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ORIGINAL ARTICLE

Differential expression of cell cycle regulators in HCV-infection and related hepatocellular carcinoma

Azza E El Bassiouny, Mona M Nosseir, Mona K Zoheiry, Noha A Ameen, Ahmed M Abdel-Hadi, Ibrahim M Ibrahim, Suher Zada, Abdel-Hakeem Saad El-Deen, Nora E El-Bassiouni

Azza E El Bassiouny, Mona K Zoheiry, Department of Immunology, Theodor Bilharz Research Institute, PO Box 30 Imbaba, Giza 12411, Egypt
Mona M Nosseir, Ahmed M Abdel-Hadi, Department of Pathology, TBRI, PO Box 30 Imbaba, Giza12411, Egypt
Noha A Ameen, Central Laboratory, Theodor Bilharz Research Institute, PO Box 30 Imbaba, Giza 12411, Egypt
Ibraheim M Ibrahim, Department of Hepato-Gastroenterology, Theodor Bilharz Research Institute, PO Box 30 Imbaba, Giza 12411, Egypt
Suher Zada, Biology Department, American University, PO Box 74, New Cairo 11835, Egypt
Abdel-Hakeem Saad El-Deen, Zoology Department, Faculty of Science, Cairo University, Giza 12613, Egypt
Nora E El-Bassiouni, Department of Hematology, Theodor Bilharz Research Institute, PO Box 30 Imbaba, Giza 12411, Egypt
Author contributions: El Bassiouny AE and El-Bassiouni NE: construction of the plan and design of work, interpretation and discussion of results, writing and revising the manuscript and financial support; Nosseir MM: interpretation and discussion of the results of histopathological and Immunohistochemical studies, close supervision of the different sets of immunohistochemistry, writing and revising the text; Zoheiry MK: help in performance of laboratory investigations, collection of data, writing and revising the manuscript; Ameen NA: collection of data, help in interpretation and discussion of the results. Performance of laboratory investigations and immunohistochemical staining; Abdel-Hadi AM: scientific interpretation of results of liver biopsies; Ibraheim IM: Providing clinical data and liver biopsy specimens; Zada S: scientific and financial support; Saad El-Deen AH: scientific supervision of the work.
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Correspondence to: Dr. Mona M Nosseir, Professor of Pathology, Pathology Department, Theodor Bilharz Research Institute 12411, PO Box 30 Imbaba, Giza 12411, Egypt. drmonanosseir@yahoo.com
Telephone: +20-2-35401019 Fax: +20-2-35408125
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Abstract

AIM: To investigate cell cycle proteins in chronic hepatitis C virus infection in order to analyze their role in the process of hepatocyte transformation and to characterize their prognostic properties.

METHODS: Subjects of the current study included 50 cases of chronic hepatitis C (CHC) without cirrhosis, 30 cases of CHC with liver cirrhosis (LC), and 30 cases of hepatitis C-related hepatocellular carcinoma (HCC) admitted to the Department of Hepato-Gastro-enterology, Theodor Bilharz Research Institute (TBRI), Giza, Egypt. Fifteen wedge liver biopsies, taken during laparoscopic cholecystectomy, were also included as normal controls. Laboratory investigations including urine and stool analysis, liver function tests and prothrombin concentration; serologic markers for viral hepatitis and ultrasonography were done for all cases of the study together with immunohistochemical analysis using primary antibodies against Cyclin D1, Cyclin E, p21, p27 and Rb/p105 proteins.

RESULTS: Normal wedge liver biopsies didn't express Cyclin E or Rb/p105 immunostaining but show positive staining for Cyclin D1, p21 and p27. Cyclin D1 expressed nuclear staining that was sequentially increased from CHC to LC ($P < 0.01$) to HCC ($P < 0.001$) cases; meanwhile, Cyclin E revealed nuclear positivity only in the case of HCCs patients that was directly correlated to Rb/p105 immuno-reactivity. The expression of p21 and p27 was significantly increased in CHC and LC cases compared to normal controls and HCCs with no significant difference between well- and poorly-differentiated tumors. p21 showed only a nuclear pattern of staining, while, p27 presented with either cytoplasmic and/or nuclear reactivity in all studied cases. Correlation analysis revealed a direct relation between Cyclin D1 and p21 in CHC cases ($P < 0.001$), between Cyclin D1 and Cyclin E in HCCs ($P < 0.01$); however, an inverse

relationship was detected between Cyclin D1 and p21 or p27 ($P < 0.001$) and between p21 and Rb/p105 ($P < 0.05$) in HCCs.

CONCLUSION: Upregulation of Cyclin D1 in CHC plays a vital role in the development and differentiation of HCC; while, Cyclin E may be a useful marker for monitoring tumor behavior. p21 and p27 can be used as predictive markers for HCC. Furthermore, higher expression of Rb/p105 as well as inverse relation with p21 and histologic grades suggests its important role in hepatic carcinogenesis.

Key words: Chronic hepatitis C; Liver cirrhosis; Hepatocellular carcinoma; Cell cycle; Cyclin D1; Cyclin E; p21; p27; Rb/p105

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INTRODUCTION

The cell cycle is divided into four sequential phases[1]. G1 is the first gap phase in which cells prepare for deoxyribonucleic acid (DNA) replication; S (synthesis) phase is the period of DNA synthesis for the reproduction of the whole genome; G2 is the second gap phase in which cells prepare mitosis; and M (mitosis) phase in which cell division occurs for the generation of two genetically identical daughter cells. Quiescent cells that have not entered the cell cycle are referred to as being in G0[2].

