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# AgNORs, PCNA and Histologic Activity Index in Chronic Liver Disease

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**Abstract:** Chronic hepatitis is a clinical and pathological syndrome, which has several causes and it is characterized by varying degrees of hepatocellular necrosis and inflammation. Several systems have been proposed to evaluate the diagnosis, grading and staging of chronic hepatitis. A histologic activity index (HAI) has been developed which generates a numerical score for liver biopsy specimens obtained from patients with chronic hepatitis. The aim of this study was to evaluate the correlation between HAI and proliferative activity markers, such as PCNA and AgNORs in biopsy specimens obtained from patients with chronic liver disease including chronic hepatitis and cirrhosis. Mean AgNOR counts and HAI were significantly higher in the chronic active hepatitis group than in the chronic persistent hepatitis group ($P<0.001$). There was a correlation between mean AgNOR values and HAI ($p<0.01$). There was no correlation between PCNA LI with conventional histologic classification, HAI and AgNOR counts ($P>0.05$). We suggest that, in contrast to PCNA, AgNORs may be a useful parameter in the histologic evaluation, like HAI.

**Key Words:** AgNORs, PCNA, Histologic activity index, chronic liver disease.

## Introduction

Chronic hepatitis is a clinical and pathological syndrome, which has several causes and it is characterized by varying degrees of hepatocellular necrosis and inflammation. Several systems have been proposed to evaluate the diagnosis, grading and staging of chronic hepatitis (1–4). A histologic activity index (HAI) has been developed which generates a numerical score for liver biopsy specimens obtained from patients with chronic hepatitis by Knodell et al (5). This consists of four separate scores for different components (periportal±bridging necrosis, intralobular degeneration and focal necrosis, portal inflammation, and fibrosis) of the lesions. The evalutation of HAI involves some disadvantages. The assessment of scores is subjective and intraobserver and interobserver variability may exist (6).

Proliferating cell nuclear antigen (PCNA) is an auxillary protein for DNA polymerase delta and has been recognized as an endogenous histologic marker for $G_1$/S–phase in the cell cycle (7). There may be a close correlation between PCNA and cell proliferation.

Nucleolar organizer regions (NORs) are structures of central importance in the transcription of DNA to ribosomal DNA (8). Silver stained NORs, reffered to as AgNORs, appear as black dots in the nucleus (9) and it has been suggested that the number of AgNOR nuclear dots may reflect the proliferative activity of cells (10).

The aim of this study was to evaluate the correlation between HAI and proliferative activity markers, such as PCNA and AgNORs in biopsy specimens obtained from patients with chronic liver disease including chronic hepatitis and cirrhosis.

## Materials and Methods

Fifty–four biopsy specimens obtained from patients with chronic hepatitis/cirrhosis and 4 necroscopy specimens obtained from normal tissues were studied. Serum HBsAg/HBeAg was positive in 24 patients, Anti–HCV was positive in 24 patients and 4 patients were both HBsAg/HBeAg and Anti–HCV positive.

Tissues were fixed in 10% formalin, embedded in paraffin wax, and 5 µm thick sections were routinely

stained with hematoxylin and eosin (H&E). The biopsy specimens with chronic liver disease were classified according to the International Group (3): chronic persistent hepatitis, chronic lobular hepatitis and chronic active hepatitis. Chronic active hepatitis was divided into three subgroups: mild, moderate and severe (1). Cirrhosis was divided into two groups according to the histologic inflammatory activity; when no significant histologic inflammatory activity was evident, it was accepted as inactive cirrhosis (2). Modified Knodell score was used for the assessment of HAI (2, 4).

Five micrometer paraffin–embedded liver sections were immunostained with the streptavidin–biotin alkaline phosphatase system (Biogenex) for PCNA (PCNA–PC10, monoclonal antibody, mouse, Biogenex). Citrate solution (Biogenex) was used for antigen retrieval with 5+5 minutes in a microwave (11) before immunostaining. Fast red was used as chromogen and nuclei that had reacted with the monoclonal antibody against PCNA were stained pink to red. The extent of PCNA positivity was evaluated by determining the percentage of positive nuclei present in 1000 cells and this percentage was expressed as Labelling Index (LI).

For AgNOR staining 3 μm sections were cut from paraffin–embedded biopsy specimens and these sections were dewaxed and rehydrated. The colloidal silver staining solution was prepared by dissolving 2% gelatin in 1% aqueous formic acid, which was then mixed in a ratio of 1:2 by volume with 50% aqueous silver nitrate. The staining was done at room temperature in darkness for 40 min. After rinsing the sections with distilled water they were dehydrated and mounted without any counterstaining. The AgNOR sites were counted in at least 200 randomly selected hepatocytes, using a X1000 objective and AgNORs were identified as recommended by Crocker et al (12). The mean number of AgNORs/nucleus was used for the analysis.

