Available online at www.tox.or.kr

# Increased Expression of Cyclin D3 are Involved in Hepatocellular Carcinoma

**Gi Jin Kim[1], Woong Sun[2], Nam-Hee Won[3] & Sun-Hwa Park[2]**

[1]Graduate School of Life Science and Biotechnology, Pochon CHA University College of Medicine, Seoul 135-081, Korea
[2]Department of Anatomy, Korea University College of Medicine, Seoul 136-705, Korea
[3]Department of Pathology, Korea University College of Medicine, Seoul 136-705, Korea
Correspondence and requests for materials should be addressed to S. H. Park (parksh@korea.ac.kr)

Accepted 7 March 2008

## Abstract

**Human cyclin D3 gene (*CCND3*) located on 6p21.1 is important for the regulation of the G1-S phase transition of the cell cycle by modulating the activity of the cyclin-dependent kinases Cdk4 and Cdk6. Because little is known about the effect of cyclin D3 in various human cancers, we evaluated the intricate relationship between expression of cyclin D3 and the process of HCC development using immunohistochemistry and TUNEL assay on 43 paraffin embedded tissues. Cyclin D3 immunoreactivity was more frequently observed in the tumors with high histologic grade and the tumors with metastasis, and more frequently expressed in HCCs with cirrhotic background and gain of 6p21.1 when compared with those with non-neoplastic tissue. Apoptotic cells were more common in tumor with cirrhotic background, amplification of 6p21.1 and expression of cyclin D3 when compared with HCCs with lower level of cyclin D3 expression. Also, we observed that some of the cyclin D3 positive cell and apoptotic cell were co-localized. From these results, it is suggested that over-expression of cyclin D3 may contribute to more rapid cell turn-over in the background of HCC, and balance between proliferation and apoptosis is a role in the progression of HCC with cirrhotic background.**

**Keywords:** Hepatocellular carcinoma, Cyclin D3, Proliferation, Apoptosis

Hepatocellular carcinoma (HCC) is one of the most frequent hepatic malignant tumors in Korea, China, and sub-Saharan Africa[1]. Some specific genetic alterations in HCC are now recognized, but the relationship between the genetic imbalances and molecular mechanisms of HCC development remains unclear until now. Especially, the most important factors that are affected in HCC were reported such as the retinoblastoma (Rb) gene, the INK4a-ARF (p14-p16) and cyclins[2]. Also, the relationship between cell cycle and apoptosis is very important for the organogenesis of normal tissues as well as progression of tumors[3]. For instance, recently we reported the genetic alterations and several recurrent chromosomal abnormalities in HCC using degenerate oligonucleotide primed PCR-comparative genomic hybridization (DOP-PCR-CGH) analysis[4]. In previous study, overrepresentation of 6p with an overlapping region 6p21-p23 was frequently observed in Korean HCC population. To date, there have been several reports demonstrating the recurring chromosomal gains of 6p in HCC by CGH[5,6]. These results suggest that gene(s) within 6p21-p23 might be involved in the HCC tumorigenesis. Because cell-cycle regulation is critical for tumorigenesis, cyclin D3 gene, which is located on 6p21, is one of the important candidates for HCC tumorigenesis.

Cyclin D3 shares considerable homology with cyclin D1 and cyclin D2 and promotes progression through the G1 phase of the cell cycle by regulating the activity of the cyclin-dependent kinases Cdk4 and Cdk6. Most studies have focused on the role of cyclin D1, which is oncogene in the mitogenic stimulation of cells. The expression patterns of cyclin D1 and cyclin D3 were different in development of normal tissues and tumorigenesis[7,8]. Cyclin D3 had a dual function in proliferation as well as differentiation[9]. The biological significance of cell-cycle control in hepatocellular carcinoma has been explored in some studies, but the significance of cyclin D3 on clinical outcome for HCC was not studied so far.