Cyclins are the prime cell cycle regulators that play a central role in the control of cell proliferation by forming complexes with different Cyclin-dependent kinases (Cdks)[3]. Members of Cyclin family are often quite distinct from each other in amino acid sequence[2]. At least, 15 different Cyclins and 10 Cdks have been identified[4]. In response to mitogenic signals, G1 Cyclins (Cyclin D1 and Cyclin E) participate in the initiation and progression of the cell cycle where Cyclin D1 is activated during the mid-G1, while Cyclin E is required for G1/S transition[5]. They can accelerate and shorten the G1-phase and reinforce the ability of cells to loose growth control[6] suggesting an oncogenic potential of G1 Cyclins. On the other hand, Cyclin-dependent kinase Inhibitors (CdkIs) are potent negative regulators of the cell cycle that inhibit the G1/S transition[6] and include two families on the basis of sequence homology: The Ink4 family including p16Ink4a, p15Ink4b, p18Ink4c and p19Ink4 that specifically binds to Cdk4 and Cdk6 and inhibits Cyclin binding[7] and the Cip/Kip family including p21Cip1, p27Kip1 and p57Kip2 that bind to and inhibit Cyclin-bound Cdks[8]. Moreover, the two main regulatory proteins of the cell cycle are the retinoblastoma proteins (pRb) and p53. The Rb gene family is composed of three members that share many structural and functional features and play a fundamental role in growth control. They include the Rb susceptibility gene which encodes a nuclear phosphoprotein (pRb/p105) and two related genes pRb/p107 and pRb2/p130[9]. The Rb/p105 gene maps to the 13q14 chromosome, where deletions and heterozygous mutations are frequent in many human malignancies[10,11]. The balance between cell cycle regulators and cell proliferation is an important determinant of tumor development and/or behavior[12].

It has been suggested that hepatocyte turnover is increased in chronic hepatitis C virus (HCV) infection as markers of cell proliferation are elevated[13] and telomere shortening is reported[14]. However, mitotic activity is usually sparse or absent as hepatocytes expressing "proliferation markers" could enter the cell cycle but have been arrested and unable to complete cell division or progress to S phase[15]. Viral replication is enhanced by induction of both cell cycle entry and cell cycle arrest by viral factors[16]. Accordingly, a relationship between viral replication and the host cell cycle state exists in HCV infection[15]. There are several potential consequences of cell cycle arrest and senescence for the liver. Cellular senescence is a risk factor for cancer development and senescent hepatocytes may act synergistically with oncogenic mutations in neighboring hepatocytes leading to the development of hepatocellular carcinoma (HCC)[17].

The present work was designed to investigate the hepatic expression of Cyclin D1, Cyclin E, p21, p27 and the retinoblastoma gene family member Rb/p105 as some regulatory molecules of the cell cycle in chronic HCV infection in a trial to assess the effect of these regulatory molecules on disease progression and development of complications in the form of liver cirrhosis and/or HCC.

PATIENTS AND METHODS

The current study enrolled 110 patients of chronic liver disease who had been admitted to Hepato-Gastroenterology Department of Theodor Bilharz Research Institute (TBRI), Giza, Egypt. They were 75 males and 35 females with a mean age of 48.7 ± 7.5 (range 22-60 years). According to the guidelines of the Institution's Human Research Ethics Committee, all patients gave informed consents before inclusion in the study. After taking their full medical history, each was

subjected to a thorough clinical examination, subjected to ultrasonography and liver biopsies using ultrasound-guided percutaneous Menghini-needle.

Also, fifteen age- and sex-matched individuals who had undergone laparoscopic cholecystectomy were included in this study as controls. This group consisted of 10 males and 5 females with a mean age of 45.0 ± 7.5. After receiving their written consent, wedge liver biopsies were obtained from these cases.

Laboratory investigations

Urine and stool samples were collected and analyzed for all cases. Liver function tests were also done, including those for alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (Alb) and prothrombin concentration (PT conc). Serological diagnosis of schistosomiasis and viral hepatitis were also carried out. Hepatitis B surface antigen and hepatitis B core antibodies were assayed using enzyme immunoassay kits (Abbott Laboratories; North Chicago, Illinois); while, circulating anti-HCV antibodies were detected using the Murex enzyme immunoassay kit (Murex anti-HCV, Version V; Murex Diagnostics; Dartford, England). Chronic hepatitis C (CHC) was confirmed by the presence of HCV-RNA viremia by reverse-transcriptase polymerase chain reaction[18].

Histopathologic study

Serial sections (5 μm thick) from formalin-fixed, paraffin-embedded blocks of either core or wedge liver biopsies which were stained with hematoxylin & eosin as well as Masson trichrome stains. Histopathologic examination of liver sections from control cases showed that they were histopathologically-free from any hepatic lesion. On the other hand, assessment of liver sections from the 110 HCV-infected patients showed features of chronic active hepatitis C in 50 biopsies, liver cirrhosis in 30 cases (according to the French METAVIR System)[19] and hepatocellular carcinoma in 30 specimens with features consistent with well-differentiated (15 cases) and poorly differentiated tumors (15 cases) according to Colecchia *et al*[20].

