

Pathological characteristics, PCNA labeling index and DNA index in prognostic evaluation of patients with moderately differentiated hepatocellular carcinoma

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Abstract

AIM: To study the relationship between prognosis and pathological characteristics, proliferating cell nuclear antigen labeling index (PCNA-LI) and DNA index (DI) in patients with moderately differentiated hepatocellular carcinoma (HCC).

METHODS: 51 cases of moderately differentiated HCC were analyzed with respect to the relation between their clinical follow-up data and pathological characteristics. Meanwhile, PCNA-LI of HCC cells was detected by immunohistochemistry assay and DI was measured by Feulgen staining and automatic image analysis technique.

RESULTS: Patients with a single tumor nodule, less than 5 cm in diameter, no tumor emboli, no daughter nodules and necrosis had relatively better prognosis; patients with euploidy HCC had better prognosis than those with aneuploidy; among the aneuploidy patients those with DI <1.5 had better prognosis than the cases with DI >1.5; The higher the PCNA-LI, the worse would be the prognosis. The increase in DI was correlated with the increase in PCNA-LI, and both of them were correlated with the pathological changes of the tumor.

CONCLUSION: A composite analysis of the pathological characteristics of tumor tissue, DI and PCNA-LI might be useful in predicting the prognosis of HCC patients.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the world. Since 1990s HCC has become

the second killer in various cancers in China^[1]. Metastasis and recurrence of the neoplasm after surgical removal is still high. The frequency of 5-year recurrence after radical resection is 61.5 % overall^[2]. So the knowledge of the indexes related to recurrence and survival is very helpful in predicting the prognosis of patients with HCC and to make decision on adjuvant therapies for those patients. It has been generally accepted that the degree of differentiation of tumor cells is one of the most important aspects affecting the prognosis, but even among patients of a similar differentiation of HCC, the survival period is quite different. To explore this difference, we analyzed 51 cases of moderately differentiated HCC with respect to their clinical follow-up data, the histopathological characteristics, proliferating cell nuclear antigen labeling index (PCNA-LI) and DNA index (DI). We expected to find some simple and applicable indexes for estimating the prognosis of HCC by examining the surgical specimens of HCC.

MATERIALS AND METHODS

Materials

Formalin-fixed and paraffin-embedded HCC tissues blocks from 51 cases of patients with moderately differentiated HCC, the clinical data, data of the specimens (tumor size, portal tumor emboli, daughter nodules and so on) and follow-up data (including ultrasound examinations of liver every half a year after surgery) were collected in Zhongshan Hospital affiliated to Fudan University during 1989-1990. We used tumor-free survival (TFS) to estimate prognosis in order to eliminate the affection of various therapies after tumor recurrence. TFS was recorded dating from the time of operation, up to the time of death or detection of HCC recurrence by ultrasound examination. The follow-up ended in December, 1999 (2 cases were lost to follow up at 67, 88 months respectively). The TFS time ranged from 8 days to more than 129 months. The probability of 1-year TFS was 62.7 %, that of 3-year TFS was 35.3 % and that of 5-year TFS was 27.5 %. 8 cases had no recurrence and were still alive at the end of the follow-up.

Histopathological inspection

4 μm tissue thick sections were cut from each block and stained with hematoxylin-eosin. We examined morphological characteristic and arrangements of tumor cells, infiltration of mononuclear cells in the stroma, tumor capsules formation and its thickness, state of tumor cells invasion, and tumor emboli and microscopic daughter nodule in vein around the cancer. The infiltration of mononuclear cells was graded as follow: 0, none or occasionally found; 1, diffuse; 2, crowded together or even lymphoid follicles formed. Tumor without capsule was defined as tumor tissue directly bordering on normal liver tissue. If ten or more layers of stratified fibrous collagen together with fibroblasts separate them, it was defined as thick

capsule; less than this was defined as thin capsule. The diameter of cancer was indicated with geometric mean of three-dimensional parameters of single cancer nodule and cube root of total volume of multiple ones.

