*Turk J Gastroenterol 2005; 16 (1): 12-16*

# P21 expression and its relation to disease activity and hepatocyte proliferation in chronic hepatitis B virus infection

## Kronik hepatit B virüs infeksiyonunda P21 proteininin hastalık aktivitesi ve hepatosit proliferasyonu ile ilişkisi

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***Background/aims:*** *P21 protein, a cell cycle regulatory protein expressed in the liver, acts as an inhibitor of cyclin dependent kinase and prevents progression of the cell cycle. In the present study, our aim was to investigate the relationships between P21 protein expression and hepatocyte proliferation, hepatitis B virus replication, and hepatitis activity.* ***Methods:*** *A total of 66 patients with chronic hepatitis B without cirrhosis were included in the study. These patients were evaluated in three different groups according to the degree of viral replication and the disease activity. Group 1: HBeAg-positive patients with active liver disease and with viral replication, group 2: HBeAg-negative patients with active liver disease and with viral replication, and group 3: HBeAg-negative inactive carriers. P21 and proliferating cell nuclear antigen were immunohistochemically stained and a labeling index was calculated for each protein.* ***Results:*** *A total of 32 (48.4%) patients were positive for nuclear P21 expression. All three groups had a similar P21 index. proliferating cell nuclear antigen-labeling index, hepatit B virus DNA levels, ALT levels, and HAI scores were not different in patients with and without P21 staining. Spearman's correlation analysis found no correlation between P21 staining and ALT and hepatit B virus DNA levels, HAI score and proliferating cell nuclear antigen-labeling index.* ***Conclusions:*** *These results suggest that the pattern of P21 expression is not associated with histological activity, hepatocyte proliferation and virus replication in patients with well-compensated chronic hepatitis B.*

**Key words:** P21 expression, cell cycle, hepatocyte proliferation, viral replication, hepatitis B virus

***Amaç:*** *P21 proteini, siklin bağımlı kinazları inhibe ederek hücre siklüsünün progresyonunu önleyen ve karaciğerde eksprese olan bir hücre siklüsü düzenleyici proteinidir. Biz bu çalışmada P21 protein ekspresyonu ile hepatosit proliferasyonu, hepatit B virüs replikasyonu ve hepatit aktivitesi arasında ilişki olup olmadığını sınamayı amaçladık.* ***Yöntem:*** *Çalışmaya siroz olmayan 66 kronik hepatit B hastası alındı. Bu hastalar viral replikasyon derecesi ve hastalık aktivitesine göre 3 gruba ayrılarak değerlendirildi. 1. grupta HBeAg pozitif ve aktif karaciğer hastalığı olanlar, 2. grupta HBeAg negatif ve aktif karaciğer hastalığı olanlar, 3. grupta HBeAg negatif ve inaktif taşıyıcılar bulunuyordu. P21 ve proliferating cell nuclear antigen boyamaları immünhistokimyasal yöntemle yapıldı ve her birinin labelling index olarak adlandırılan boyanma indeksleri hesaplandı.* ***Bulgular:*** *Toplam 66 hastanın 32'sinde (%48.4) P21 nükleer boyanması pozitifti. Tüm gruplar P21 boyanma indeksleri yönünden benzerdi. P21 boyanan ve boyanmayan hastalar arasında proliferating cell nuclear antigen boyanma indeksi, hepatit B virüs DNA düzeyleri, ALT düzeyleri ve HAİ skorları açısından fark yoktu. Spearman korelasyon analizi yapıldığında P21 boyanma durumu ile ALT düzeyleri, hepatit B virüs DNA düzeyleri, HAİ skorları ve proliferating cell nuclear antigen boyanma indeksleri arasında korelasyon bulunmadı.* ***Sonuç:*** *Bu çalışmanın sonucunda, kronik hepatit B infeksiyonlu hastalarda P21 ekspresyonu ile histolojik aktivite, hepatosit proliferasyonu ve virüs replikasyonu arasında ilişki saptanmadı.*