Immunohistochemistry for cell cycle markers

The 5-μm thick sections from formalin-fixed, paraffinem-bedded blocks were collected on microscopic slides which had been coated with 3-amino propyl triethoxysilane (Sigma Chemicals; St. Louis, USA) both for proper fixation of tissue sections on the slides and minimization of staining artifacts. Following deparaffinization, rehydration and endogenous peroxidase inactivation, antigen retrieval was performed by microwaving in 10 mmol/L citrate buffer, pH 6.0 (Dako, Denmark). Non-specific antibody binding was hindered by pre-incubation with 100 μL blocking serum for 30 min at room temperature. Liver sections were incubated overnight, at 4℃, with the primary mouse anti-human monoclonal antibodies for Cyclin D1, Cyclin E, p21, p27 (Santa Cruz Biotechnology, Inc, USA) and Rb1/p105 (BioGenex, USA) at 1:25, 1:40, 1:20, 1:20, and 1:20 dilution, respectively. After thorough rinsing in PBS, the biotinylated secondary antibody was applied, followed by streptavidin peroxidase conjugation. Peroxidase activity was developed, using diamino-benzidine as the chromogen, and Mayer's hematoxylin as the counterstain. Negative controls were stained appropriately with each setting.

Scoring was performed by counting 500 hepatocytes in each biopsy. Results are shown as labeling index (LI), which represents the percentage of the hepatocyte nuclei that were positive for the antigen under high power magnification of X40. For Cyclin D1, 5% nuclear positivity was considered as overexpression[21] and the immunohistochemical (IHC) reactivity was divided into mild (less than 5%), moderate (5%-30%) and marked expression (> 30%). If the positive rate for Cyclin E protein was over 5%, it was, also, defined as over expression[22]. The IHC reactivity of cyclin E was divided into mild (< 5%), moderate (5%-49%) and marked expression (50%-100%)[23]. These cut-off levels were chosen in order to achieve distinct separation between patients with high and low cyclin E expression[24]. For p21 only cells with distinct nuclear staining were considered positive[25]; while, those considered positive for p27 expressed either a nuclear and/or cytoplasmic staining pattern[7] that may have been either weak or marked. The results were therefore, stratified into cases showing p27 staining in less than 50% of cells (weak) and those with more than 50% positive cell staining (marked)[26]. Finally, for Rb1/p105, immunostaining was quantified by counting the cells exhibiting positive staining in 10 randomly selected high-power fields within the site of the most severe lesion in the biopsy, and the results were expressed as percentage of positive cells in these areas[27] as follows: 0, undetectable level; 1: Low expression level (positive cells = 1%-30%); 2: Medium expression level (positive cells = 31%-60%); and 3: High expression level (positive cells = 61%-100%)[9].

Statistical analysis

The Statistical Package for Social Sciences (SPSS) for Windows (version 11) computer software was used for the statistical analysis. Means of different groups were compared using one-way ANOVA. A "*P*" value < 0.05 was considered statistically significant. Pearson correlation coefficient "*r*" was used to measure the relationship between 2 variables.

RESULTS

The one hundred and ten (110) HCV-infected patients had elevated liver enzymes, circulating anti-HCV antibodies and/or HCV-RNA viraemia. Moreover, they were sero-negative for hepatitis B virus or Schistosoma infection. The liver function tests of the fifteen control subjects were within normal range. They had no

Table 1 Clinical and laboratory data of all studied cases (mean ± SD) *n* (%)

Parameters	Control (*n* = 15)	CHC without cirrhosis (*n* = 50)	CHC with cirrhosis (*n* = 30)	HCV-related HCC (*n* = 30)
Age	45.0 ± 7.5	47.4 ± 9.3	51.3 ± 5.9	48.9 ± 7.2
Male/female ratio	2/1	7/3	2/1	2/1
Pallor	0 (0)	2 (4.0)	5 (16.5)	8 (26.6)
Jaundice	0 (0)	3 (6.0)	6 (20.0)	13 (43.3)
Palmer erythema	0 (0)	0 (0)	15 (50.0)	17 (56.6)
Spider naevi	0 (0)	0 (0)	13 (43.3)	16 (53.3)
Lower limb edema	0 (0)	0 (0)	16 (53.3)	10(33.3)
Child classification				
A	0 (0)	50 (100)	8 (26.6)	1 (3.3)
B		0 (0)	9 (30.0)	11 (36.6)
C		0 (0)	13 (43.3)	18 (60.0)
ALT (IU/L)	32.6 ± 4.2	63.2 ± 29.7	51.8 ± 4.3	78.1 ± 16.3
AST (IU/L)	31.1 ± 5.1	49.1 ± 18.3	46.4 ± 5.1	68.3 ± 12.4
Albumin (g/dL)	4.4 ± 0.5	3.80 ± 0.3	2.90 ± 0.7	2.80 ± 0.4
PT conc	97.6 ± 3.4	89.7 ± 5.8	53.4 ± 11.2	68.5 ± 3.8

serologic evidence of hepatitis B and/or C virus infection (Table 1).

In the current study, 3 normal wedge liver biopsies expressed mild Cyclin D1 immunostaining (Figure 1A). Positive nuclear expression was found in the hepatocytes of HCV-infected groups with sequential increase from CHC without cirrhosis (66%) to CHC with cirrhosis (70%) (Figure 1B and C) to HCV-related HCC (100%) (Table 2). Hepatic expression of Cyclin D1 in HCC was correlated with the histological grade, being significantly higher in the poorly differentiated tumors than the well-differentiated ones ($P < 0.001$) (Figure 1D). Marked staining intensity was only observed in poorly-differentiated neoplasms (Table 2).

For Cyclin E, no staining reaction was found in normal patients in the control group. CHC without cirrhosis and LC specimens showed a cytoplasmic pattern of staining that was considered negative. A strong nuclear staining was detected in cancerous livers (Figure 2A) with marked enhancement of expression in poorly differentiated cases (Table 3 and Figure 2B).

