Immunohistochemical assessment of proliferating cell nuclear antigen in primary hepatocellular carcinoma and dysplastic nodules

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Abstract

A complementary way for the assessment of HCC prognosis is represented by the analysis of molecular markers. Thus, immunohistochemical assessment of proliferation can describe tumor aggressiveness, probability of local recurrence or metastasis potential, being very useful for the assessment of recurrence-free survival and survival until death. The aim of our study was to assess proliferating cell nuclear antigen activity in HCC and dysplastic nodules as compared with surrounding non-neoplastic areas. Immunohistochemical techniques were thus performed on the samples obtained by ultrasound-guided liver biopsies or intraoperative biopsies, in 32 patients with HCC, as well as in 3 patients with dysplastic nodules occurring in liver cirrhosis. Expression of PCNA within extranodular areas of the HCC patients in the absence or presence of cirrhosis, was increasing from 40% to 70%, respectively. PCNA expression further increased within intranodular areas of dysplastic nodules and HCC, to 100% and 96.88%, respectively. A progressive increase of the mean values of PCNA-LI was also observed from extranodular areas without or with cirrhosis, towards intranodular areas of dysplastic nodules and HCC (4.2%, 6.8%, 27.9%, 31.9%, respectively). Dysplastic nodules can thus be considered lesions with a high-proliferation rate, representing an early stage of hepatocarcinogenesis. This supported the current recommendations for borderline hepatocellular nodules identified by ultrasound, which indicate an aggressive treatment similar to malignant lesions. In summary, we demonstrated a progressively increasing rate of cellular proliferation, from extranodular non-neoplastic areas to intranodular areas (dysplastic nodules and HCC), as reflected by an increased expression of proliferating cell nuclear antigen labelling index.

Keywords: peroxisome • gene • β -oxidation • biogenesis • PPAR • fibrates • fatty acids • hormones

Introduction

Conventional prognostic factors in primary hepatocellular carcinoma (HCC) include the TNM (tumor-

node-metastasis) system and the establishment of HCC grade (based on the cellular differentiation). Pathological and biological factors involved in HCC prognosis were also extensively studied. Thus, macroscopical and microscopical characteristics of the tumors are correlated with the survival of the patients and tumor recurrence. However, a complementary way of analysis of HCC prognosis is

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represented by the analysis of molecular markers [1]. Different markers are clearly associated with the aggressiveness of HCC and hold prognostic information, being very useful for the establishment of the cellular malignant phenotype: cell proliferating activity, tumor suppressor and promoter genes, proteinases that attack the extracellular matrix, adhesion molecules, angiogenic factors.

Cell proliferation is an important mechanism in oncogenesis. Immunohistochemical assessment of proliferation can describe tumor aggressiveness, probability of local recurrence or metastatic potential, thus being very useful for the assessment of recurrence-free survival and survival until death [2]. To study proliferation in HCC we have chosen the "proliferating cell nuclear antigen" (PCNA), which is an auxiliary protein of DNA polymerase with an increased expression in proliferating cells. However, PCNA is present also in non-proliferating cells, because most of the tumors have active processes of reparation for DNA. PCNA is present only in the G1, S and G2 phase in the cycling cells and it is partly correlated with other proliferation markers such as %S phase fraction, Ki67 staining and mitotic count [2].

The aim of our study was to assess proliferative activity in HCC and dysplastic nodules, as compared with surrounding cirrhosis or normal liver tissue, by means of immunohistochemical analysis of the expression of PCNA.

Patients and methods

Patients

During the study period (01.01.1996 - 01.01.2001), we examined 75 patients consecutively diagnosed with HCC in our Department of Internal Medicine, Division of Gastroenterology. Liver biopsies were performed in 43 HCC patients with 17 G automatic needles (Autovac, BARD-Angiomed, Germany). In other 8 HCC patients, intraoperative biopsies were performed during open laparotomy for other indications. Both intranodular and extranodular biopsies were performed in patients with HCC. For the patients with multiple HCC nodules, we performed a single biopsy from the dominant nodule, which was described concerning dimensions and precise location.