**Anahtar kelimeler:** P21 ekspresyonu, hücre siklüsü, hepatosit proliferasyonu, viral replikasyon, hepatit B virüs

## INTRODUCTION

Chronic hepatitis B infection is a major risk factor for the development of cirrhosis and hepatocellular carcinoma (1). Recurrent immune attacks against infected hepatocytes and continuing necroinflammation are thought to cause increased hepatocyte proliferation and liver cancer development. On the other hand, maintaining the liver mass and function by regenerating hepatocytes is criti-

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**Manuscript received:** 05.10.2004 **Accepted:** 14.12.2004

cal. In fact, diminished hepatocyte proliferation has been associated with a poor prognosis in liver diseases, especially in the setting of fulminant hepatic failure (1). Thus, the regulation of hepatocyte proliferation may be important in determining the outcome of any type of liver injury.

Current studies have suggested that hepatocyte cell cycle may also determine replication efficacy of hepatitis B virus (HBV) and thus may be involved in the pathogenesis of HBV-related liver disease. Likewise, HBV replication is increased in quiescent hepatocytes in the absence of liver injury and hepatocyte proliferation; it is reduced in patients with increased inflammatory activity and cell turnover (2, 3). Therefore, understanding the regulation of hepatocyte proliferation and the factors interacting with cell cycle in HBV infection may be crucial.

The regulation of hepatocyte proliferation is not completely understood (4). Cyclin and cyclin-dependent kinase (CDK) complexes are known to play a key role in cell proliferation and differentiation (5). Current studies have shown that P21, a cyclin-CDK inhibitor, plays an important regulatory role in hepatocyte proliferation (6, 7). P21 protein prevents entry into S phase by inhibiting CDK complexes and directly inhibits DNA replication by inhibiting proliferating cell nuclear antigen (PCNA) (7, 8).

In this study, P21 expression pattern was investigated in patients with chronic HBV infection. The relationships between P21 expression, hepatocyte proliferation, viral load, and inflammatory activity were also evaluated.

## MATERIALS AND METHODS

In this retrospective study, a total of 66 patients (39 male, 27 female; mean age 36.3±10.4, range: 15 to 62 years) with chronic HBV infection were studied. None of the patients had antibodies against hepatitis D (anti-HDV), hepatitis C (anti-HCV), or HIV (anti-HIV), and there was no biochemical, clinical, or histological evidence of other causes of chronic liver injury such as metabolic or autoimmune liver disease. Weekly alcohol consumption was less than 40 g in all patients.

These patients were divided into three groups. Group 1 patients were HBeAg-positive patients with active liver disease (high ALT levels and active liver histology) and with detectable HBV DNA by a hybridization assay. Group 2 consisted of HBeAg-negative patients with active liver disease with detectable HBV DNA. Group 3 patients were HBeAg-negative inactive carriers (IC) with normal ALT levels and undetectable HBV DNA by the same assay.

### Hepatitis Serology and HBV DNA Assay

HBsAg, antibody against hepatitis B surface antigen (anti-HBsAg), HBeAg, anti-HBe, anti-HDV, anti-HCV, and anti-HIV were measured by enzyme immunoassay (Abbott Laboratories, Chicago, IL). HBV DNA levels were determined by a liquid hybridization assay (Digene, Gaithersburg, MD). Reference values of HBV DNA assay were 5 to 2000 pg/ml.

### Evaluation of Liver Biopsy Specimens

Liver biopsy specimens were fixed in 10% formaldehyde and embedded in paraffin. After routine tissue processing, the histological diagnosis was made on the H&E stained sections. Histopathological findings were evaluated and numerically scored according to the histological activity index (HAI) scoring system proposed by Knodell et al. (9).

### Immunohistochemical Detection of P21 Protein and Proliferating Cell Nuclear Antigen

4-6-μm paraffin sections were obtained from each liver biopsy specimen for immunohistochemical analysis. The expressions of PCNA and P21 were studied by avidin-biotin immunoperoxidase method [mouse anti-PCNA and mouse anti-P21 (WAF1), Neomarkers, Fremont, CA]. Nuclear labeling indexes (LI) for PCNA and P21 were determined by random evaluation of at least 1000 hepatocytes with distinct nuclei. Positive and negative controls were also included for each antibody staining. Assessment of immunohistochemical staining was performed by a single pathologist who was blind with regard to the clinical and histological diagnosis.

