Original paper

Steatosis influences hepatocytes proliferative potential in chronic hepatitis C patients

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The study evaluates the influence of steatosis on hepatocytes proliferative potential, reflected by proliferating cell nuclear antigen (PCNA) expression in chronic hepatitis C (CHC) patients both in steatotic and non-steatotic areas of lobules. The liver histology was evaluated according to Kleiner's score. Nonalcoholic steatohepatitis (NASH) was also defined as the presence of lobular inflammation, hepatocyte ballooning and steatosis. Expression of PCNA was significantly in patients with definite NASH compared to those with simple steatosis, but not to those with borderline NASH. Advanced steatosis negatively influenced PCNA expression. NASH not only affects PCNA expression in staetotic, but also in non-steatotic lobule areas. Expression of PCNA could be an independent indicator of changes in hepatocyte metabolism in CHC patients. High NAS values and low PCNA expression may be a negative prognostic factor in predicting the further course of the disease.

Key words: chronic hepatitis C, PCNA, nonalcoholic fatty liver disease, steatosis, hepatocyte ballooning.

Introduction

Chronic hepatitis C (CHC) and non-alcoholic fatty liver disease (NAFLD) are the two most common liver diseases in well developed countries, coexisting on several occasions. Hepatitis C virus (HCV) has been suggested to be a strong contributor of hepatic steatosis and insulin resistance (IR), which finally leads to metabolic abnormalities including glucose intolerance or type 2 diabetes mellitus [1]. Insulin resistance is strictly associated not only with hepatic lipid accumulation, but also with more aggressive fibrosis progression [2, 3, 4, 5]. It has to be noted, however, that as many as 30% of patients with fatty liver who do not drink alcohol have normal body mass index (BMI) and insulin sensitivity [6]. It is well established that HCV may interfere with lipid metabolism at three levels: impaired secretion,

increased neosynthesis and impaired degradation [7]. Experimental studies have shown that HCV core protein is localized on the surface of lipid droplets, and its over-expression seems to further stimulate the formation of lipid droplets [8]. HCV core protein has been suggested to play a pivotal role in HCV-host cell interplay by altering gene expression through interaction with the transcriptional machinery and through enzymes that modify the chromatin [9].

Proliferating cell nuclear antigen (PCNA) is a highly conserved acidic nuclear protein with a molecular weight of about 36 kDa. It is synthesized in the late G1- and in the S-phase of the cell cycle and encircle the DNA duplex and tetherthe replicative polymerases to the genomic template. Later, it was found that PCNA serves as a docking platform where other proteins dock to carry out different DNA metabolic processes [10]. Proliferating cell nuclear antigen immunoreactivity has been widely used for the assessment of proliferative activity in normal regenerative and neoplastic liver in rodents and human [11, 12]. The regeneration process appears to be essential for restoration and/or maintaining liver function. The proliferative activity of hepatocytes is evaluated by estimation of PCNA labeling index [13]. Only several studies analyzed the expression of PCNA in chronic viral hepatitis.

Aim of the study was to analyze the relationship between hepatocyte proliferation disorders reflected by PCNA expression and the extent of fat accumulation in lobules in patients with coexisting CHC and NAFLD.

Materials and methods

In a group of 47 patients with genotype 1b HCV infection liver biopsies were performed before antiviral treatment. The infection was confirmed by detection of HCV-RNA in serum with reverse polymerase chain reaction (RT-PCR) (Amplicor Roche/ Promega v.2 Diagnostic Test, New Jersey, USA), while the HCV genotype with reverse hybridization line probe assay (LiPA Versant test, Milwaukee, WI, USA). Patients with other HCV genotypes, toxic liver damage, autoimmune, neoplastic and thyroid diseases, renal or heart failure and with concomitant hepatitis B virus and/or human immunodeficiency virus infections were excluded from the study. Additional exclusion criteria were diagnosis of type 2 diabetes mellitus, alcohol use more than 20 g/day and BMI above 35 kg/m^2.

Liver histology

Liver biopsy was performed with Hepafix kit (B. Braun, Melsungen AG, Germany) and tissue specimens were fixed in 5% buffered formalin and embedded in paraffin. Histopathological preparations were made using haematoxylin-eosin staining, and with methods according to Gomory and Azan (fibrotic stage evaluation). Five-micrometer thick tissue sections were cut. For further immunohistochemical examinations each paraffin block was used to make at least two preparations including 4 biopsy sections. Histopathological examination was carried out retrospectively by two experienced pathologists using a double-headed microscope.