Only 32 patients of the initial cohort of 75 patients consecutively diagnosed with HCC were included in our

study, due to various conditions which prevented the immunohistochemical analysis because of missing or inadequate biopsy material. These conditions included: advanced age or stage of the disease (Child-Pugh stage C of the underlying liver cirrhosis), unfeasibility of liver biopsy (large ascites, coagulopathy, large tumors, refusal of the liver biopsy procedure), inadequate material for immunohistochemical studies, etc. The diagnosis of HCC was confirmed by usual histological and immunohistochemical techniques according to a standard protocol, based on Edmonson-Steiner classification [3]. Accordingly the patients were classified with well differentiated (grade I), moderate differentiated (grade II), poor (grade III) and undifferentiated (grade IV) HCC nodules.

We also included three patients with liver cirrhosis and dysplastic nodules, diagnosed as a function of liver biopsy results. Thus, three high-grade dysplastic nodules, identified by transabdominal ultrasound, with dimensions below 3 cm, had cytological and architectural atypia insufficient for the diagnosis of HCC. Because HCC diagnosis could not be excluded, the patients were consequently followed by repeated transabdominal ultrasound examinations performed in three months intervals.

Consequently, the total number of patients included in the study was 35, consisting of 32 patients with HCC and 3 patients with dysplastic nodules.

Immunohistochemical reactions

Immunohistochemical techniques were performed in 32 patients with HCC. We thus included 21 men and 11 women, with an age of 49.7 ± 14.3 years (mean $\pm$ standard deviation). HCC nodules identified in these patients had variable dimensions, between 1.3 and 8.1 cm (mean $\pm$ SD = 4.3 ± 1.9 cm), but most of the patients were diagnosed in advanced stages. Thus, in 25 patients (78.12%), HCC nodules were identified in advanced stages, with dimensions of more than 3 cm. The diagnosis of liver cirrhosis was confirmed in 27 patients (84.38%), based on the histopathologic exam of intraoperative or needle biopsies from extranodular non-neoplastic areas. The patients were classified as a function of the degree of differentiation (according to Edmondson Steiner classification) in well-differentiated (5 patients), moderate differentiated (12 patients), poor differentiated or undifferentiated (15 patients).

Table 1. Peroxisome metabolic pathways.

PCNA-LI	Patients	Mean	SD	Min	Max
Extranodular	N=35				
Without cirrhosis	N=5	4.2	2.7	1	12
With cirrhosis	N=30*	6.8	3.2	1	17
Intranodular†	N=35				
High-grade dysplastic nodules	N=3	27.9	24.4	9	47
Well differentiated HCC	N=5	18	13.5	9	41
Moderate differentiated HCC	N=12	27.8	12.1	14	52
Poor/undifferentiated HCC	N=15	39.6	13.7	20	60

*: $P < 0.05$; †: $P < 0.001$.

For the differential diagnosis between high-grade dysplastic nodules and well-differentiated HCC we have used the following criteria: prominent nuclear atypia, increased nucleo-cytoplasmic ratio, absence of portal tracts, presence of isolated arteries or mitoses. The patients with dysplastic nodules were followed up as the patients with HCC, because dysplastic nodules can contain foci of carcinoma and because the diagnosis of HCC could not be excluded based on needle biopsies due to sampling errors.

The formalin-fixed endoscopic biopsy samples obtained for each patient were immunostained with a monoclonal antibody for PCNA (PC10, Dako). The tissues were fixed for 8-12 hours, in 10% buffered formalin (pH=7) and routinely processed for paraffin embedding at 56°C. Paraffin sections of 3 microns were cut. For immunohistochemistry, paraffin sections were deparaffinized in xylene and rehydrated in ethyl alcohol. The sections were incubated for 30 min with 1% H_2O_2 in methanol and then incubated for 20 min at room temperature with non-immune rabbit cell. Afterwards, the serial sections were incubated at 4°C with a monoclonal antibody for PCNA (PC10, Dako). After washing with PBS the bound antibody was visualized by the peroxidase ABC method using diaminobenzidine (Sigma Chemical Co.) as developer. The sections were rinsed with water and counterstained with Mayer's hematoxylin.