### Statistical Methods

All data were analyzed using the SPSS statistical software. Mann-Whitney U test, Kruskal-Wallis test, chi-square, and Spearman's correlation tests were used where appropriate and a p value of $<0.05$ was accepted as significant.

## RESULTS

Clinical characteristics of patients are shown in (Table 1). Group 1 patients were younger than group 2 and 3 patients ($p<0.05$). Similar sex distribution with female predominance was present in all

**Table 1.** Clinical characteristics of the patient groups. Mann-Whitney U tests were used for group compaisons

| | N | % | Age (years)* | Sex female | Sex male | HbeAg | ALT (U/L)* | HBV DNA (pg/ml)† | HAI* |
|---|---|---|---|---|---|---|---|---|---|
| Group 1 | 27 | 40.9 | 30±12 | 22 | 5 | positive | 97±60 | >2000 | 6.8±3.8 |
| Group 2 | 22 | 33.3 | 42±8 | 13 | 9 | negative | 135±132 | 100 | 9.4±4.1 |
| Group 3 | 17 | 25.8 | 37±11 | 13 | 4 | negative | 16±4 | <5 | 2±1.1 |

HAI: Histological activity index
*Mean (±SD)
†Median

**Table 2.** PCNA and P21 indexes of patient groups. Mann-Whitney U tests were used for comparisons. Data were expressed as mean (±SD)

| | PCNA(LI) | P21(LI) | p values |
|---|---|---|---|
| Group 1 | 0.163±0.140 | 0.083±0.130 | NS |
| Group 2 | 0.301±0.240 | 0.048±0.111 | NS |
| Group 3 | 0.277±0.323 | 0.03±0.06 | NS |

LI: Labeling index; PCNA: proliferating cell nuclear antigen

groups. (Table 2) shows the PCNA and P21 labeling indexes of the groups. PCNA and P21 indexes were similar in all three groups.

### Relationship between nuclear P21 expression (P21-LI) and disease activity

A total of 32 (48.4%) patients were positive for nuclear P21 expression. Total HAI scores (inflammation and fibrosis) as well as inflammatory activity score were not different in patients with or without P21 expression (Table 3). In addition, when HAI of 8 or greater was accepted as the cut-off value for significant liver injury, P21 expression was not different between patients with low activity (HAI<8) and with significant activity (HAI>8) (Table 4). ALT levels were also similar in patients with and without P21 staining. Correlation analysis did not show a correlation between P21 indexes, HAI scores and ALT levels.

**Table 3.** Comparison of disease activity (ALT and HAI), hepatocyte proliferation (PCNA) and viremia level (HBV DNA) in patients with and without P21 positivity. Chi-square test was used for comparisons

| | N | % | ALT (U/L) * | HBVDNA (pg/ml) † | PCNA (LI)* | HAI* |
|---|---|---|---|---|---|---|
| P21 (+) | 32 | 48.4 | 100±129 | 198,5 | 0.219±0.246 | 6.5±4.2 |
| P21 (-)‡ | 34 | 51.6 | 79±50 | 73,0 | 0.256±0.230 | 6.4±4.8 |

LI: Labeling index; PCNA: Proliferating cell nuclear antigen; HAI: Histological activity index
* Mean (±SD)
† Median
‡ P21 (-): None of the hepatocytes is stained for P21

### Relationship between nuclear P21 expression and the degree of hepatocyte proliferation (PCNA-LI)

PCNA labeling indexes were similar in patients with and without P21 staining (Table 3). In addition, no correlation was found between P21 and PCNA labeling indexes.

### Relationship between nuclear P21 expression and viral load

Patients with and without P21 staining had similar HBV DNA levels (Table 3). P21 index did not correlate with HBV DNA level.

