI between the LC lesions of the treated and untreated HCC cases, although the HCC lesions of treated cases were more or less necrotic (Okayasu et al. [25]). The fact that whether HCC had been treated or not had no significant influence on the surrounding liver tissue indicates that there should be no problem in using cases of HCC both with and without anti-cancer treatment together in this work.

Accordingly, it can be said that it is not helpful to use only one marker as a reference for histological diagnosis. For this reason, the observation of several markers as used in this work are required for the differential diagnosis of AH and HCC GI, particularly on needle biopsy specimens, in which available histopathological findings are limited. In comparing the tumor to non-tumor tissue ratios of each value in the individual cases, HCC GI showed a significantly greater thickness of cell cord than AH did. This result suggests that it is worthwhile to compare the histologic findings between tumorous and non-tumorous lesions within a histologic section of a needle biopsy.

In conclusion, AH is considered to be in a different category from HCC GI, on the basis of histopathological findings, cellularity, thickness of cell cord, and Ki-67 LI. Particularly, in addition to the conspicuous structural atypism, represented by microacinus formation, we proposed that it is important and critical to observe non-fatty lesions, when making a differential diagnosis between AH and HCC GI with the above described markers. The premalignant potential of AH is supported only by its frequent coexistence with HCC GI or GII. However, this concept does not appear to be consistent with morphometrical, immunohistochemical and histological data obtained in the present study. In other words, most of the malignant hepatic lesions seem to develop from the liver tissue having a background of CAH or LC without passing through AH, as in the case of gastric cancer proposed by Nakamura (Nakamura [26]).

Acknowledgments

The authors express their appreciation to Prof. Kyoichi Nakamura, 1st Department of Pathology, Tokyo Medical and Dental University, for his valuable criticism and suggestions, and to Dr. Nishimata, Department of Pathology, Nanpuh Hospital, Kagoshima and Dr. K. Saito, Department of Pathology, International Medical Center of Japan, Tokyo, for providing the histologic materials and also for making the arrangements, that enabled us to perform this study.

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