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Hepatocellular Carcinoma : Correlation of Histological Grading with Immunohistochemical Identification of Proliferative Hepatocytes with PC 10

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Abstract : Sections from 31 liver needle biopsies of hepatocellular carcinomas (HCCs) were examined for the histological grading of tumors with the expression of a labelling index, using monoclonal antibodies to proliferating cell nuclear antigen PC-10. The correlation between histological grading and the labelling index showed a statistically significant increase in the labelling index in grades III to IV of HCCs. A cut-off point of the labelling index at 49 per cent was proposed to indicate the prognosis of the patient. HCCs with a labelling index below 49 per cent has a better prognosis than HCCs with a labelling index above 49 per cent. A combination of histological grading and the labelling index indicated the prognosis of the patients with HCCs.

เรื่องย่อ : การศึกษาความสัมพันธ์ระหว่างอัตราการเติบโตของเซลล์มะเร็งในมะเร็งเซลล์ตับกับการแบ่ง
เกรดของเซลล์มะเร็งในมะเร็งเซลล์ตับโดยวิธีทางอิมมูโน
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ชิ้นเนื้อของมะเร็งตับจากผู้ป่วยจำนวน 31 รายได้รับการศึกษา โดยการแบ่งชนิดของเซลล์มะเร็งเป็นเกรดตามลักษณะจุลกายวิภาค และการตรวจหาอัตราการเจริญเติบโตของเซลล์มะเร็งในมะเร็งเซลล์ตับโดยใช้โมโนโคลนัลแอนติบอดีชนิดแอนติบอดี PCNA (PC10) ซึ่งค่าดัชนีการเจริญเติบโตของเซลล์มะเร็ง (labelling index) ที่ได้นำมาเปรียบเทียบกับความสัมพันธ์กับชนิดของเซลล์มะเร็งที่แบ่งเกรดตามลักษณะจุลกายวิภาค พบว่ามะเร็งเซลล์ตับที่มีเกรดสูงตามลักษณะจุลกายวิภาค จะให้ค่าดัชนีการเติบโตของเซลล์มะเร็งสูงกว่ามะเร็งเซลล์ตับที่มีเกรดต่ำตามลักษณะจุลกายวิภาคอย่างมีนัยสำคัญทางสถิติ นอกจากนี้ได้เสนอค่าดัชนีการเติบโตของเซลล์มะเร็งตับที่ระดับ 49% เป็นระดับที่ใช้แบ่งมะเร็งเซลล์ตับเพื่อบ่งชี้การพยากรณ์โรค โดยมะเร็งเซลล์ตับที่มีค่าดัชนีการเติบโตของเซลล์มะเร็งต่ำกว่า 49% จะมีการพยากรณ์โรคที่ต่ำกว่ามะเร็งเซลล์ตับที่มีค่าดัชนีการเติบโตของเซลล์มะเร็งสูงกว่า 49%

INTRODUCTION

Cell kinetic information is now an important potential prognostic indicator in cancer treatment as it provides a method for evaluating response to radiotherapy and chemotherapy regimens.^{1,2} There are many methods for assessing cellular proliferation.³ Proliferating cell nuclear antigen (PCNA) is one proliferative marker that is distributed in the cell cycle from the G1 phase, peaking at the G1/S-phase and decreasing through the G2 phase. The application of immunohistochemical methods using monoclonal antibodies to PCNA is widely used to measure the percentage of the labelling index (LI%) in many tumour tissues.⁴

Hepatocellular carcinoma (HCC), with an incidence of 2 per cent of all malignant tumours in Thailand, is the third most common tumour in males⁵ and most patients die within four months after histopathological diagnosis.⁶ The most common precursor conditions leading to HCCs in Thai patients are liver cirrhosis and chronic hepatitis following infection with hepatitis B virus and, increasingly, hepatitis C virus. Prognosis depends on the size, invasion and metastasis, histological grading and the presence of cirrhosis.⁷ However, patients with clear cell HCC have a better prognosis^{8,9} and the smaller size of the tumour yield a better prognosis.^{10,11} Correlation of histological grading of HCCs with proliferative activity had been considered by many studies to indicate the prognosis.¹²⁻¹⁵ This study investigates immunohistochemical staining of PCNA in biopsy specimens of HCCs and correlates the histological grading with the labelling index.