The results were expressed as mean values±standard error of mean (SEM). Pearson coefficient of correlation and Student's test were performed and a p value less than 0.05 was considered statistically significant.

## Results

The histological findings of the biopsy specimens form 54 patients with chronic liver disease were classifed as follows: chronic persistent hepatitis 14, chronic lobular hepatitis 1, chronic active hepatitis 27 (mild 7, moderate 8, severe 12) and cirrhosis 12 (active 8, inactive 4). The mean HAI, PCNA LI and AgNOR values of the patients according to conventional histologic classification are summarized in the table.

The HAI score and AgNOR values were correlated with the conventional histological classification as shown in the table. The mean AgNOR values and HAI score progressively increased from normal lives tissue to active cirrhosis. The mean AgNOR counts and HAI were significantly higher in the chronic active hepatitis group than in the chronic persistent hepatitis group ($P<0.001$). The mean AgNOR value and HAI score of the patients with inactive cirrhosis were 11.27 and 4.25, respectively. There was a correlation between mean AgNOR values and HAI ($P<0.01$, Table 1).

There was no correlation between PCNA LI with conventional histologic classification, HAI and AgNOR counts ($P>0.05$, Table 1).

The representative examples of AgNOR and PCNA staining are shown in Figures 1 and 2, respectively.

Table 1. HAI score, AgNOR counts and PCNA LI in chronic hepatitis and cirrhosis.

| | n | HAI score | AgNOR | PCNA LI (%) |
|---|---|---|---|---|
| Normal | 4 | 0 | 5.85±0.15 | 0.62±0.12 |
| CPH | 14 | 4.36±0.43 | 8.32±0.35 | 11.46±3.37 |
| CLH | 1 | 5 | 8.84 | 2.30 |
| CAH | | | | |
| mild | 7 | 7.71±0.18 | 9.47±0.64 | 8.10±1.54 |
| moderate | 8 | 9.37±0.18 | 10.32±0.43 | 14.68±3.81 |
| severe | 12 | 11.25±0.25 | 11.50±0.40 | 10.00±1.89 |
| Cirrhosis | | | | |
| active | 8 | 13.25±0.88 | 11.86±1.38 | 16.51±4.08 |
| inactive | 4 | 4.25±0.25 | 11.27±1.81 | 4.15±1.53 |

CPH : Chronic persistent hepatitis, CLH: Chronic lobular hepatitis, CAH: Chronic active hepatitis. Results are expressed as mean±standard error of mean.

## Discussion

The correlation between proliferative activity and AgNORs in human diseases has been shown by many authors. It has also been suggested that the counting of AgNORs may be useful both for discriminating between malign and benign tumors and for estimating histologic tumor grades in a wide range of malign neoplasms (8, 9, 13–16). The prognastic importance of AgNOR counting in human liver disease has also been emphasized (13, 17, 18). There are few studies evaluating AgNOR counting in

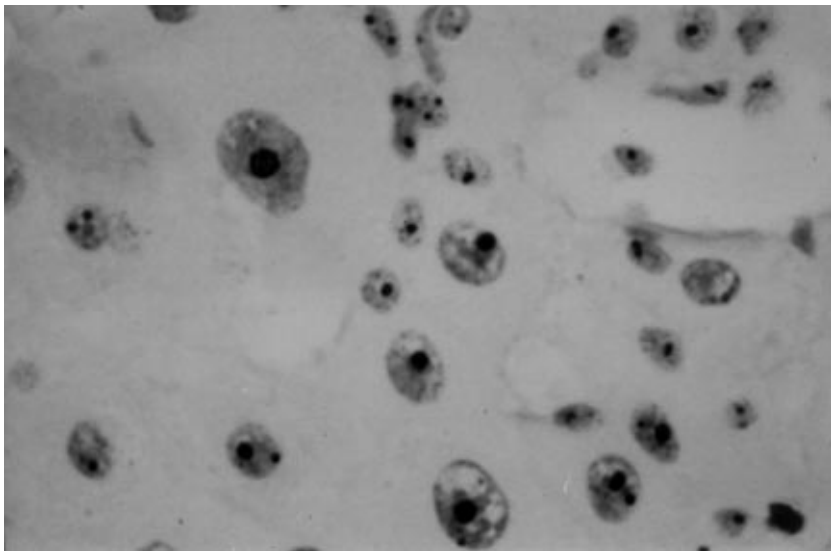

Figure 1. AgNOR staining of hepatocytes in chronic liver disease (x1000).