Immunoreactivity was detectable for p21 in all HCV-infected livers with minimal expression in the normal patients of the control group. In CHC without cirrhosis, p21 was expressed predominantly in hepatocytes, although occasional positive lymphocytes, sinusoidal lining cells and bile duct cells were also seen. p21 positive hepatocytes were more numerous in areas of intense inflammation and spotty necrosis as well as areas close to fibrosis. In biopsies with less inflammation or fibrosis, most p21 positive hepatocytes were located in the periportal areas rather than the central region. p21 expression in LC was higher than that observed in CHC without cirrhosis with no significant difference; however, it was significantly ($P < 0.01$) down-regulated in HCC cases, particularly in poorly-differentiated tumors (Table 4 and Figure 2C).

p27 was expressed in all cases of the study. The LI for p27 in HCC (Figure 2D) was 34.1 ± 2.7 which was significantly lower than that of the non-tumoral lesions ($P < 0.001$) and normal controls ($P < 0.01$). Furthermore, the LI of p27 in CHC without cirrhosis and LC were, also, significantly higher than those in normal patients in the control group ($P < 0.01$). The expression of p27 was either nuclear alone or mixed with cytoplasmic staining in HCC, LC and normal controls. While, the staining reaction was only cytoplasmic in CHC without cirrhosis (Table 5).

Immunoreactivity was detectable for Rb/p105 in some patients in the HCV-infected groups, and it was absent in normal control livers. The expression was mild to moderate in cases of CHC without cirrhosis and in LC cases (Figure 3A and B). Marked expression was found only in malignant cases. Hepatic expression of Rb/p105 was significantly lower in poorly differentiated HCCs than well-differentiated tumors (Table 6 and Figure 3C).

Correlation analysis revealed a highly significant correlation between Cyclin D1 and p21 in CHC without cirrhosis ($r = 0.70$, $P < 0.001$, Table 7).

In HCC, Cyclin E showed a direct correlation with Cyclin D1 ($P < 0.01$) and Rb/p105 ($P < 0.01$); however, Cyclin D1 showed an inverse correlation with both CdkIs p21 and p27 ($P < 0.001$). An inverse relation was, also, detected between p21 and Rb/p105 or Cyclin E in cancerous cells.

DISCUSSION

Hepatocyte cell cycle phase distribution is altered in chronic HCV infection[15]. Chronic necrosis and inflammation of the liver in HCV infection constituted an important driving force in the multistep process of hepatocarcinogenesis[28] and the majority of HCCs develop in cirrhotic livers[29].

In the current study, Cyclin D1 expression was found to be very low in control cases. However, the expression was significantly elevated in patients with chronic hepatitis C, emphasizing the increased hepatocyte turnover in chronic HCV infection[15]. The upregulation of Cyclin D1 in cirrhotic livers and HCC cases suggests that its expression may play an important role in the process of tumorigenesis. This goes in hand with the findings of other investigators[30] who reported that Cyclin D1 overexpression accelerates and shortens the G1-phase of the cell cycle, leading to a more rapid entry into the S-phase and also increases the number of cell cycle divisions. The upregulation of Cyclin D1, was also shown to be related to the histologic grade of HCC and reflects the aggressiveness of hepatic tumors. These data appear to be consistent with the results of previous studies[31, 32].

Immunolabeling localizes Cyclin E to the nucleus in the majority of human neoplasms. Although, the protein is synthesized and degraded in the cytoplasm, it is ordinarily transferred rapidly to the nucleus, where it carries out its functions[33]. This study revealed that Cyclin E was only expressed in the nuclei of cancerous hepatocytes and was marked in liver biopsies from patients with

Table 2 Immunohistochemical reactivity for Cyclin D1 in all studied cases (mean ± SE) *n* (%)

Groups *n* = 125	Staining pattern	Positive expression	Imunohistochemical reactivity			Hepatic expression
			Mild (< 5%)	Moderate (5%-30%)	Marked (> 30%)	
Control (*n* = 15)	Nuclear	3 (20.0)	3 (100)	0 (0)	0 (0)	1.33 ± 0.33
CHC without cirrhosis (*n* = 50)	Nuclear	33 (66.0)	25 (75.8)	8 (24.2)	0 (0)	6.60 ± 0.72[a]
CHC with cirrhosis (*n* = 30)	Nuclear	21 (70.0)	15 (71.4)	6 (28.6)	0 (0)	9.95 ± 1.70[a,b]
HCV-related HCC (*n* = 30)	Nuclear	30 (100)	12 (40.0)	12 (40.0)	6 (20.0)	19.85 ± 3.90[a,b,c]
Well-differentiated HCC (*n* = 15)	Nuclear	15 (100)	9 (60.0)	6 (40.0)	0 (0)	10.10 ± 2.20[a,b]
Poorly-differentiated HCC (*n* = 15)	Nuclear	15 (100)	3 (20.0)	6 (40.0)	6 (40)	31.60 ± 6.20[a,b,c d]

[a]$P < 0.001$ *vs* controls; [b]$P < 0.01$ *vs* CHC without cirrhosis; [c]$P < 0.001$ *vs* LC; [d]$P < 0.001$ *vs* well differentiated HCC.