**Table 4.** P21 and PCNA expressions according to the activity of liver disease. HAI > 8 was accepted as significant liver injury. Chi-square tests were used for comparisons

| | PCNA(LI) | P21(LI) | p value |
|---|---|---|---|
| HAI≤8 | 0.235±0.255 | 0.019±0.052 | NS |
| HAI>8 | 0.243±0.209 | 0.103±0.152 | NS |

LI: Labeling index; PCNA: Proliferating cell nuclear antigen; HAI: Histological activity index
Mean (±SD)

## DISCUSSION

Hepatocyte proliferation is a response to liver injuries caused by viruses, toxins, and partial hepatectomy, and plays an important role in maintaining hepatic function in chronic liver diseases (2). P21, as a regulator of hepatocyte proliferation, inhibits CDKs and PCNA and thus acts as a cell cycle inhibitor (4, 8). In the present study, the expression pattern of P21 in liver biopsy specimens and its relation to hepatocyte proliferation (PCNA index) were investigated in patients with chronic hepatitis B. Because of a possible control of HBV replication by hepatocyte cell cycle, we also aimed to evaluate the relationships between P21 expression, histological activity and viral load.

In our study, there was no relationship between P21 expression and hepatic inflammatory activity and hepatocyte proliferation. P21 protein is not

only expressed in patients with hepatocellular carcinomas but also in patients with non-tumoral liver diseases such as nonalcoholic steatohepatitits, chronic hepatitis C infection and chronic hepatitis B infection (10). The results of previous reports suggested that P21 expression correlates with the degree of liver injury, inflammation and liver regeneration (4, 10, 11). The absence of such a correlation in our study may be related to the fact that our study included only chronic hepatitis B patients. However, different mechanisms may play roles in the pathogenesis of liver diseases in different etiologies such as HCV, alcoholic and nonalcoholic steatohepatitis and autoimmune liver disease. Therefore, expression patterns of cell cycle regulators such as P21, P53, and PCNA may vary according to the specific etiology. In fact, Wagayama et al. reported that the expression level of P21 protein in chronic liver disease with type-C hepatitis is significantly higher than in those with type-B hepatitis (11). Furthermore, Crary and Albrecht reported low P21 expression in nonalcoholic steatohepatitis (4). Therefore, further studies comparing P21 expression pattern in patients with liver disease caused by different etiologies are needed. In addition, relationships between P21 expression and inflammatory activity and hepatocyte proliferation need to be determined in patients with liver disease caused by different etiologies.

We did not include cirrhotic patients in our study to compare hepatocyte proliferation (PCNA) and P21 expression. Ongoing necro-inflammation results in fibrosis and nodular regeneration, namely liver cirrhosis, in most types of chronic liver diseases. Cell proliferative activity is expected to be higher in cirrhotic patients compared to non-cirrhotics. In parallel, a cell cycle regulatory protein (e.g. P21) could be more remarkably takes place in the control of cell cycle. The results of the present study reflect the relationship between P21 protein and the inflammatory activity in the absence of advanced liver disease. It should be kept in mind that the expression pattern of P21 and its relation to hepatocyte proliferation may be completely different in more advanced liver disease. This can also explain the different results obtained in different studies, since some studies included the patients with more advanced liver disease when compared to our study.

We found no correlation in this study between P21 expression and HBV replication. To date, there have been no reports investigating a possible association between P21 expression and HBV replication. Current studies have reported that replication of HBV has been associated with hepatocyte cell cycle (2). Our previous study also showed that replication of HBV may be affected by hepatocyte proliferative activity (3). Absence of a relationship between P21 expression and viral load is not surprising since hepatocyte proliferation is not solely controlled by a single regulatory protein (e.g. P21). Complex interacting mechanisms regulate cell cycle and cell proliferation. Causes and type of liver injury, stage of the disease, several other host factors and presence of other concomitant factors may also have an impact.

In conclusion, expression pattern of P21 protein is not correlated with liver injury, hepatocyte proliferation or virus replication in patients with chronic hepatitis B infection. Because of the complexity of liver cell injury and cell cycle regulation, further investigations dissecting each factor are needed. In this respect, our study provides evidence that there is no association between P21 expression, hepatocyte injury and proliferation in early stages of chronic hepatitis B. Further studies including patients with more advanced disease may provide more evidence of a possible role of cell cycle regulators in the pathogenesis of liver injury.

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