Immunohistochemical staining

Formalin-fixed and paraffin-embedded liver tissue sections of 4 μ m thickness were dewaxed in xylene and graded alcohols, hydrated, and washed in phosphate-buffered saline. The endogenous peroxidase was inhibited by 0.6 % H_2O_2 for 30 minutes, and the sections were incubated with normal goat serum (1:20 for 20 minutes). A 1:40 diluted rabbit polyclonal antibody to PCNA was applied 1 hour at 37 °C and then at 4 °C overnight in a moist chamber. Phosphate-buffered saline replaced the first antibody as blank control. A standard avidin-biotin peroxidase technique (ABC assay) was applied. Briefly, biotinylated goat anti-rabbit Ig and avidin-biotin peroxidase complex were applied for 1 hour each, with 15 minute washing in phosphate-buffered saline. The reaction was finally developed with 3-3' diaminobenzidine solution in 0.003 % H_2O_2 buffer phosphate (PH 7.8) for 10 minutes. Sections were counterstained with hematoxylin, dehydrated in alcohol, cleared in xylene, and mounted in DPX. The antibody and ABC kit were purchased from Dako Co. Copenhagen, Denmark.

To get average positive level of each case, six microscopic fields of 400 \times magnification were selected which included two representative fields each of considerable, medium and a few positive cells. PCNA-LI is the percentage of immunohistochemical staining positive cells in 1 000 HCC cells counted. The criterion to evaluate the expression of PCNA-LI was as following: 1, >15 % (high); 2, 5-15% (medium); 3, <5 % (low).

Determine of DNA-index

Feulgen staining was applied on 4 μ m thick sections, and IMS Cell Analysis System (Shenteng Co. Shanghai, P.R. China) was used to detect DNA index. In each case six representative microscopic fields were chosen and the integral optical density (IOD) of 100 discrete HCC cells was measured with lymphocyte nuclei in stroma as diploidy control. Then the DNA index was automatically calculated.

Statistical analysis

SPSS for windows 10.0 software was used in statistical analysis. Data were expressed as means \pm SD in the presence of continuous variables and as counts (percentages) for the categorical variables. Continuous variables were compared with the student *t* test, and categorical variables were compared with χ^2 test. The probability of TFS time was calculated using the Kaplan-Meier approach and differences between survival curves were tested by the Log-rank test.

RESULTS

General conditions

51 patients were from 27 to 71 years old, median 53 years old. 48 cases were male (94.1 %). The probability of 3-year TFS in single nodule group was 44.4 %, significantly higher than multiply nodules group 13.3 % ($P < 0.05$). The probability of 5-year TFS in tumor size ≤ 5 cm was 43.3 %, higher than group with tumor size > 5 cm 4.8 % ($P < 0.01$) (Table 1).

Inspection of tissue section

Tumor emboli were found in 19 cases and tumor necrosis in 16 cases during surgical operation and/or in pathological

sections. The probability of 5-year TFS in those without tumor emboli was obviously higher than those with them ($P < 0.01$). The probability of 1-year TFS in those without tumor necrosis was significantly higher than those with it ($P < 0.01$). 45 of 51 cases had well-preserved wax blocks and had one or two sections available to show capsules and tissues around tumor. We found that the probability of 1-year TFS in those with daughter nodules was lower than those without them ($P < 0.01$) and the probability of TFS in those with thick capsule was slightly higher than those with thin capsule or no capsule. The mononuclear cells infiltrating in the tumor and surroundings were mainly lymphocytes, mixing with fibrous collagen and fibroblasts, which were mostly distributed at the interface between HCC and the surroundings (Figure 1). They formed thin or thick capsule, some of which extended to tumor tissue and connected with stroma in HCC. Various degrees of lymphocytic infiltration occurred in tumor stroma (Figure 2), coordinated with that of the interface area. Only very few of the lymphocytes infiltrating tumor parenchyma showed direct contact with tumor cells. The probability of TFS in those with more heavy lymphocytic infiltration was slightly higher. (Table 1).