Immunohistochemical reactions for PCNA were considered positive when it was observed a clear, unequivocal, diffuse or granular brown coloration of the nucleus. We have assessed at least 1000 hepatocyte nuclei in 10-

15 high-power optical fields with a large objective (Ob40), from the most evenly labeled areas of HCC nodules, as well as extranodular non-neoplastic regions of surrounding parenchima. Values of PCNA were expressed as a percent of positive hepatocytes reported to total hepatocytes, representing PCNA labelling index (PCNA-LI).

Statistical analysis

All the results were expressed as mean $\pm$ standard deviation (SD). For assessing the differences between the patients with HCC as a function of the degree of differentiation we have used the ANOVA test, which allows the concomitant analysis of data from multiple sources. A value of $P < 0.05$ was considered statistically significant.

Results

The expression of PCNA in hepatocytes was limited to the nucleus and appeared as a diffuse or granular brown coloration (Fig. 1). Occasionally, we encountered Kupffer cells, lymphocytes and biliary epithelial cells with a positive PCNA reaction, which were not considered.

The expression of PCNA was demonstrated for extranodular non-neoplastic areas of the patients without cirrhosis (Fig. 2) in 40% of the cases (2 of 5

Fig. 1 Positive reaction for PCNA (Arrow) at the level of hepatocyte nuclei as compared with cells with unstained nuclei (Arrow head) [immunostaining, Ob40].

Fig. 2 Positive reaction for PCNA at the level of hepatocyte nuclei in extranodular regions of the patients with HCC in the absence of liver cirrhosis, for a patient with macrovacuolar liver steatosis [immunostaining, Ob20].

Fig. 3 Positive reaction for PCNA at the level of hepatocyte nuclei for extranodular regions of the patients with HCC and liver cirrhosis (regenerative nodule) [immunostaining, Ob20].

Fig. 4 Positive reaction for PCNA at the level of hepatocyte nuclei, with a predominance towards the periphery of regenerative nodules in the non-neoplastic regions of the patients with HCC and liver cirrhosis [immuno-staining, Ob20]

Fig. 5 Positive reaction for PCNA at the level of hepatocyte nuclei, for intranodular regions of the patients with high-grade dysplastic nodules [immuno-staining, Ob20].

Fig. 6 Positive reaction for PCNA at the level of hepatocyte nuclei in the patients with well-differentiated HCC [immuno-staining, Ob20].

patients). PCNA-LI in these cases had variable values between 1% and 12% (mean $\pm$ SD, 4.2 ± 2.7).

For the patients with liver cirrhosis, the expression of PCNA for extranodular non-neoplastic areas (Fig. 3) was evident in 70% of patients (21 of 30 patients). PCNA-LI in these patients had variable values, between 1% and 17% (mean $\pm$ SD, 6.8 ± 3.2). Hepatocytes with PCNA expression were located towards the periphery of the regenerative nodules, and were concentrated in the areas with inflammation, sometimes in all nodules, isolated or in small groups (Fig. 4).

All three patients (100%) with dysplastic nodules expressed PCNA inside high-grade dysplastic nodules (Fig. 5). PCNA-LI had variable values between 9% and 47% (mean $\pm$ SD, 27.9 ± 24.4).

Almost all the patients with HCC had a positive PCNA reaction inside HCC nodules (31 of 32 patients, 96.88%), while PCNA-LI had values between 9 and 60% (mean $\pm$ SD, 31.9 ± 15.1). The values of PCNA-LI increased with the progressive dedifferentiation of HCC nodules. Thus, the patients with well-differentiated HCC (Fig. 6) had relatively low values of PCNA-LI, between 9 and 41% (mean $\pm$ SD, 18 ± 13.5). The patients with moderate differentiated HCC (Fig. 7) had variable values of PCNA-LI, between 14% and 52% (mean $\pm$ SD, 27.8 ± 12.1). The patients with poor differentiated or undifferentiated (Fig. 8) HCC had relatively higher values of PCNA-LI, between 20 and 60% (mean $\pm$ SD, 39.6 ± 13.7).

