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

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.

Supported by Theodor Bilharz Research Institute (Grant # 74D) in collaboration with the American University of Cairo 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

Received: March 9, 2009 Revised: October 10, 2009
Accepted: October 17, 2009
Published online: January 27, 2010

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-Gastroenterology, 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.

© 2010 Baishideng. All rights reserved.

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

**Peer reviewers:** Patricia Cristina Baré, PhD, Virology Section, Instituto de Investigaciones Hematologicas, Academia Nacional de Medicina, Pacheco de Mello 3081, Buenos Aires, 1425, Argentina; Melchiorre Cervello, Dr, Istituto di Biomedicina e Immunologia Molecolare “Alberto Monroy”, Consiglio Nazionale delle Ricerche (CNR), Via Ugo La Malfa 153, Palermo 90146, Italy

El Bassiouney AE, Nosseir MM, Zoheiry MK, Ameen NA, Abdel-Hadi AM, Ibrahim IM, Zada S, Saad El-Deen AH, El-Bassiouni NE. Differential expression of cell cycle regulators in HCV-infection and related hepatocellular carcinoma. *World J Hepatol* 2010; 2(1): 32-41 Available from: URL: http://www.wjgnet.com/1948-5182/full/v2/i1/32.htm DOI: http://dx.doi.org/10.4254/wjhe.v2.i1.32

**INTRODUCTION**

The cell cycle is divided into four sequential phases: 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.

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). Members of Cyclin family are often quite distinct from each other in amino acid sequence. At least, 15 different Cyclins and 10 Cdks have been identified. 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. They can accelerate and shorten the G1-phase and reinforce the ability of cells to loose growth control suggesting an oncogenic potential of G1 Cyclins. On the other hand, Cyclin-dependent kinase Inhibitors (Cdks) are potent negative regulators of the cell cycle that inhibit the G1/S transition and include two families on the basis of sequence homology: The Ink4 family including p16Ink4a, p15Ink4b, p18Ink4c and p19Ink4 that specifically binds to Cdks and inhibits Cyclin binding and the Cip/Kip family including p21Cip1, p27Kip1 and p57Kip2 that bind to and inhibit Cyclin-bound Cdks. 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/p106. The Rb/p105 gene maps to the 13q14 chromosome, where deletions and heterozygous mutations are frequent in many human malignancies. The balance between cell cycle regulators and cell proliferation is an important determinant of tumor development and/or behavior.

It has been suggested that hepatocyte turnover is increased in chronic hepatitis C virus (HCV) infection as markers of cell proliferation are elevated and telomere shortening is reported. 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. Viral replication is enhanced by induction of both cell cycle entry and cell cycle arrest by viral factors. Accordingly, a relationship between viral replication and the host cell cycle state exists in HCV infection. 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).

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.

**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) 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.

**Immunohistochemistry for cell cycle markers**

The 5-μm thick sections from formalin-fixed, paraffin-embedded blocks were collected on microscopic slides which had been coated with 3-aminopropyl 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 mM 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°C, 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 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 overexpression. The IHC reactivity of cyclin E was divided into mild (< 5%), moderate (5%-49%) and marked expression (50%-100%). These cut-off levels were chosen in order to achieve distinct separation between patients with high and low cyclin E expression.

For p21 only cells with distinct nuclear staining were considered positive; while, those considered positive for p27 expressed either a nuclear and/or cytoplasmic staining pattern 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). 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 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%).

**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 viremia. 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
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 (60%) 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 perportal 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[13]. 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[30]. 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[31, 32] 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[30, 31].

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

| 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             | 21/2            | 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)      |
| Palmar 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)       | A | 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 ± 0.4       | 2.90 ± 0.7       | 2.80 ± 0.4     |
| PT conc    | 2/1            | 7/3             | 21/2            | 2/1            |
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. 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. 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 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. In the present study, minimal p21 expression was detected in normal livers, which is consistent with a previous immunohistochemical study, but was significantly increased in HCV-infected patients. Wagayama et al. 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. Stimuli causing increased hepatocyte p21 expression raise the threshold of Cyclin/Cdk inhibition and thereby, diminish mitogen-induced hepatocyte proliferation. However, p21 expression induces transcription of profibrotic factors such as connective tissue growth factor and fibronectin-1, thus enhancing the progression to cirrhosis. Crary and Albrecht and Wagayama et al. reported that the p21 labeling index in patients with liver
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, Cdns 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[7,23,30-32] 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 3  Immunohistochemical reactivity for cyclin E in patients with well-differentiated and poorly-differentiated (HCC) (mean ± SE) n (%) |
|---------------------------------|----------------|----------------|----------------|
| Groups                          | Positive expression |                |                |
| Well-differentiated HCC (n = 15) | 6 (40.0)          | 2 (33.3)       | 4 (66.7)       |
| Poorly-differentiated HCC (n = 15) | 6 (40.0)          | 0 (0)          | 0 (0)          |