Table 1 The relationship between pathological characteristics and TFS

Variables	n	The probability of TFS (%)		
		1-year	3-year	5-year
Tumor nodule				
Single	36	72.2	44.4	33.3
Multiple	15	40.0 ^a	13.3 ^a	6.7
Tumor diameter				
≤ 5 cm	30	86.7	53.3	43.3
> 5 cm	21	33.3 ^b	9.5 ^b	4.8 ^b
Tumor necrosis				
No	35	77.1	42.9	34.3
Yes	16	31.3 ^b	18.8	12.5
Tumor emboli				
No	26	76.9	50.0	42.3
Yes	19	36.8 ^b	10.5 ^b	0 ^b
Daughter nodules				
No	30	80.0	40.0	30.0
Yes	15	20.0 ^b	20.0	13.3
Infiltrated lymphocytes				
Many	9	88.9	66.7	44.4
A few or none	42	59.5	31.0	23.8
Capsule				
Thick	13	69.2	46.2	30.8
None or thin	32	56.3	28.1	21.9

^a $P < 0.05$ vs the above group, ^b $P < 0.01$ vs the above group.

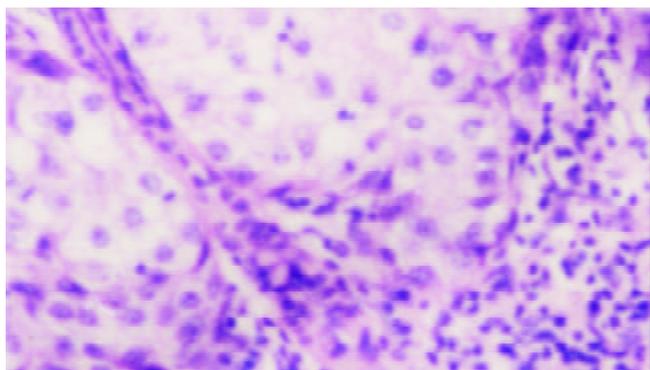


Figure 1 Massive lymphocytes infiltrating the interface between HCC and surroundings (HE, ×100)

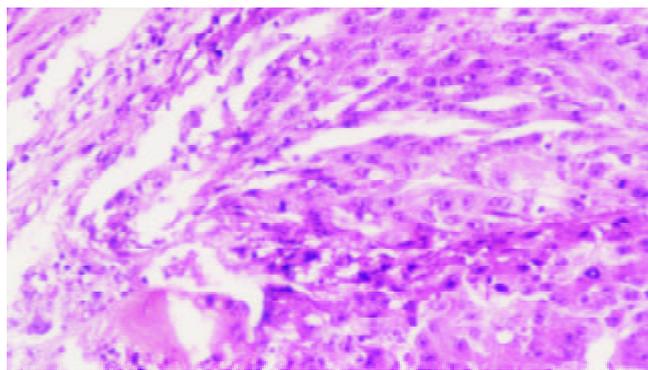


Figure 5 PCNA positive cells (the continuous section of Figure 4, PCNA immunohistochemical staining, ×100)

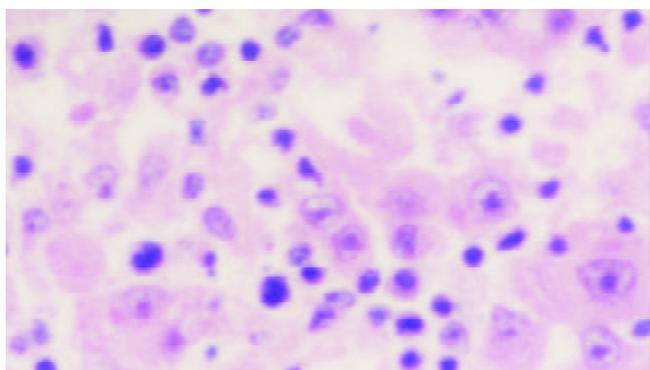


Figure 2 Many lymphocytes infiltrating tumor stroma (HE, ×100)