Stage of fibrosis and grade of inflammatory activity were evaluated semi-quantitatively according to Scheuer's scoring system (0-4 pts.) [14]. Steatosis and hepatocyte ballooning were assessed according to Kleiner's score with area of fat accumulation in lobules grading as follows: 5-33% – grade 1, ≥ 33 < 66% – grade 2 and ≥ 66% – grade 3. For further analysis patients with steatosis were divided into two subgroups – steatosis < 33% of lobule hepatocytes affected – low grade and ≥ 33% – high grade. NAS was estimated on the basis of common assessment of fat accumulation (0-3 points), hepatocyte ballooning (0-2 points), inflammatory activity (0-3 points). Values below 3 points indicate simple steatosis, value of 3 or 4 points "borderline" nonalcoholic steatohepatitis (NASH), whereas values equal of 5 or more points correspond to definite NASH [15]. Additionally NASH was also assessed as a simultaneous appearance of steatosis, lobular inflammation and hepatocyte ballooning independently of their grades. The further analysis compared all three subgroups of patients with respect to fibrosis stage and PCNA expression in lobules.

Immunochistochemistry

For this procedure formalin-fixed paraffin-embedded sections were deparaffinized, dehydratated and demasked in a microwave oven for 20 minutes in 0,01 M sodium citrate buffer (pH 6.0).Method consist of two steps using LSAB 2 system –HRP. Assessment of proliferative potential of the hepatocytes was determined using as the primary antibody. monoclonal anti- PCNA (Dako, Glostrup, Denmark, MO 879). The antigen – antibody reaction was visualized with LSAB 2 System – HRP (K0675, DAKO, Glostrup, Denmark) using 3,3 diaminobenzidine (DAB) as a chromogen. The sections were counter stained with Mayer's hematoxylin. Reaction were performed using automated staining system Dako Autosteiner Plus. Proliferating cell nuclear antigen expression was then evaluated in 5 view fields in 400× magnification, separately in areas of steatosis and areas without steatosis within the lobules. The final result were calculated as an average value of PCNA expression from 5 view fields, and defined as PCNA expression index.

Statistical analysis

The results were presented as the mean values with standard deviation (± SD). The distribution of the values were assessed using Shapiro-Wilk test. Due to abnormal distribution of the values, nonparametric methods were used to calculation. Differences between groups were tested using U Mann-Whitney and ANOVA rang Kruskal-Wallis tests for independent groups. The Spearman rank correlation coefficient was used to calculate the correlation between different values. $P < 0.05$ was considered to be statistically significant. Analysis was carried out with Statistica v.10 software (StatSoft Inc. Tulsa, OK.,USA).

Results

Liver biopsies were conducted in a group of 47 patients (28 females and 19 males) with CHC infected with HCV genotype 1b. The mean age of patients was 49.5 ±9.6 years and BMI 27.7 ±3.8 kg/m^2. The histopathologic evaluation of liver tissue specimens according to NAS revealed < 3 points in 17 patients (simple steatosis, "not NASH"), 3-4 points in 22 patients ("borderline" NASH), and ≥ 5 points in 8 patients (definite NASH). When analyzed simultaneous appearance of steatosis, lobular inflammation and hepatocyte ballooning independently of their grades NASH was found in 11 patients. The detailed results of histopathological evaluation was shown in Table I. There were no significant differences in staging of fibrosis between "not NASH" patients and those with "borderline NASH" as well as between "borderline" and "definite NASH" subjects. The comparison of PCNA lobule expression in particular subgroups are collected in Tables II and III.