Stained cells
25.3 ± 5.9
82.5 ± 2.1

aP < 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         | 6.3 ± 0.2a      |
| CHC with cirrhosis (n = 30)     | Nuclear         | 7.2 ± 0.8b      |
| HCV-related HCC (n = 30)        | Nuclear         | 4.9 ± 0.1a      |
| Well-differentiated HCC (n = 15) | Nuclear         | 5.6 ± 0.16      |
| Poorly-differentiated HCC (n = 15) | Nuclear         | 4.2 ± 0.13      |

aP < 0.01 vs controls; bP < 0.05 vs LC.
Table 5  Immunohistochemical reactivity for p27 in all studied cases (mean ± SE) $n$ (%)

| Groups $n = 125$ | Staining pattern | Immunohistochemical 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 ± 2.3 $^{a,b}$ |
| 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 | Immunohistochemical reactivity | Hepatic expression |
|------------------|------------------|---------------------|------------------------------|-------------------|
|                   |                  | Mild (1%-30%) | Moderate (31%-60%) | Marked (61%-100%) |  |
| Control ($n = 15$) | Negative | 0 (0) | 0 (0) | 0 (0) | 0.00 ± 0.00 |
| CHC without cirrhosis ($n = 50$) | Nuclear | 30 (60.0) | 27 (54.0) | 12 (24.0) | 28.7 ± 1.1 |
| CHC with cirrhosis ($n = 30$) | Nuclear | 15 (50.0) | 5 (33.3) | 10 (66.7) | 40.2 ± 2.8 |
| HCV-related HCC ($n = 30$) | Nuclear | 21 (70.0) | 0 (0) | 12 (57.1) | 49 (92.9) | 61 ± 3.7 $^{a,b}$ |
| Well-differentiated HCC ($n = 15$) | Nuclear | 12 (80.0) | 0 (0) | 12 (100) | 0 (0) | 67.3 ± 4.8 $^{a,b,c}$ |
| 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.[58]. 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.[56]. The protein p27 can bind to and inhibit the active Cyclin/Cdk complexes in the nucleus,[57] but some tumors expressed increased level of p27 because of increased cytoplasmic expression of this protein, especially in their early stages.[58]. However, this may be regulated by self stabilization through attenuating the activity of the proteasome pathway for p27, contributing to tumor development.[59]. 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.[53]. 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[54] 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[51] 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[60]. 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...
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 HCCs. 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.

ACKNOWLEDGMENTS

The authors are greatly indebted to Mrs. Hoda Abu-Taleb for performing the statistical analysis of this work.

REFERENCES

1. Owa T, Yoshino H, Yoshimatsu K, Nagasu T. Cell cycle...
EL Bassiouney et al. Cell cycle regulatory molecules in HCV/HCC

regulation in the G1 phase: a promising target for the development of new chemotherapeutic anticancer agents. Curr Med Chem 2001; 8: 1487-1503

2 Morgan DO. The cell cycle control system. In: E Lawrence. The Cell Cycle: Principles of control. London: New Science Press Ltd, 2007: 29-55

3 Bornfeldt KE. The cyclin-dependent kinase pathway moves forward. Circ Res 2003; 92: 345-347

4 Rosania GR, Chang YT. Targeting hyperproliferative disorders with cyclin dependent kinase inhibitors. Expert Opin on Therapeutic Patents 2000; 10: 215-230. Available from: URL: http://www.informapharmascience.com/ddi.