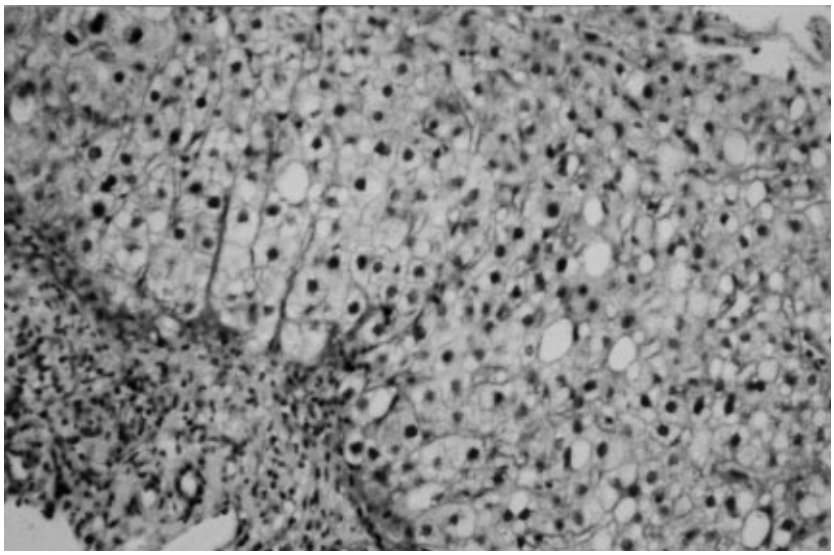

Figure 2. Intranuclear immunostaining for PCNA, chronic active hepatitis, positive (red), negative (blue) (Fast Redx200).

human liver disease (13–15, 19) and the results are contradictory. In the study of Crocker et al (14), the mean AgNOR values in normal, cirrhotic and carcinomatous livers were found to be significantly different and similar in adenoma and chronic active hepatitis respectively. In their study, there was no overlap between the ranges of AgNOR counts in normal, cirrhotic and malignant liver specimens, and quantitation of staining for AgNORs may be a diagnostically useful method in liver disease. Zalatnai et al (13) have concluded that AgNOR counts reflect only the proliferative activity of a given cell population and they cannot discriminate between the hyperplastic, benign and neoplastic lesions in liver disease. Shiro et al (20) have suggested that AgNORs may be useful for evaluating the progress of hepatocellular carcinoma. Derenzini et al (16) have reported that high AgNOR values are associated with an increased risk of hepatocellular carcinoma in patients with chronic liver disease.

As regeneration or repair is one of the consequences of inflammation, the regenerative growth rate of hepatocytes could be critical for the prognosis of chronic liver disease and the degree of inflammation may predict the regenerative growth rate. To our knowledge, the correlations between AgNOR values with HAI and PCNA in chronic liver disease have not been studied previously. Our study showed that there is a correlation between mean AgNOR counts and HAI in chronic liver disease, including chronic persistent hepatitis, chronic active hepatitis and active cirrhosis. Because assessment of HAI

does not give any information about proliferative activity in chronic liver disease, AgNOR counts may be a useful parameter in the evaluation of liver biopsies. AgNOR staining is a simple procedure and its price is low; these may be additional advantages.

Immunostaining of PCNA involves some difficulties. Monoclonal antibodies such as PC10, 19A2, and 19F4 may be used in the evaluation of PCNA (21–23). The experience with 19A2 and 19F4 monoclonal antibodies is limited and most of the commercially available monoclonal antibody for PNCA assessment is PC10 and it is the most common antibody used in the literature. PCNA (PC10) is fixation dependent and its positivity in proliferating cells is reduced after 48 hours fixation and is almost eliminated after 72 hours (24). Antigen retrieval form fixed material using protease treatment and microwave heating is an alternative method to increase the sensitivity of PC10 (25). The number of studies evaluating a correlation between PCNA LI and inflammatory activity in chronic liver disease is limited (26, 27). Nakamura et al (26) have shown that PCNA LI of hepatocytes in chronic persistent hepatitis had a significant relationship with HAI scores and did not exceed 3.0%. Our results did not show any relationship between PCNA LI with conventional histological diagnosis, HAI score and AgNOR values in patients with chronic liver disease.

We suggest that, in contrast to PCNA, AgNORs may be a useful parameter in the histologic evaluation and response to the treatment in chronic liver disease, like HAI.

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