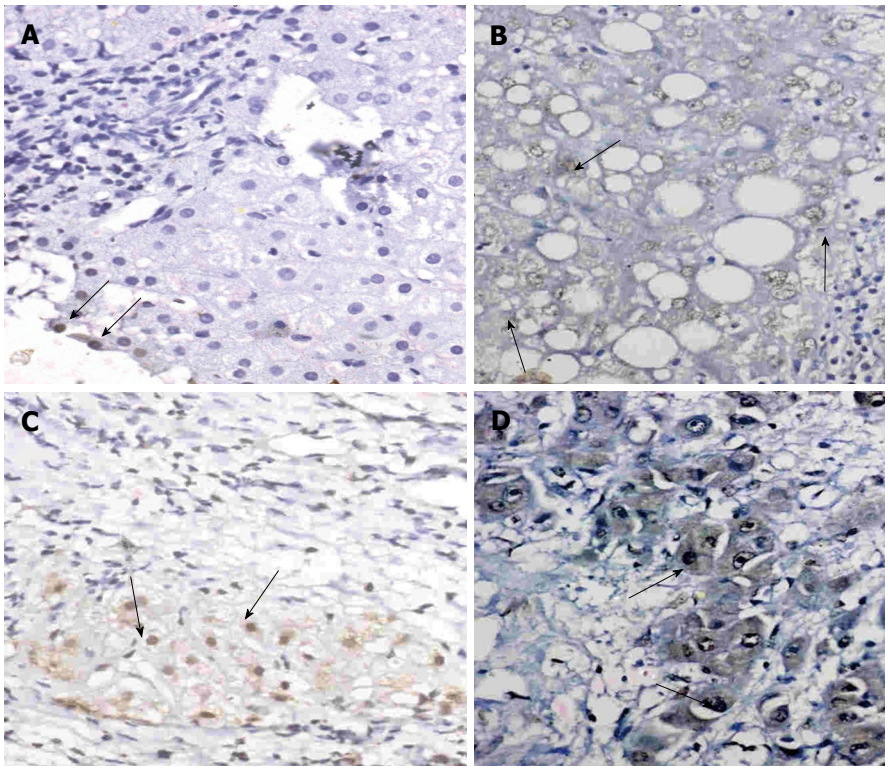

Figure 1 Immunohistochemical staining for Cyclin D1 in liver sections of patients from the control group as well as HCV-infected patients and HCV-related HCC patients. A: Liver section from a control case showing mild nuclear staining for Cyclin D1 (arrows) (Immunoperoxidase × 40); B: Liver section from a case of CHC showing mild nuclear expression of Cyclin D1 (arrows). The portal tract is completely free from immunostaining (Immunoperoxidase × 40); C: Liver section from a case of LC showing moderate number of positively stained hepatocytes in a cirrhotic nodule (arrows) (Immunoperoxidase × 40); D: Liver section from a case of poorly-differentiated HCC showing marked nuclear expression of Cyclin D1 (arrows) (Immunoperoxidase × 40).

poorly differentiated tumors. This indicates that Cyclin E overexpression is associated with tumor aggressiveness and may be considered as one of the markers for outcomes. Keyomarsi *et al*[34] suggested that high Cyclin E expression conveys additional negative consequences for the malignant cell, besides high proliferation. Our results corroborate the previous findings of Jung *et al*[5] who reported that Cyclin E protein was found to be overexpressed in HCC, whereas its expression was hardly detectable in their normal counterparts. Also, other investigators[23,26,32] observed that overexpression of Cyclin E was associated with poor differentiation, invasiveness and metastasis in HCC.

Deregulated cell-cycle progression is one of the most significant alterations in cancer cells. Because G1- to S-phase is the key target of tumorigenesis, it is, in part, negatively regulated by p21 protein, which is a universal inhibitor of Cyclin-dependent kinases and cell cycle progression[35]. In the present study, minimal p21 expression was detected in normal livers, which is consistent with a previous immunohistochemical study[36], but was significantly increased in HCV-infected patients. Wagayama *et al*[37] demonstrated that CdkIs which arrest or slow cell cycle progression are increased in chronic HCV infection. The upregulated p21 expression may play a role as a guard to prevent hepatocytes from tumorigenicity in HCV hepatitis. The highly expressed p21 may hold hepatocytes against transformation by inducing enough G1 span to evoke apoptosis or repair DNA mismatches under the activated cell cycle progression[38]. Stimuli causing increased hepatocyte p21 expression raise the threshold of Cyclin/Cdk inhibition and thereby, diminish mitogen-induced hepatocyte proliferation[25]. However, p21 expression induces transcription of profibrotic factors such as connective tissue growth factor and fibronectin-1[39], thus enhancing the progression to cirrhosis. Crary and Albrecht[25] and Wagayama *et al*[37,40] reported that the p21 labeling index in patients with liver