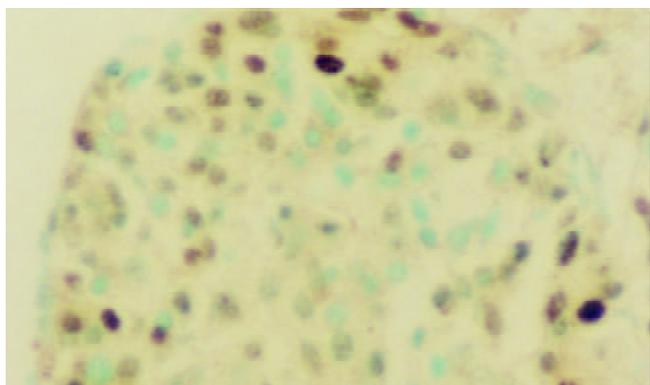


Figure 3 PCNA positive cells mainly distributed in the peripheral area of tumor nest (PCNA immunohistochemical staining, ×200)

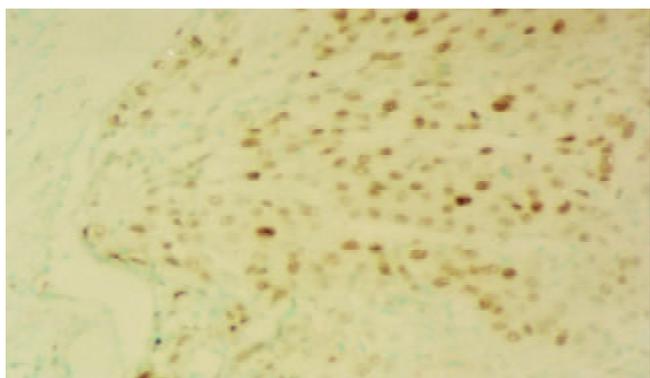


Figure 4 HCC invasion in capsule (HE, ×100)

PCNA-Labeling Index

PCNA positive granules were found in the nuclei, and all cells with recognizable nuclear stain were considered as positive. PCNA positive cells were mainly distributed in the peripheral area of tumor nest and also in tumor emboli and at the site of HCC invasion (Figure 3,4,5). The prognosis in the case with higher PCNA-LI was worse, $P < 0.01$ (Figure 6). With a higher PCNA-LI, the tumor size was usually larger and the probability of tumor emboli, daughter nodules and tumor necrosis was also higher ($P < 0.05$, Table 2).

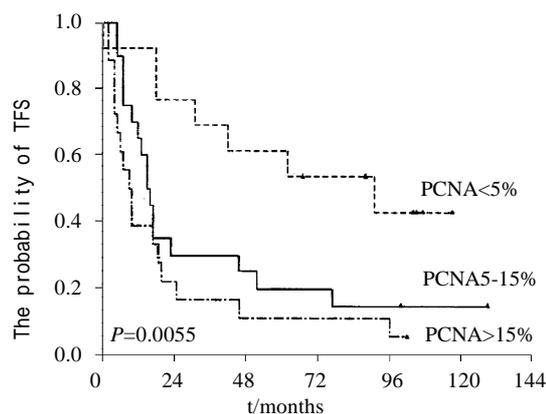


Figure 6 The Kaplan-Meier curves of different PCNA-LI groups

Table 2 The relationship of pathological characteristics to DI and PCNA-LI

Variables	n	Diameter (cm)	Multiple nodules	Tumor necrosis	Tumor emboli ^a	Daughter nodules ^a
DI						
Euploid	8	4.30±1.97	2	1	0/6	0/6
Aneuploid DI<1.5	22	3.64±1.29	5	4	8/19	4/19
Aneuploid DI>1.5	21	6.34±2.65 ^{bc}	8	11	11/20	11/20 ^d
PCNA-LI						
< 5 %	13	4.09±1.44	0	1	0/10	2/10
5-15 %	20	4.53±2.50	10 ^c	4	8/18	3/18
>15 %	18	5.78±2.60 ^b	5	11 ^{ce}	11/17 ^c	10/17 ^e