We have found significant differences between PCNA-LI values for the extranodular areas in patients without cirrhosis, as compared with the patients with cirrhosis ($P < 0.05$). Although the values of PCNA-LI increased progressively with HCC dedifferentiation, we could not find statistically significant differences between the patients with high-grade dysplastic nodules, and the groups of patients with HCC (well-differentiated, moderately differentiated and poor differentiated / undifferentiated). However, there were significantly statistic differences between the values of PCNA-LI for intranodular (dysplastic nodules and HCC) as compared with extranodular non-neoplastic areas ($P < 0.001$).

The values of PCNA-LI for extranodular areas in the patients without cirrhosis (N=7) or with cirrhosis (N=28), as compared with intranodular areas, in the patients with high-grade dysplastic nodules (N=3), well-differentiated (N=5), moderate differ-

entiated (N=12) and poor differentiated / undifferentiated are represented in Table 1. A progressive increase of the values of PCNA-LI was observed from extranodular areas of the patients without liver cirrhosis, to extranodular areas of the patients with liver cirrhosis, and to the intranodular areas of the patients with high-grade dysplastic nodules and well-differentiated, moderately differentiated and poor differentiated / undifferentiated HCC.

Discussions

The results of our study indicated the presence of rapid cellular proliferation in HCC. Moreover, a progressive increase of cellular proliferation was observed from extranodular to intranodular areas (dysplastic nodules and HCC). Thus, expression of PCNA was increasing from 40% and 70% in extranodular areas of the patients without or with cirrhosis, to 100% and 96.88% within intranodular areas from the patients with dysplastic nodules and HCC, respectively (Fig. 9).

Mean values of PCNA-LI were also progressively increasing from extranodular areas of the patients without liver cirrhosis, to extranodular areas of the patients with liver cirrhosis, and further to intranodular areas of the patients with high-grade dysplastic nodules, well-differentiated, moderate differentiated, and poor differentiated / undifferentiated HCC, respectively. The difference between the mean values of PCNA-LI for extranodular areas (patients without or with liver cirrhosis and HCC) and intranodular areas (dysplastic nodules and HCC) were significantly statistic ($p < 0.001$) (Fig. 10). In the extranodular non-neoplastic areas, the patients with liver cirrhosis and HCC had significantly higher values of PCNA-LI as compared to the patients with HCC without liver cirrhosis. High-grade dysplastic nodules had similar values of PCNA-LI with HCC nodules. Extranodular areas are thus characterized by a low proliferation, while dysplastic nodules are lesions with high proliferation, which might represent an early stage of hepatocarcinogenesis.

The results of our study suggested that the areas surrounding dysplastic nodules and HCC had relatively lower values of proliferation indices as com-

Fig. 7 Positive reaction for PCNA at the level of hepatocyte nuclei, inside intranodular regions of the patients with moderate differentiated HCC [immunostaining, Ob20].

Fig. 8 Positive reaction of PCNA at the level of hepatocyte nuclei for the patients with poor differentiated HCC [immunostaining, Ob10].

pared with dysplastic nodules and HCC. The same results were obtained in previously published studies [4-6]. However, other authors suggested that the tissue nearby HCC has intermediate values of proliferation indices as compared with non-neoplastic liver tissue at distance [7].

The present study also demonstrated that dysplastic nodules had similar values of proliferation indices as compared with HCC, results confirmed by the previously published studies. However, the results are difficult to assess because of the small number of patients with dysplastic nodules identified and included (N=3). Another important limitation of the study was that the positive diagnosis of dysplastic

nodules was based on liver biopsy (which is frequently hampered by sampling errors, due to the presence of HCC foci in the patients with dysplastic nodules), and not on the liver explants obtained by hepatectomy, liver transplant or necropsy.