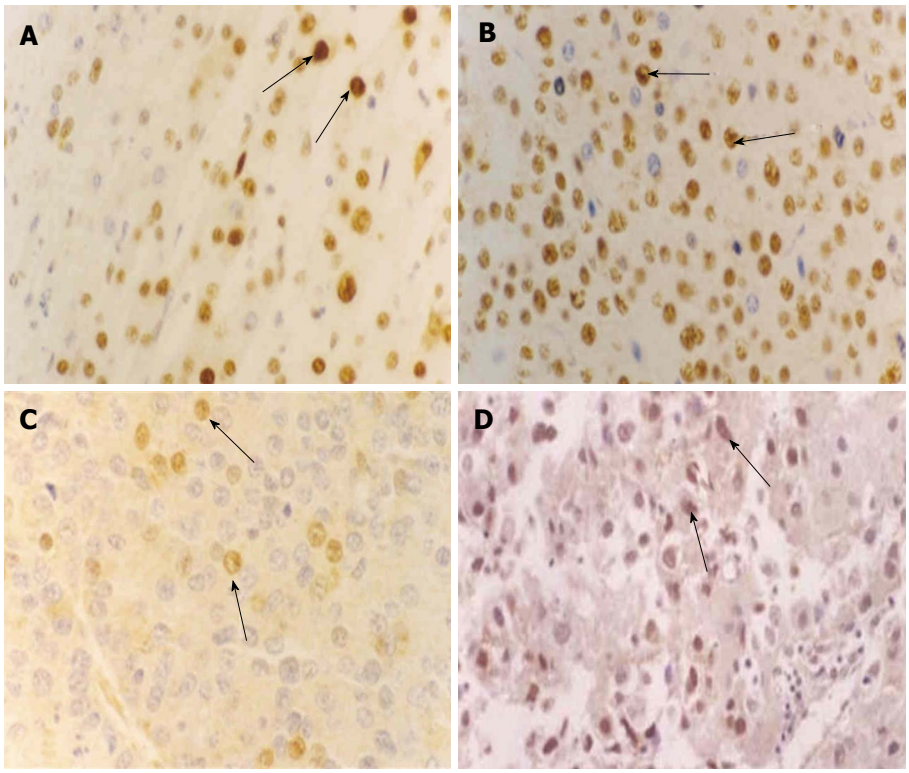

Figure 2 Immunohistochemical staining of liver sections from different differentiated HCC cases (Immunoperoxidase × 40). A: Well differentiated HCC showing moderate nuclear staining of cyclin E (arrows); B: Poorly-differentiated HCC showing marked nuclear staining of cyclin E (arrows); C: Well-differentiated HCC revealing nuclear expression of p21Cip1/Waf1 (arrows) in the hepatocytes; D: Poorly-differentiated HCC showing p27Kip1 expression in a mixed pattern (arrows).

Table 3 Immunohistochemical reactivity for cyclin E in patients with well-differentiated and poorly-differentiated (HCC) (mean ± SE) *n* (%)

Groups	Positive expression	Imunohistochemical reactivity			Stained cells
		Mild < 5%	Moderate 5%-49%	Marked 50%-100%	
Well-differentiated HCC (*n* = 15)	6 (40.0)	2 (33.3)	4 (66.7)	0 (0)	25.3 ± 5.9
Poorly-differentiated HCC (*n* = 15)	6 (40.0)	0 (0)	0 (0)	6 (100)	82.5 ± 2.1[a]

[a]*P* < 0.01 *vs* well differentiated HCC.

Table 4 Immunohistochemical reactivity for p21 in all studied cases (mean ± SE)

Groups	Staining pattern	Hepatic expression
Control (*n* = 15)	Nuclear	1.5 ± 0.5
CHC without cirrhosis (*n* = 50)	Nuclear	6.5 ± 0.2[a]
CHC with cirrhosis (*n* = 30)	Nuclear	7.2 ± 0.8[a]
HCV-related HCC (*n* = 30)	Nuclear	4.9 ± 0.1[a, b]
Well-differentiated HCC (*n* = 15)	Nuclear	5.6 ± 0.16
Poorly-differentiated HCC (*n* = 15)	Nuclear	4.2 ± 0.13

[a]*P* < 0.01 *vs* controls; [b]*P* < 0.05 *vs* LC.

cirrhosis was significantly higher than that in patients with chronic hepatitis, and concluded that p21 expression was upregulated by the stress of inflammation and fibrosis, and influenced by viral proteins. It was found that in normal cells, p21Cip1 is associated with quaternary complexes of most Cyclins, Cdks and proliferation cell nuclear antigen, but is absent from these complexes in most transformed cells[41]. These observations suggest that the reduction or loss of p21 expression plays an important role in the process of tumorigenesis that has also been shown in other reports[42] and supported in this study by the expression of elevated G1 cyclins. As a cause for decreased p21 mRNA expression in tumorous tissues of human cancers, p53 gene mutations are mostly suspected[43] because induction of the p53 tumor suppressor gene after DNA damage inhibits the G1 Cyclins/Cdk activity *via* p21Cip1[44,45] and this inhibition causes cell cycle arrest which, in turn, facilitates DNA repair[46,47].

In this study, expression of p27Kip1 in hepatic cells was significantly upregulated in patients with CHC and LC compared to control cases. On the other hand, it was significantly decreased in HCC cases compared to all groups. Matasuda *et al*[48] found that p27 is abundantly expressed in quiescent cells and is downregulated in many aggressive cancers. It has been recently found[49], that p27 is frequently inactivated in HCC, and is now considered to be a potent tumor suppressor as it is a negative regulator of G1-S phase transition through inhibition of the kinase activities of Cdk2/Cyclin E. Other series[7,23,50-52] have reported that the decreased expression of p27Kip1 is related to tumor progression and could be used as a potential predictor for HCC. The loss or decrease of p27

Table 5 Immunohistochemical reactivity for p27 in all studied cases (mean ± SE) *n* (%)

Groups *n* = 125	Staining pattern	Imunohistochemical reactivity		Hepatic expression
		Weak (< 50%)	Marked (> 50%)	
Control (*n* = 15)	Nuclear/mixed	9 (60.0)	6 (40.0)	44.4 ± 1.9
CHC without cirrhosis (*n* = 50)	Nuclear/cytoplasmic	5 (10.0)	45 (90.0)	59.8 ± 1.9[a]
CHC with cirrhosis (*n* = 30)	Nuclear/mixed	0 (0)	30 (100)	65.9 ± 1.6[a]
HCV-related HCC (*n* = 30)	Nuclear/mixed	21 (70.0)	9 (30.0)	34.1 ± 2.7[a,b,c]
Well-differentiated HCC (*n* = 15)	Nuclear	7 (46.7)	8 (53.3)	36.2 ± 5.2[a,b,c]
Poorly-differentiated HCC (*n* = 15)	Mixed	15 (100)	0 (0)	32.0 ± 1.7[a,b,c]

[a]$P < 0.01$ *vs* controls; [b]$P < 0.001$ *vs* CHC without cirrhosis; [c]$P < 0.001$ *vs* LC.