5 Jung YJ, Lee KH, Choi DW, Han CJ, Jeong SH, Kim KC, Oh JW, Park TK, Kim CM. Reciprocal expressions of cyclin E and cyclin D1 in hepatocellular carcinoma. Cancer Lett 2001; 168: 57-63

6 Mann CD, Neal CP, Garcia G, Manson MM, Dennison AR, Berry DP. Prognostic molecular markers in hepatocellular carcinoma: a systematic review. Eur J Cancer 2007; 43: 979-992

7 Nan KJ, Jing Z, Gong L. Expression and altered subcellular localization of the cyclin-dependent kinase inhibitor p27Kip1 in hepatocellular carcinoma. World J Gastroenterol 2004; 10: 1425-1430

8 Polak J, Pekova S, Schwarz J, Kozak T, Haskovec C. Expression of cyclin-dependent kinase inhibitors in leukemia. Cas Lek Cesk 2003; 142: 25-28

9 Baldi A, Esposito V, De Luca A, Howard CM, Mazzarella G, Baldi F, Caputi M, Giordano A. Differential expression of the retinoblastoma gene family members pRb/p105, p107, and pRb2/p130 in lung cancer. Clin Cancer Res 1996; 2: 1239-1245

10 Nevins JR. Toward an understanding of the functional complexity of the E2F and retinoblastoma families. Cell Growth Differ 1998; 9: 585-593

11 Sàrseverinò F, Torrecelli M, Petragna F, Giordano A. Role of the retinoblastoma family in gynecological cancer. Cancer Biol Ther 2003; 2: 636-641

12 Lu ZL, Luo DZ, Wen JM. Expression and significance of tumor-related genes in HCC. World J Gastroenterol 2005; 11: 3850-3854

13 Lake-Bakaa G, Mazzoccoli V, Ruffini L. Digital image analysis of the distribution of proliferating cell nuclear antigen in hepatocitis C virus-related chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Dig Dis Sci 2002; 47: 1644-1648

14 Miura H, Horikawa I, Nishimoto A, Ohmura H, Ito H, Hirohashi S, Shav JW, Oshimura M. Progressive telomere shortening and telomerase reactivation during hepatocellular carcinogenesis. Cancer Genet Cytogenet 1997; 93: 56-62

15 Marshall A, Rushbrooke S, Davies SE, Morris LS, Scott IS, Powler SL, Coleman N, Alexander G. Relation between hepatocyt G1 arrest, impaired hepatic regeneration, and fibrosis in chronic hepatitis C virus infection. Gastroenterology 2005; 128: 33-42

16 Flemington EK. Herpesvirus lytic replication and the cell cycle: arresting new developments. J Virol 2001; 75: 4475-4481

17 Krtolica A, Parrinello S, Lockett S, Desprez PY, Campisi J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. Proc Natl Acad Sci USA 2001; 98: 12072-12077

18 Hodinka RL. Detection of HCV RNA in serum by reverse transcriptase-PCR and radiolabeled liquid hybridization. Method Mol Med 1998; 19: 29-45

19 Intraobserver and interobserver variations in liver biopsy interpretation in patients with chronic hepatitis C. The French METAIVIR Cooperative Study Group. Hepatology 1994; 20: 15-20

20 Colecchia A, Scailoi E, Vestito A, Grazi GL, Festi D. Tumor grading of hepatocellular carcinoma by preoperative needle biopsy: Is it useful for choosing the best therapeutic strategy? Dig Liver Dis 2009; 41: A18

21 Khan AA, Abel PD, Chaudhary KS, Gulzar Z, Stamp GW, Lalani EN. Inverse correlation between high level expression of cyclin E and proliferation index in transitional cell carcinoma of the bladder. Mol Pathol 2003; 56: 353-361

22 Peng SY, Chou SP, Hsu HC. Association of downregulation of cyclin D1 and of overexpression of cyclin E with p53 mutation, high tumor grade and poor prognosis in hepatocellular carcinoma. J Hepatol 1998; 29: 281-289

23 Zhou Q, He Q, Liang LJ. Expression of p27, cyclin E and cyclin A in hepatocellular carcinoma and its clinical significance. World J Gastroenterol 2005; 9: 2450-2454

24 Lindahl T, Landberg G, Ahlgren J, Nordgren H, Norberg T, Klaar S, Holmberg L, Bergh J. Overexpression of cyclin E protein is associated with specific mutation types in the p53 gene and poor survival in human breast cancer. Carcinogenesis 2004; 25: 375-380

25 Crazy GS, Albrecht JH. Expression of cyclin-dependent kinase inhibitor p21 in human liver. Hepatology 1998; 28: 738-743