Proliferating cell nuclear antigen expression index reached 21.9 ±14.8 in whole analyzed group, 20.9 ±12.7 in the subgroup of patients with low grade steatosis and 10.8 ±7.0 in those with high grade of steatosis, respectively. When analyzed separately all steatosis grades a very evident

Table I. Results of histopathological examination of the liver tissue in analyzed CHC patients

Histopathological features	Patients	
	Number	%
Steatosis G 0/1/2/3	47 3/12/19/13	100
Portal inflammation Grades 1/2-4	9 9/0	19
Lobular inflammation Grades 1/2/3	17 10/5/2	36
Hepatocyte ballooning Grades 1/2	15 14/1	32
Fibrosis Stage 1A/1B/1C/2/3/4	33 3/1/13/10/2/4	70
Mallory bodies	0	0
NASH Definite/Uncertain (borderline)	30 8/22	64 17/47
NASH according to presence of steatosis, hepatocyte ballooning and lobular inflammation	11	23

Table II. Comparison of PCNA expression within lobules between patients with different steatosis, hepatocyte ballooning and lobular inflammation grades. Steatosis grade

Steatosis	Mean PCNA index	PCNA in areas within lobules with steatosis	PCNA in areas within lobules without steatosis
5-33% (grade 1)	15.3 ±8.4	4.7 ±5.5	28.9 ±14.7
34-66% (grade 2)	17.4 ±6.7	9.0 ±4.9	25.9 ±10.2
> 66% (grade 3)	5.1 ±6.0	5.2 ±3.3	5.0 ±10.0
Hepatocyte ballooning			
Grade 1		20.1 ±8.3	
Grade 2*		15.2 ±8.3	
Lobular inflammation			
Grade 1		18.8 ±10.6	
Grade 2		15.4 ±8.3	
Grade 3		14.8 ±7.3	

**Grade 2 was found only in one patient; PCNA – proliferating cell nuclear antigen.*

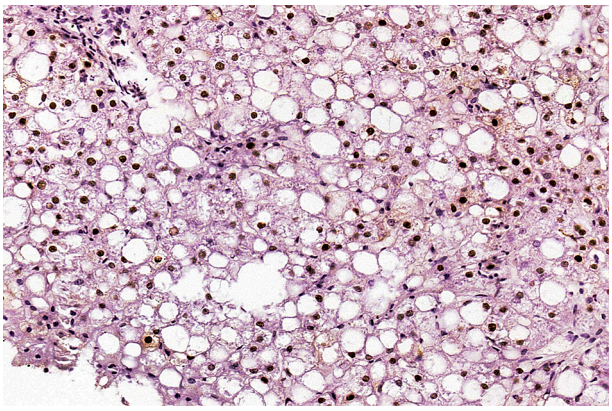

Fig. 1. NASH, high grade steatosis, a very low PCNA expression within ballooning hepatocytes and steatosis areas (magnification 100×)

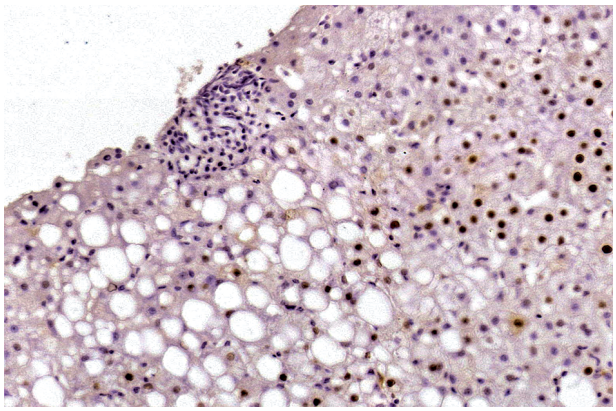

Fig. 2. Lobular inflammation, low PCNA expression in areas without steatosis (magnification 200×)

decrease in PCNA lobule expression was found in patients with the highest steatosis grade (grade 3, > 66% of hepatocytes affected) with mean value 5.1 ±6.0 (Table II, Figs. 1-4). Evident down-regulation of PCNA expression was observed in areas of hepatocyte ballooning independently of steatosis grade (Fig. 5).

Subsequently, PCNA expression was compared in arears of lobules with and without lipid droplets independently of general steatosis grade. In this case PCNA expression in the liver from patients with NAS<3 points appeared to be higher in areas within lobules affected with lipid droplets. Contrary, in patients with borderline and definite NASH PCNA expression was found to be up-regulated in lobule areas without lipid droplets (Tables III and IV, Fig. 6).