Table 6 Immunohistochemical reactivity for Rb/p105 in all studied cases (mean ± SE) *n* (%)

Groups *n* = 125	Staining pattern	Positive expression	Imunohistochemical reactivity			Hepatic expression
			Mild (1%-30%)	Moderate (31%-60%)	Marked (61%-100%)	
Control (*n* = 15)	Negative	0 (0)	0 (0)	0 (0)	0 (0)	0.00 ± 0.00
CHC without cirrhosis (*n* = 50)	Nuclear	30 (60.0)	27 (90.0)	3 (10.0)	0 (0)	28.7 ± .1
CHC with cirrhosis (*n* = 30)	Nuclear	15 (50.0)	5 (33.3)	10 (66.7)	0 (0)	40.9 ± 2.8[a]
HCV-related HCC (*n* = 30)	Nuclear	21 (70.0)	0 (0)	12 (57.1)	9 (42.9)	61 ± 3.7[a,b]
Well-differentiated HCC (*n* = 15)	Nuclear	12 (80.0)	0 (0)	12 (100)	0 (0)	67.3 ± 4.8[a,b]
Poorly-differentiated HCC (*n* = 15)	Nuclear	9 (60.0)	0 (0)	0 (0)	9 (100)	55.7 ± 1.4[a,b c]

[a]$P < 0.01$ *vs* CHC without cirrhosis; [b]$P < 0.001$ *vs* LC; [c]$P < 0.01$ *vs* well-differentiated carcinoma.

Table 7 Correlation analysis of different parameters in HCCs

Parameter	HCV-related HCC	
	r	*P*
Cyclin D1 # Cyclin E	0.61	< 0.01
Cyclin D1 # p21	-0.51	< 0.001
Cyclin D1 # p27	-0.65	< 0.001
Cyclin E # Rb/p105	0.62	< 0.01
Cyclin E # p21	-0.64	< 0.01
p21 # p27	0.47	< 0.001
p21 # Rb/p105	-0.42	< 0.05

protein may lead to reduction or disappearance of its cell cycle negative regulation; thus the cells pass the G1 into S-phase, resulting in division and autonomous program[53]. Moreover, in these studies, reduced p27Kip1 expression in HCC both at protein and mRNA levels was associated with tumor invasiveness, advanced clinical stage and poor cellular differentiation grade, as it may be involved in the anchor free survival of malignant cells resulting in viable metastatic tumor nests. However, cells with preserved or increased p27Kip1 expression are not able to proliferate and are driven to apoptotic death[26]. The protein p27 can bind to and inhibit the active Cyclin/Cdk complexes in the nucleus[7], but some tumors expressed increased level of p27 because of increased cytoplasmic expression of this protein, especially in their early stages[54]. However, this may be regulated by self stabilization through attenuating the activity of the proteasome pathway for p27, contributing to tumor development[54]. Results of the current study revealed the decreased p27 expression associated with increased Cyclin D1 expression that was previously explained[49] in some cases of HCC with increased cell proliferation, where p27 is overexpressed but inactivated by sequestration into Cyclin D1-Cdk4-containing complexes.

The retinoblastoma family of growth-inhibitory proteins act by binding and inhibiting several proteins with growth stimulatory activity, the most prominent of which is the cellular transcription factor E2F[55]. Phosphorylation of retinoblastoma family proteins by Cyclin-dependent kinases leads to release of the associated growth stimulatory proteins, which in turn mediate progression through the cell division cycle[56] that was supported in this study by the finding of a direct correlation between Rb/p105 and Cyclin E expression in HCCs. Bagui *et al*[57] suggested that Cyclin D1 combine with Cdk at mid- to late G1, forming complexes that phosphorylate the pRb and sequester p21Cip1 and p27Kip1 which when activated elicit additional events required for the initiation and execution of the S phase.

The p105 protein is important in the synthesis and transport of RNA[58]. It was detected in proliferating cells only where its concentration is elevated during G2-phase and mitosis[58]. Variable staining intensities of the tumor suppressor gene Rb/p105 have been demonstrated in different groups of the present study; and its presence in 60% of CHC and 50% of LC cases may help to protect cells against malignant transformation. When the degree of malignant potential (grading) of HCC cases was compared with the expression of this protein, lower expression was found in poorly differentiated tumors. The highest percentage of detectable levels of Rb/p105