26 Armengol C, Boix L, Bachs O, Sole M, Fuster J, Sala M, Llovet JM, Rodes J, Bruix J. p27(Kip1) is an independent predictor of recurrence after surgical resection in patients with small hepatocellular carcinoma. J Hepatol 2003; 39: 59-57

27 Santopietro R, Shahalova I, Petrovichew N, Kozachenko V, Zakhvoroba T, Pajandi J, Podistov J, Chemeris G, Sozaeva L, Lipova E, Tsidaeva I, Ivanchenko O, Pshepurko A, Zakhorenko S, Nerovnya R, Klujikina L, Erokhsina O, Branovskaja K, Nikitina M, Grunberg A, Brunso TM. Cell cycle regulators p105, p107, and pRb2/p130 in lung cancer. Clin Cancer Res 1996; 2: 1239-1245

28 Di Bisceglie AM. Hepatitis C and hepatocellular carcinoma. Semin Liver Dis 1995; 15: 64-69

29 Bruno S, Silini E, Cresignani A, Porzio F, Leandri G, Bonf O, Asti M, Rossi S, Larghi A, Cerino A, Podda M, Mondeli MU. Hepatitis C virus genotypes and risk of hepatocellular carcinoma in cirrhosis: A prospective study. Hepatology 2003; 25: 754-758

30 Fu M, Wang C, Li Z, Sakamaki T, Pestell RG. Mini review: Cyclin D1: normal and abnormal functions. Endocrinology 2004; 145: 5439-5447

31 Nishida N, Fukuda Y, Komeda T, Kita R, Sando T, Furukawa M, Amenomori M, Shibagaki I, Nakao K, Ikenaga M. Amplification and overexpression of the cyclin D1 gene in aggressive human hepatocellular carcinoma. Cancer Res 1994; 54: 3107-3110

32 Masaki T, Shiratori Y, Rengifo W, Ishiguro K, Yamagata M, Kurokohchi K, Uchida N, Miyayoshi Y, Yoshii H, Watanabe S, Omata M, Kuriyama S. Cyclins and cyclin-dependent kinases: comparative study of hepatocellular carcinoma versus cirrhosis. Hepatology 2003; 37: 534-543

33 Donnellan R, Chetty R. Cyclin E in human cancers. FASEB J 1999; 13: 773-780

34 Keyomarsi K, Tucker SL, Buchholz TA, Callister M, Ding Y, Horebagy GN, Bedrosian I, Knickerbocker C, Toyoafuku W, Lowe M, Herlitzek TW, Baccus SS. Cyclin E and survival in patients with breast cancer. N Engl J Med 2002; 347: 1566-1576

35 Shi YZ, Hui AM, Takayama T, Li X, Cui X, Makuuchi M. Reduced p21WAF1/CIP1 protein expression is predominant in small hepatocellular carcinoma of the bladder. British J Cancer 2000; 83: 59-65

36 Frederders S, Milne AW, Hall PA, Lu X. Characterization
of a panel of novel anti-p21Waf1/Cip1 monoclonal antibodies and immunochemical analysis of p21Waf1/Cip1 expression in normal human tissues. *Am J Pathol* 1996; 148: 825-835

37 *Wagayama H,* Shiraki K, Yamakana T, Sugimoto K, Ito T, Fujikawa K, Takase K, Nakano T. p21WAF1/CTP1 expression and hepatitis virus type. *Dig Dis Sci* 2001; 46: 2074-2079

38 *Kobayashi S,* Matsushita K, Saigo K, Urashima T, Asano T, Hayashi H, Ochiai T. P21WAF1/CIP1 messenger RNA expression in hepatitis B, C virus-infected human hepatocellular carcinoma tissues. *Cancer* 2001; 91: 2096-2103

39 *Chang BD,* Watanabe K, Broude EV, Fang J, Poole JC, Kalinchenco TV, Roninson IB. Effects of p21Waf1/Cip1/Sdi1 on cellular gene expression: implications for carcinogenesis, senescence, and age-related diseases. *Proc Natl Acad Sci USA* 2000; 97: 4291-4296

40 *Wagayama H,* Shiraki K, Sugimoto K, Ito T, Fujikawa K, Yamakana T, Takase K, Nakano T. High expression of p21WAF1/CIP1 is correlated with human hepatocellular carcinoma in patients with hepatitis C virus-associated chronic liver diseases. *Hum Pathol* 2002; 33: 429-434

41 *Xiong Y,* Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature* 1993; 366: 701-704