Proliferating cell nuclear antigen liver expression was significantly higher in patients with "not NASH" compared to those with defined NASH, both in lobule areas with and without steatosis. Despite, significant decrease of PCNA in lobule areas with steatosis in patients with "not NASH", it was still significantly

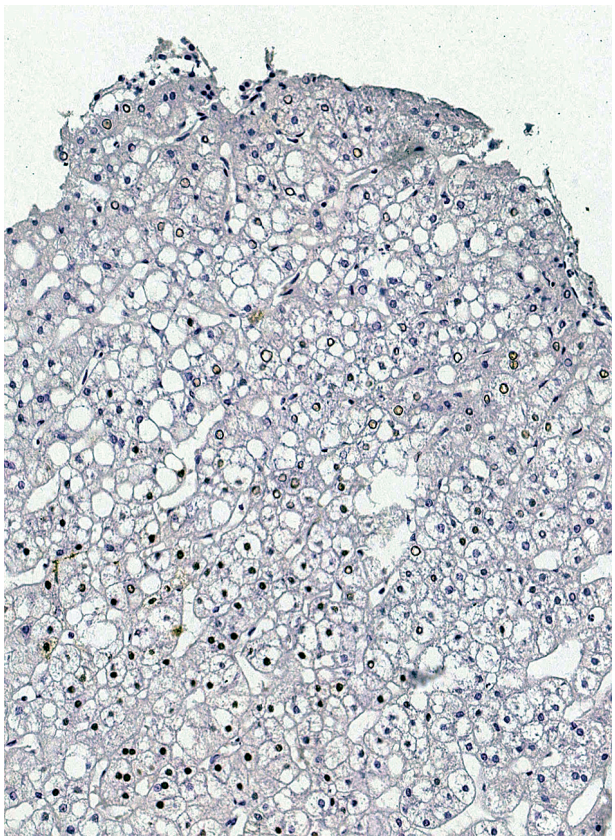

Fig. 3. High grade steatosis and diffuse hepatocytes ballooning, a very low expression of PCNA (magnification 100×)

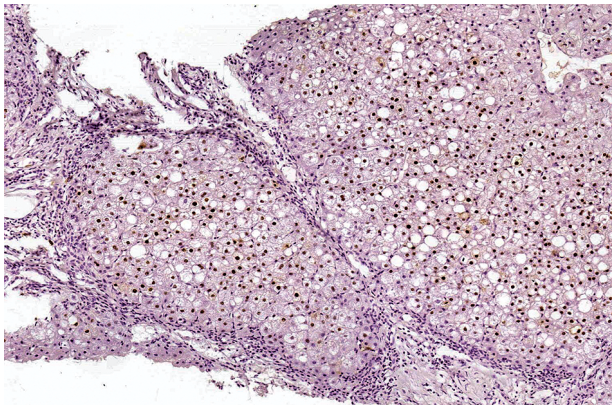

Fig. 4. Cirrhosis, low PCNA expression in lobular areas without steatosis and hepatocytes ballooning (magnification 100×)

higher than expression in such areas of subjects with definite NASH. Also, PCNA index was significantly up-regulated in patients with "not NASH" compared to those with defined NASH. Detailed results were described in Table III. As mentioned above, PCNA expression in patients with borderline and definite NASH was increased in lobule areas without steatosis. However, there was no difference when compared these two subgroups of patients regarding both PCNA index and PCNA expression in areas with and without steatosis (Table IV).

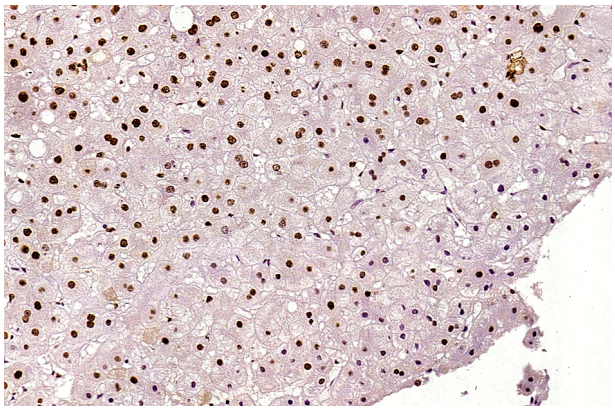

Fig. 5. Lobular areas without steatosis. Absent and a very low expression of PCNA in ballooning hepatocytes (magnification 200×)