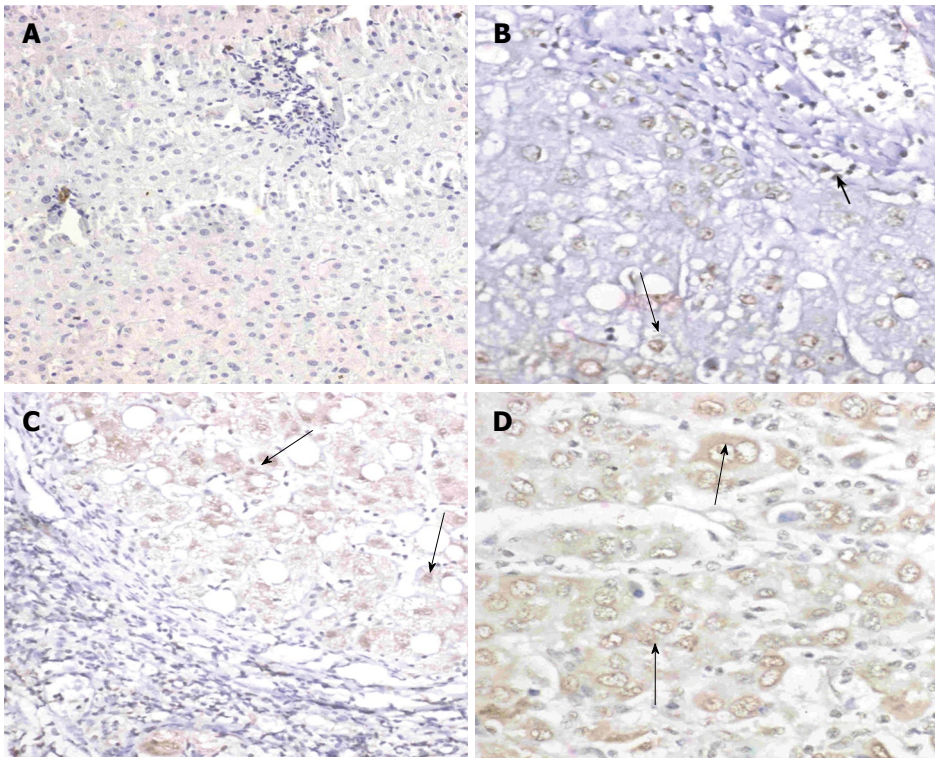

Figure 3 Immunohistochemical staining for Rb1/p105 in liver sections of the control group patients as well as HCV-infected patients and HCV-related HCC patients. A: Liver section from a case of the control showing negative staining for Rb1/p105 either in the cytoplasm or in the nuclei of hepatocytes (IP × 20); B: Liver section from a case with CHC showing moderate nuclear expression for Rb1/p105 in the hepatocytes (long arrow) and in few scattered inflammatory cells (short arrow) of the portal tract (IP × 40); C: Liver section from a case with LC showing moderate nuclear expression for Rb1/p105 in hepatocytes (arrows) and some inflammatory cells of the portal tract in LC (IP × 20); D: Liver section from a case with poorly-differentiated HCC showing marked nuclear expression for Rb1/p105 in the hepatocytes (arrows) (IP × 40).

in HCCs (70%) and the inverse correlation with p21 and histologic grading suggest an important role of Rb/p105 in the pathogenesis and progression of HCCs.

In conclusion, in HCV infection, the up-regulation of Cyclin D1 expression plays an important role in the development of tumorigenesis and in differentiation of HCC. Cyclin E; however, play no role in HCV infection and may be considered as a marker of differentiation and aggressiveness in HCCs. Further studies are needed to elucidate the mechanism of interaction among different Cyclins and the pathway for their regulation.

The p21 and p27 are independently increased in CHC and LC. They mediate hepatocyte cell cycle arrest and accumulation of growth-arrested hepatocytes which impairs hepatocellular function and limits hepatic regeneration. However, both of them are negative regulators of G1-S phase transition and may be considered as predictive factors in HCC. Decreased p21, p27Kip1, Rb/p105 and increased Cyclin D1 and Cyclin E stress the presence of invasive and highly proliferating tumors.

Our results offer insights into the highly complex mechanisms of cell cycle regulation and need to be confirmed by further large scale studies. Understanding the effect of interference at multiple points may well be the foundation upon which designing novel strategies for improving therapeutic approaches in CHC and HCC might begin.

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COMMENTS

Background

An increased risk of hepatitis C virus(HCV)-related cirrhosis and/or hepatocellular carcinoma (HCC) can be explained by the highly complex mechanisms of cell cycle regulation.

Research frontiers

The current study was designed to investigate the hepatic expression of some regulatory molecules of the cell cycle in chronic HCV infection to assess their effect on disease progression and the development of complications.

Innovations and breakthroughs

The role of Cyclins, which are the prime cell cycle regulators, Cyclin-dependent kinase Inhibitors, which are the potent negative regulators of the cell cycle, and retinoblastoma proteins (pRb), as one of the main regulatory proteins of the cell cycle in HCV infection and associated cirrhosis or HCC were thoroughly addressed and correlated in this work. This is the first study to report high expression of Rb/p105 in HCV-induced liver disease and associated complications.

Applications

By understanding the interrelated factors involved in the expression or repression of cell cycle regulatory molecules, new strategies can be developed for improving the therapeutic treatment of chronic hepatitis C and its sequel.

Peer review

The authors have analyzed, through immunohistochemical staining, the expression of cell cycle regulatory molecules in the hepatic tissue of patients with different stages of hepatitis C-related disease, from chronic hepatitis to cirrhosis to hepatocellular carcinoma. Interestingly, they found that cyclin D1 expression increased from chronic hepatitis to cirrhosis to cancer, and thus its expression can be associated with the development of cancer, while cyclin E was over-expressed only in overt cancer with a strict correlation with cancer grading and Rb/p105. Cyclin-dependent kinase inhibitors, p21 and p27, showed an inverse correlation with Cyclin D1 in cancer and thus, as well as Cyclin D1, could be used as predictors of cancer onset. The article is well written, the population size, is large and data are convincing, also from a statistical point of view. The authors did a massive and great job by scrupulously analysing slides and counting positive stained cells.

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