Proliferation associated antigens like Ki-67 and MIB-1 partly correlate with PCNA and other proliferation markers like %S phase cells and mitotic count. The Ki-67 antigen could only be studied initially in frozen sections, and is expressed in the G1, S and G2 phase in cycling cells. The MIB antibodies are a series of antibodies that work on paraffin embedded tumour material after antigen retrieval techniques. Of these, MIB-1 was studied widely

Fig. 9 Positive reaction of PCNA in extranodular regions (for the patients with HCC without, or with liver cirrhosis, respectively) as well as intranodular regions (for the patients with dysplastic nodules and HCC).

Fig. 10 Mean values of PCNA-LI for extranodular areas (patients without or with liver cirrhosis and HCC) and intranodular areas (dysplastic nodules and HCC).

being regarded as a reliable marker of proliferating cells. An assessment of PCNA-LI and MIB-1 for hepatocellular nodules indicated a progressive increase of labeling indices from regenerative nodules, to dysplastic nodules and HCC [4]. The values of these two markers of proliferation were significantly correlated ($P < 0.001$). However the differences between low-grade dysplastic nodules, high-grade dysplastic nodules, HCC smaller than 2 cm and HCC greater than 2 cm were not significant. Most of the authors obtained similar values of PCNA-LI between high-grade dysplastic nodules and HCC [8, 9]. Other authors observed a progressive increase of PCNA-LI from regenerative nod-

ules, towards low-grade dysplastic nodules, high-grade dysplastic nodules and HCC [5, 10]. These differences reflect probably sampling errors and size of the biopsies, but they do not seem to indicate a significant difference between the results [4]. Recent studies also suggested that apoptosis is slightly higher in HCC as compared to normal liver, which implies that it is less involved in the development and progression of this disease [11-13]. Thus, although a high index of proliferation seems to be characteristic for HCC, this is not associated with an increased rate of apoptosis.

Several published studies indicated that large regenerative nodules (> 0.5 cm) have low values of

proliferation indices, i.e. PCNA-LI [5, 8, 14] and Ki-67 [15], even when compared to ordinary regenerative nodules. It is possible that macroregenerative nodules might represent neoplastic lesions that are not susceptible to the necro-inflammatory process responsible for the remodeling of the surrounding hepatic parenchyma. The premalignant potential of macroregenerative nodules is thus determined by a survival advantage generated by a decrease of apoptosis, and less by an increase of the proliferating activity [14]. Different authors postulated that some of the hepatocellular nodules from the cirrhotic liver have a premalignant potential and already have the necessary genetic alterations necessary for hepatocarcinogenesis, before the microscopical separation of the nodules from the adjacent hepatocyte parenchyma by fibrotic septa [14, 16]. Clonal proliferation of the hepatocytes inside macroregenerative nodules was demonstrated by several studies, which suggested a clonal proliferation of HBV (hepatitis B virus) [17, 18] and HCV (hepatitis C virus) [16] within the cirrhotic nodules. Moreover, two studies suggested that there are no histopathological differences between a monoclonal and polyclonal hepatocyte proliferation [16, 17]. All these studies support the hypothesis of a monoclonal expansion of hepatocytes.

However, other authors suggested that hepatocellular nodules are early lesions in the multistage progression of hepatocarcinogenesis [19-24]. Thus, it is considered that high-grade dysplastic nodules are already malignant or they might contain foci of HCC, if they are assessed as a function of size (> 1.5 cm) or different morphological criteria [21, 24]. The succession low-grade dysplastic nodules → high-grade dysplastic nodules → HCC, is currently considered a way of progression towards HCC [8, 25]. However, this succession is not the only mechanism of progression, because HCC is also identified in patients without liver cirrhosis.