MATERIALS AND METHODS

Patients

Liver needle biopsies of hepatocellular carcinoma were obtained from 31 patients. The mean age of the patients was 49.2 ± 11.2 years (ranging from 17 to 67 years), of whom 22 were males with a mean age of 49.2 ± 12.5 years (range: 17 to 67 years) and nine were females with a mean age of 49.9 ± 7.6 years (range: 39 to 64 years).

Tumours histopathology

The liver needle biopsy specimens were immediately fixed in modified Millonig's phosphate buffer formalin and were processed within 24 hours for conventional section. The 3-micron paraffin sections were cut and stained with hematoxylin-eosin, Masson's trichrome, Gomori's methenamine silver and Periodic Acid Schiff stains. Classification and histological grading of HCC were performed according to AFIP classification and Edmonson-Steiner's classification.⁷ The distribution of the tumours among the different histological grading was 16 cases of grade II and 15 cases of grade III and IV.

Immunohistochemistry

To detect a proliferative cell nuclear antigen (PCNA), a mouse monoclonal antibody to PCNA (PC 10:NOVA Castra Lab) was used at a dilution of 1:200. Bound monoclonal antibodies were located by the avidin-biotin peroxides complex technique with 3-3'-diaminobenzidine as chromogen (Vectastain ABC Kit, Vector Laboratories, Burlingame, California) for two to four minutes under microscopy, with a methyl green counterstain. In negative control staining, non-immune mouse im-

munoglobulin was used instead of the specific antibody. The result of the positive stains was expressed by the nuclei of the percentage of positive stained nuclei in 1,000 tumour cells, using a standard light microscope equipped with an index squared grid. In addition, positive cells in five representative high powerfields from 10 sampling areas were counted. The fraction of positive tumour cells was determined as the labelling index (LI %).

Statistical analysis

The percentages of the labelling index were reported as mean values $\pm$ standard deviation (SD). The variables were normal distribution. Correlation between the labelling index and histological grading was evaluated by the Mann-Whitney U test. A Cut-off point of the labelling index was suggested as the mean of the total observation, using the chi-square test to confirm the statistical significance. The test calculation was made with the Stat View software (Abacus Concepts, Inc., Berkeley, California.)



Figure 1. PC10 immunostaining of a histological grade II HCC, showing many positive nuclei with dark brown intranuclear granules (PC10 on methyl green counterstain x 400).

RESULTS

The staining reaction of the monoclonal antibody to PCNA was confined to the nuclei of the tumour cells (Figure 1). The mean percentages and the SD of the labelling index in the total observation for grade II HCCs and grade III to IV HCCs are shown in table 1. The correlation between the percentage of the labelling and histological grading showed a sta-

tistically significant difference ($p < 0.05$).

Using the mean percentage of the labelling index for the total observation as the cut-off point, that is, 49 per cent, two subgroups ($LI\% < 49$ and $LI\% > 49$) were correlated with the histological grading (Table 2).

The results also showed a statistically significant difference in the correlation between histological grading and the subgroups ($p < 0.05$).

Table 1. Labelling index LI% and histological grading of tumours in 31 HCCs.

	No. of tumours	LI% (mean $\pm$ SD)	Significance of difference, p
Total observation	31	49.0 $\pm$ 19.0	
Histological grade			
grade II	16	42.3 $\pm$ 18.7	p=0.048, p<0.05
grade III to IV	15	56.3 $\pm$ 17.0	
Mann Whitney U test			

Table 2. Subgroup of the labelling index (LI% <49 and LI% >49) and the histological grading of tumours in 31 HCCs.