^aThe denominator was the number of observed cases, the numerator was the number of those presenting tumor emboli or satellite nodules. ^b $P < 0.05$, ^c $P < 0.01$ compared with the first group; ^d $P < 0.05$, ^e $P < 0.01$ compared with the second group.

DNA-index

In diploidy $DI=1 \pm 0.1$, and in tetraploidy $DI=2 \pm 0.1$. DI out of the above range was aneuploidy. The prognosis of HCC cases with euploidy was better than those with aneuploidy, and in the latter $DI<1.5$ was better than $DI>1.5$, $P<0.001$ (Figure 7). In the group of $DI>1.5$ aneuploidy, tumor size was larger and the probability of tumor emboli, daughter nodules and tumor necrosis was higher (Table 2).

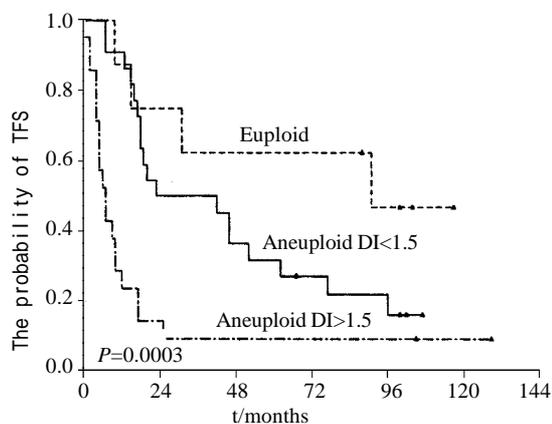


Figure 7 The Kaplan-Meier curves of different DI groups

The relationship between PCNA-LI and DI

The PCNA-LI in $DI<1.5$ aneuploidy group was obviously higher than in euploidy group ($P<0.05$), and that in $DI>1.5$ aneuploidy group was also higher than $DI<1.5$ aneuploidy group ($P<0.05$, Table 3). Aneuploidy $DI>1.5$ was seen more in higher PCNA-LI cases ($P<0.01$), and no euploidy was found in 18 cases with high PCNA-LI (Table 4). The TFS time in 13 aneuploidy cases with high PCNA-LI and $DI>1.5$ was from 2 to 25 months; their average TFS time was 8.1 months and the probability of 1-year TFS was only 15 %, much lower than that of the total cases (62.7 %, $P<0.01$).

Table 3 PCNA-LI in different DI groups

DI	n	PCNA-LI (%)
Euploid	8	5.076±3.904
Aneuploid $DI<1.5$	22	9.300±6.896 ^a
Aneuploid $DI>1.5$	21	15.113±9.527 ^{bc}

^a $P<0.05$, ^b $P<0.01$ compared with the first group, respectively; ^c $P<0.05$ compared with the second group.

Table 4 The DNA-index distribution in different PCNA groups

PCNA-LI	n	Euploidy	Aneuploidy $DI<1.5$	Aneuploidy $DI>1.5$
<5%	13	5	6	2
5-15%	20	3	11	6
>15%	18	0	5	13

χ^2 test $P<0.01$, compared aneuploidy $DI>1.5$ and the others among the groups.