Hepatocellular nodules detected by transabdominal ultrasound and followed-up by serial ultrasound examinations or ultrasound-guided biopsies, have an increased rate of malignant transformation [19, 23]. The frequency of malignant transformation reaches 68% of the nodules followed-up for 31 months [19] or 22%, 50% and 80% of the nodules followed-up for 1, 2 and 3 years, respectively [23]. Current recommendations for borderline lesions identified by ultrasound include either frequent

ultrasound monitoring [26], or an aggressive treatment as premalignant lesions [10], or malignant lesions [23]. A progression of proliferative indices, from regenerative nodules towards dysplastic nodules and HCC, is clearly demonstrated in the present study, as well as in previously published studies with a similar design [4, 8]. A high degree of overlapping for the values of proliferative indices does not permit definitive conclusions concerning therapeutic decisions. A type II statistical error appears, because there is a clear difference between the values of PCNA-LI between regenerative nodules, dysplastic nodules and HCC (well differentiated, moderate differentiated and poor differentiated / undifferentiated), but the difference is too small to be detected because of the large variability of the results. Moreover, a clear progression of low-grade dysplastic nodules to high-grade dysplastic nodules has not been demonstrated until now. Some of the patients with cirrhosis and HCC do have high-grade dysplastic nodules, but low-grade dysplastic nodules are usually absent. However, low-grade dysplastic nodules are frequently encountered in the patients with liver cirrhosis in the absence of HCC [24].

The presence of cytological and architectural atypias indicates an increased possibility of malignant transformation [4]. Macroregenerative nodules can be a fertile ground for initiation and promotion of hepatocarcinogenesis [27]. However, another factor (a "2nd hit") might be necessary for the development of HCC, because the incidence of HCC is much lower than predicted by the relatively frequently diagnosed dysplastic nodules in liver cirrhosis [28]. A recent study also suggested that the high liver cell proliferative activity in liver cirrhosis is the strongest independent predictor of HCC development as assessed by multivariate analysis [29]. Thus, the yearly incidence of HCC was 5.2% for the patients with PCNA-LI > 2.0% as compared to 1.1% for the patients with low PCNA-LI (relative risk 4.90; 95% confidence interval 2.63-9.55). Survival was significantly lower in cirrhotic patients with high liver cell proliferative activity than in those with low proliferative rates, which implied that the development of HCC in patients with compensated cirrhosis seems to be reliably predicted by liver cell proliferation rate.

Assessment of the proliferative activity is undoubtedly very important for the early diagnosis

of HCC in cirrhotic patients, as well as for evaluation of the prognosis of the patients diagnosed with HCC. However, the need of liver biopsy for confirmation of HCC diagnosis in cirrhotic patients is currently contested in view of the clear progresses of imaging methods (computed tomography and magnetic resonance), as well as inherent risks of liver biopsy (bleeding, needle tract metastases, etc.). The same limitation was encountered in our study because of the impossibility to perform the immunohistochemical analysis due to unfeasibility of routine liver biopsy in all patients with HCC. Biopsy material was available in less than half of the patients (32 patients), out of a total of 75 patients consecutively admitted and diagnosed with HCC.

Conclusions

1. Our study indicated a progressively increasing rate of cellular proliferation, from extranodular non-neoplastic areas to intranodular areas (dysplastic nodules and HCC), reflected by an increased expression of PCNA-LI. Future studies will have to establish if a high proliferative activity is a good prognostic indicator for these patients.

2. Although the values of PCNA-LI have increased progressively with the dedifferentiation of HCC, the differences have not reach statistical significance between the patients with high-grade dysplastic nodules, and the three groups of HCC (well-differentiated, moderate differentiated, poor differentiated and undifferentiated). Dysplastic nodules can be considered lesions with a high-proliferation rate, representing an early stage of hepatocarcinogenesis. However, it is highly unlikely that multistage progression is the only mechanism of hepatocarcinogenesis.

3. Current recommendations for borderline hepatocellular nodules identified by ultrasound (macroregenerative nodules, low-grade or high-grade dysplastic nodules) include an aggressive treatment similar to the malignant lesions (liver transplant, partial hepatectomy, ultrasound-guided percutaneous ethanol ablation or radio-frequency ablation, and chemoembolisation).

4. Both hypotheses concerning the pathogenesis of HCC (the theory of monoclonal expansion and the theory of multistage hepatocarcinogenesis) are

still discussed in the literature, with pros and cons. Our study seems to support the theory of multistage progression for HCC, although it cannot exclude the theory of monoclonal expansion.

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