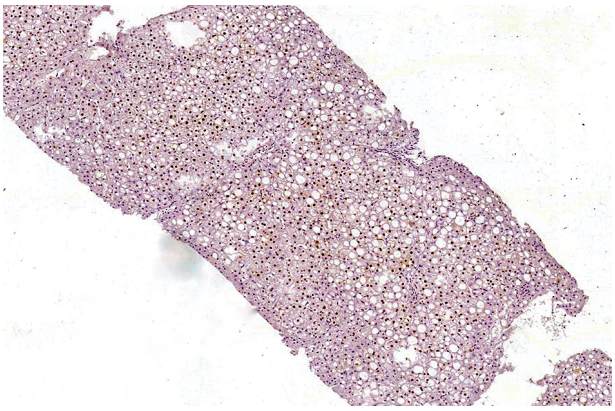

Fig. 6. Borderline NASH. Low PCNA expression in steatotic areas and high expression in hepatocytes without steatosis (magnification 50×)

Table III. Comparison of PCNA expression in lobules between patients without NASH and definite NASH (NASH defined according to NAS)

	NO NASH	DEFINITE NASH	P
Number of patients	17	8	
Mean fibrosis stage	2.1 ±1.2	2.5 ±1.0	> 0.05
PCNA in areas within lobules with steatosis	50.5 ±30.2	13.0 ±4.3	< 0.05
PCNA in areas within lobules without steatosis	34.3 ±14.5	20.1 ±15.3	< 0.05
Mean PCNA index	31.6 ±29.5	14.1 ±10.1	< 0.05

PCNA – proliferating cell nuclear antigen; NASH – nonalcoholic steatohepatitis; NAS – nonalcoholic fatty liver disease Activity Score.

Table IV. Comparison of PCNA expression in lobules between patients with "borderline" NASH and definite NASH (NASH defined according to NAS)

	BORDERLINE NASH	DEFINITE NASH	P
Number of patients	22	8	
Mean fibrosis stage	2.1 ±1.1	2.5 ±1.0	> 0.05
PCNA in areas within lobules with steatosis	7.5 ±20.8	13.0 ±4.3	> 0.05
PCNA in areas within lobules without steatosis	26.8 ±13.2	20.1 ±16.2	> 0.05
Mean PCNA index	19.3 ±12.3	14.1 ±10.1	> 0.05

PCNA – proliferating cell nuclear antigen; NASH – nonalcoholic steatohepatitis; NAS – nonalcoholic fatty liver disease Activity Score.

Table V. Comparison of PCNA expression in lobules between patients with NASH and without NASH. Nonalcoholic steatohepatitis defined as the presence of steatosis, hepatocyte ballooning and lobular inflammation

	NASH	NO NASH	P
Number of patients	11	36	
Mean fibrosis stage	2.7 ±1.1	2.2 ±1.1	> 0.05
PCNA in areas within lobules with steatosis	7.8 ±4.3	12.3 ±14.1	> 0.05
PCNA in areas within lobules without steatosis	13.0 ±16.2	30.0 ±14.2	< 0.05
Mean PCNA index	13.1 ±10.4	21.2 ±16.8	> 0.05

Additional analysis assessed the difference in PCNA expression between patients with NASH defined as simultaneous appearance of steatosis, lobular inflammation and hepatocyte ballooning independently of their grades and those without NASH. Macrovesicular steatosis in particular in severe form is associated with an increase in lobular size/diameter. Considering the above fact, in addition to the areas of steatosis, areas without steatosis were also evaluated independently in lobules. As shown in Table V the PCNA expression values in these fields was significantly lower in patients with NASH morphological features compared to those without NASH. The difference disappeared in areas with steatosis.

Steatosis grade was found to be negatively associated with PCNA expression in areas of steatosis within lobules ($r = [-0.41]$; $p < 0.05$).

Discussion

Some studies focused on the expression of PCNA in chronic viral hepatitis showed that there was a correlation between its intensity and the values of hepatitis activity index (HAI). Hamada *et al.* showed that the PCNA index increased alongside with the progression of the liver disease in CHC patients [16]. Authors described the role of hepatocyte proliferation in the development of liver cirrhosis and hepatocellular carcinoma (HCC). High proliferative rate of hepatocytes subject to the persistent liver cell injury in chronic active hepatitis may be related to a reconstruction pattern of the liver in cases of progression to cirrhosis and development of HCC. Similar results were showed by Nakamura *et al.* [17]. PCNA index in hepatocytes in chronic hepatitis B (CHB) and CHC revealed a significant relationship with HAI score, suggesting a contribution of lobular hepatocyte necrosis and/or portal inflammation to the regenerative rate of hepatocytes. In another report higher expression of PCNA was found in patients with elevated alanine aminotransferase (ALT) activity when compared to those with ALT activity in normal range [18]. There are no studies evaluating the proliferative potential of hepatocytes in the presence of morphological features of metabolic disorders in the liver such as steatosis and hepatocyte ballooning in CHC patients. Our study showed that there is a relationship between expression of PCNA and the extent of steatosis, with very evident depletion in severe steatosis. Down-regulation of PCNA expression was observed in areas of hepatocyte ballooning independently of steatosis grade. Hepatocyte ballooning results from energetics disorders within hepatocytes which lead to their decreased proliferative potential.