DISCUSSION

According to pathomorphological inspection of HCC, the TFS of patients with single tumor nodule (diameter ≤ 5 cm, no tumor

emboli, no daughter nodules and tumor necrosis) was obviously high and these factors are indicators of better prognosis, which are consistent with results of other retrospective researches^[3-6]. The mononuclear cells reaction may represent the response to local irritation by the tumor, but it also may be a host immunological defense mechanism against the neoplastic process. Tumor-infiltrating lymphocytes are important in immunotherapy for tumors^[7-9]. The presence of collagen I and III in tumor tissues may inhibit tumor cell infiltration and metastasis and such patients may have a better prognosis^[10]. The expression of MMPs (a matrix-degrading enzyme family) in tumor and its relation to prognosis is one of hot points in tumor research^[11-16]. Our studies also implied that patients with more mononuclear cells infiltrating in the tumor and surrounding tissue had better prognosis, and those with a thick capsule also had a longer TFS, though the differences were not statistically significant because of small number of cases.

Detection of DNA content and ploidy of tumor cells is still a common method to evaluate tumor malignancy and patient's prognosis^[17-22]. Our studies demonstrated that the prognosis of patients with euploidy HCC was better than that of patients with aneuploidy HCC, and in the latter the prognosis of $DI<1.5$ cases was better than that of $DI>1.5$ ones. In aneuploidy, $DI=1.5$ seemed to be a borderline, and the probability of 3-year TFS in $DI>1.5$ cases (13.6 %) was obviously lower than in $DI<1.5$ cases (52.4 %, $P<0.01$).

PCNA, auxiliary protein of DNA polymerase delta, accumulates in the nuclei during the S-phase of the cell-cycle, i.e., proliferating cells^[23]. PCNA-LI can explicitly reflect proliferating degree of tumor. It was reported that PCNA-LI>2.0 % was the strongest independent predictor of HCC in patients with compensated cirrhosis^[24]. Previous research found that PCNA-LI was closely related with HCC differentiation, but others reached a conclusion that PCNA was not related with patients survival^[25]. We found that PCNA-LIs were very different even in the similar moderately differentiated HCC cases, and the prognosis was worse in patients with higher PCNA-LI, and the probability of 5-year TFS in PCNA-LI>15 % group was only 11.1 % which was far lower than that in PCNA-LI<5 % group (61.5 %, $P<0.01$). Closset el at reported that PCNA-LI<30 % appeared to be a good prognostic factor in patients without cirrhosis^[26]. Besides differentiation of HCC, the difference of PCNA-LI borderline is most closely due to fields selection. We noticed that PCNA positive cells often mainly distributed in the peripheral area of tumor nest and also in tumor emboli and at the site of HCC invasion, while seldom in the middle of tumor nest. Such uneven distribution caused difficulty in selecting fields for immunohistochemistry. We applied an objective way to solve the problem: firstly, we went over the whole section, then selected six microscopic fields of 400× magnification, including two representative fields for each one of a considerable number, medium and a few positive cell areas, and finally calculated positive ones in 1 000 tumor cells.

Both PCNA-LI and DI can reflect the degree of tumor proliferation^[19]. Studies in 51 cases HCC showed a certain relationship between them: the PCNA-LI in aneuploidy group was higher than in euploidy group, and a higher PCNA-LI had a higher proportion of aneuploidy. In 18 cases with a high PCNA-LI not a single case of euploidy was detected and the proportion of $DI>1.5$ aneuploidy was also high ($P<0.05$). However, it should be pointed out that merely judged by PCNA-LI or DI a few exceptions were found, for example, the TFS of 2 cases in the high PCNA-LI group was more than 5 years and one of them was still alive at the end of follow-up, and similarly 2 cases in $DI>1.5$ aneuploidy group were alive

at the end of follow-up. But all 13 cases with high PCNA-LI and $DI > 1.5$ aneuploidy had bad prognosis. So we consider that PCNA-LI and DI should be applied together in evaluating HCC patient's prognosis.

Surely other indexes, for instance, microvessel density, etc, are also helpful for evaluating prognosis through pathological examinations of HCC surgical specimens^[2, 27-30]. We believe that a composite prospective analysis of the pathological characteristics of tumor tissue, DI and PCNA-LI is a simple and objective means to evaluate the risk of recurrence and prognosis for those patients with HCC after surgery.

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