	No. of tumours LI% <49	No. of tumours LI% >49	Significance of difference, p
Histological grade			
grade II	10	6	p=0.045, p<0.05
grade III to IV	4	11	

Chi-square test

Table 3. Sensitivity and specificity of different cut-off points found in the correlation of the labelling index and histological grading of tumours in 31 HCCs.

	No. Of grade II HCCs	No. Of grade III to IV HCCs	Sensitivity	Specificity
Cut-off points of LI %				
<30	5	1	31.3 %	93.3%
>30	11	14		
<40	8	2	50.0%	86.7%
>40	8	13		
<50	11	5	68.8%	66.7%
>50	5	10		
<60	13	9	80.3%	40.0%
>60	3	6		
<70	15	11	93.8%	26.7%
>70	1	4		

Chi-square test

DISCUSSION

HCC is generally known to have a poor prognosis depending on the size, invasion and metastasis, presence of cirrhosis and histological grading.⁷ The histopathological finding of HCCs evaluated in liver needle biopsy may show the presence of cirrhosis and histological grading, but rarely the invasion or metastasis or size of the tumour. However, the degree of cancer cell differentiation can be assessed according to Edmonson-Steiner's classification¹⁶ which provides an indication of the prognosis of the

disease at one level. Cell kinetic information, using immunohistochemical methods of assessing cell proliferation, has been widely used as an adjunct to histological tumour classification in understanding and indicating neoplastic prognosis.³ Using determination by this method enables hepatocellular carcinoma to be characterized more accurately than by the means of histological typing and grading only.

Using monoclonal antibody to PCNA, the receptors in the HCCs were assayed with histological grading. Our study yielded a good correlation between LI and histological grading with a statisti-

cally significant difference. The mean LI was higher in grade III to IV HCCs or high DNA synthesis HCCs than in grade II HCCs or low DNA synthesis HCCs (Table 1). Similar results were obtained by previous studies.^{12-15,17} Tarao et al also showed a strong correlation between LI expressed by BrdU and histological fraction in HCCs. Furthermore, the correlation between LI and clinical findings was also evaluated. Tarao studied 17 liver biopsy specimens of HCCs and concluded that DNA synthesis in HCCs was higher in males than in females. Chao also showed that while over-expression of Cyclin A (PCNA) had a strong correlation with increased alpha-fetoprotein it was not associated with the patient's age, tumour size, cirrhosis or positive detection of HBsAg.^{19,20} Takai concluded that moderately differentiated or poorly differentiated HCCs had high PCNA-LI with frequent portal and capsular invasion.¹⁷ Hino proposed another evaluation of PCNA-LI as a PCNA-LI ratio (PCNA-LI ratio=PCNA-LI of cancer/PCNA-LI of surrounding liver cancer tissue) and concluded from his study of 61 HCCs the statistically significant increasing ratios between well differentiated and

poorly differentiated HCCs.¹⁵ The 1-2 year survival rate in high DNA synthesis HCCs was poorer than in low DNA synthesis HCCs, but there was no significant difference in the recurrent rate of the tumour.^{14,19}

In our study, the mean percentage of LI of the total studied specimens was also used as the cut-off point of percentage of LI. This was used to divide the HCCs into subgroups such as LI%<49 and LI%>49. The correlation between LI%<49 and LI%>49 with histological grading also showed a statistically significant difference (Table 2). Sensitivity and specificity of different cut-off points of LI correlated with histological grading which showed that the range of LI%<50> yielded 68 per cent of sensitivity and 66.7 per cent of specificity. Thus, it appears reasonable to postulate that using the mean percentage of the labelling index of total observation as the cut-off point could provide a potentially important prognostic factor in dividing subgroups of HCCs. However, because of the small number of cases in our initial study, we intended to study more cases of HCCs.

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