42 *Kao JT,* Chuah SK, Huang CC, Chen CL, Wang CC, Hung CH, Chen CH, Wang JH, Lu SN, Lee CM, Changchien CS, Hu TH. P21/WAF1 is an independent survival prognostic factor for patients with hepatocellular carcinoma after resection. *Liver Int* 2007; 27: 772-781

43 *Furutani M,* Arii S, Tanaka H, Mise M, Niwano M, Harada T, Higashitsuji H, Imamura M, Fujita J. Decreased expression and rare somatic mutation of the CIP1/WAF1 gene in human hepatocellular carcinoma. *Cancer Lett* 1997; 111: 191-197

44 *Di Leonardo A,* Linke SP, Clarkin K, Wahl GM. DNA damage triggers a prolonged p53-dependent GI arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes Dev* 1994; 8: 2540-2551

45 *Gartel AL,* Serfas MS, Tyner AL. p21--negative regulator of the cell cycle. *Proc Soc Exp Biol Med* 1996; 213: 138-149

46 *Pellegrata NS,* Antoniono RJ, Redpath JL, Stanbridge EJ. DNA damage and p53-mediated cell cycle arrest: a reevaluation. *Proc Natl Acad Sci USA* 1996; 93: 15209-15214

47 *Fotedar R,* Fitzgerald P, Rouselle T, Cannella D, Dorée M, Messier H, Fotedar A. p21 contains independent binding sites for cyclin and cdk2: both sites are required to inhibit cdk2 kinase activity. *Oncogene* 1996; 12: 2155-2164

48 *Matsuda Y,* Ichida T, Genda T, Yamagisawa S, Aoyagi Y, Asakura H. Loss of p16 contributes to p27 sequestration by cyclin D(1)-cyclin-dependent kinase 4 complexes and poor prognosis in hepatocellular carcinoma. *Clin Cancer Res* 2003; 9: 3389-3396

49 *Matsuda Y,* Molecular mechanism underlying the functional loss of cyclin-dependent kinase inhibitors p16 and p27 in hepatocellular carcinoma. *World J Gastroenterol* 2008; 14: 1734-1740

50 *Jing Z,* Nan KJ, Hu ML. Cell proliferation, apoptosis and the related regulators p27, p53 expression in hepatocellular carcinoma. *World J Gastroenterol* 2005; 11: 1910-1916

51 *Lei PP,* Zhang ZJ, Shen LJ, Li JY, Zou Q, Zhang HX. Expression and hypermethylation of p27kip1 in hepatocarcinogenesis. *World J Gastroenterol* 2005; 11: 4587-4591

52 *Shehata MA,* Nosseir HR, Nagy HM, Farouk G. Cyclin dependent kinase inhibitor p27(kip1) expression and subcellular localization in relation to cell proliferation in hepatocellular carcinoma. *Egypt J Immunol* 2006; 13: 115-130

53 *Filipits M,* Puhlalla H, Wbra F. Low p27Kip1 expression is an independent prognostic factor in gallbladder carcinoma. *Anticancer Res* 2003; 23: 675-679

54 *Sgambato A,* Ratto C, Faraglia B, Merico M, Ardito R, Schinazi G, Romano G, Cittadini AR. Reduced expression and altered subcellular localization of the cyclin-dependent kinase inhibitor p27(kip1) expression in human colon cancer. *Mol Carcinog* 1999; 26: 172-179

55 *Beijersbergen RL,* Bernards R. Cell cycle regulation by the retinoblastoma family of growth inhibitory proteins. *Biochim Biophys Acta* 1996; 1287: 103-120

56 *Classon M,* Harlow E. The retinoblastoma tumour suppressor in development and cancer. *Nat Rev Cancer* 2002; 2: 910-917

57 *Bagui TK,* Mohapatra S, Haura E, Pledger WJ. P27Kip1 and p21Cip1 are not required for the formation of active D cyclin-cdk4 complexes. *Mol Cell Biol* 2003; 23: 7285-7290

58 *Yonemura Y,* Ohyama S, Kimura H, Kamata T, Matsumoto H, Yamaguchi A, Kosaka T, Miwa K, Miyazaki I. The expression of proliferative-associated nuclear antigen p105 in gastric carcinoma. *Cancer* 1991; 67: 2525-2528

S-Editor Zhang HN L-Editor Herholdt A E-Editor Liu N