PCNA as a central component of DNA replication machinery is widely used as a marker of proliferation, however, today its role is understood much wider. It is able to cooperate with molecules other than polymerases and takes part in DNA repair and cell-cycle control, cell survival, genes transcription and epigenomic maintenance [19, 20]. The cell expression of PCNA is significantly elevated during the S and G2 phases of the cell cycle, but is very low in quiescent cells [21]. The question remains, which molecular factors trigger its activity in the liver of CHC patient. Animal experiment revealed that the HCV core and myc-F proteins could induce hepatocyte proliferation in the transgenic mice possibly through β-catenin signaling pathway [22]. Core protein is the most highly conserved of all the HCV proteins which can interact with a wide range of viral and cellular proteins [9]. Many of these interactions result in hepatocyte metabolic disturbances and lipid accumulation which can in turn contribute to the reprogramming of cell growth and hepatic steatosis [23, 24]. In addition, the HCV core protein may interact with transcriptional regulator, retinoid X receptor (RxRα), that controls many aspects of cell proliferation [25]. Our results revealed significantly higher PCNA expression in the HCV infected livers with the lowest grade of steatosis. This effect diminished along with increasing steatosis grade, NAS value and fibrosis stage. Proliferating cell nuclear antigen expression decreased in patients with NASH, defined according to NAS, both in steatotic and non-steatotic areas of lobules. Significant PCNA expression decrease in lobules areas without steatosis was also observed when NASH was assessed as simultaneous appearance of steatosis, lobular inflammation and hepatocyte ballooning independently of their grades. Pointing to the negative relationship between steatosis and PCNA expression, low grade of steatosis in some patients qualified to NASH according to the presence of all its hallmarks may results in the lack of significant decrease of PCNA expression in steatotic areas. However, the relatively low PCNA expression in steatotic areas may suggest that in the case of NASH proliferative activates are also impaired in non-steatotic hepatocytes. The study showed high PCNA expression in hepatocytes localized in steatotic areas in patients without NASH, while PCNA evidently declined in areas of steatosis in those with NASH. These results suggest that simple steatosis does not affect proliferative abilities of hepatocytes. However, evident lobular inflammatory process co-existing with steatosis reduce proliferative potential of hepatocytes both in steatotic and non-steatotic areas, decreasing regenerative abilities of hepatocytes independently of HCV infection. Additional aspect of impaired proliferative potential of hepatocytes in inflammed area with steatosis may be associated with hypoxia requiring new blood vessels formation [5, 26]. To confirm our suggestion the study of a larger group of CHC

patients need to be carried out, together with analysis of expression of additional molecules involved in cell growth and proliferation. Lowering PCNA expression in areas of more advanced steatosis and hepatocyte ballooning may suggest decreasing proliferative and regenerative abilities of hepatocytes affected with oxidative stress and mitochondrial impairment. These observations also explain, at least partially, the negative impact of steatosis and hepatocyte ballooning on fibrosis progression. Finally, we suggest that determination of PCNA expression may be an independent marker in determining the severity of hepatocyte metabolism changes in CHC patients.

In conclusion expression of PCNA was significantly lower in NASH patients compared to those with simple steatosis, but not to those with borderline NASH. Advanced steatosis negatively influenced PCNA expression. Nonalcoholic steatohepatitis not only affects PCNA expression in staetotic, but also in non-steatotic lobule areas. Expression on PCNA could be an independent indicator of changes in hepatocyte metabolism in CHC patients. High values of NAS and the low expression of PCNA seem to be a negative prognostic factor in predicting the further course of the disease.

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The authors declare no conflict of interest.

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