In this recurrent study, we selected cyclin D3 on chromosome 6p21.1 region with overrepresentation and evaluated the expression pattern of cyclin D3 on 43 HCCs including twenty-seven of HCC with cir-

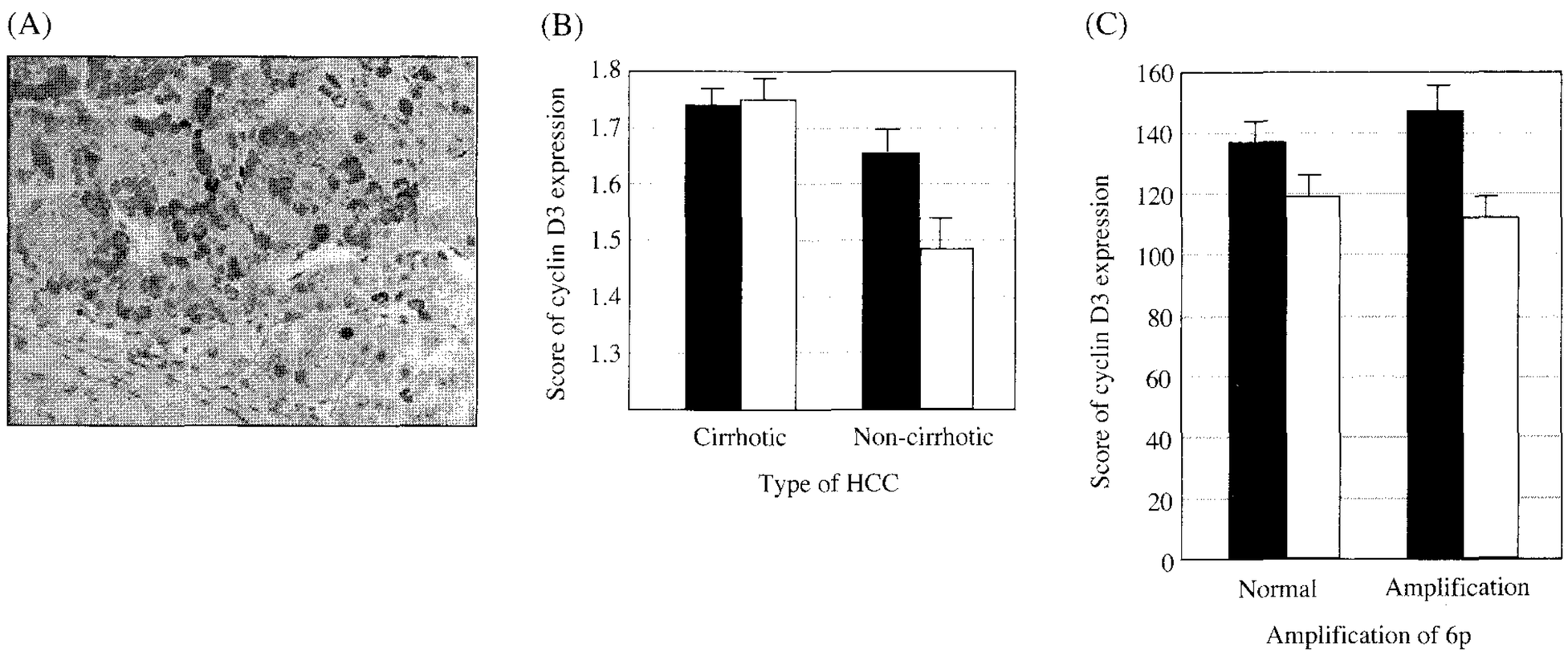


**Figure 1.** Expression of cyclin D3 in hepatocellular carcinoma. Representative immunohistochemical staining for cyclin D3 (A, arrow), the correlation for cyclin D3 expression between cirrhotic and non-cirrhotic background (Black), and tumor region (White) (B), expression of cyclone D3 between normal and 6p amplification in cirrhotic and non-cirrhotic background (Black), and tumor region (White), (C). Original magnification 200X.

rhosis (cirrhotic HCC) and sixteen of HCC without cirrhosis (non-cirrhotic HCC) using immunohistochemical staining and TUNEL assay with special reference to its possible relationship with apoptosis.

The objective of this study is to examine whether cyclin D3 protein is increased in the HCC tissues, and to further characterize functional role of cyclin D3 in the HCC tumorigenesis.

## Expression of Cyclin D3 in Hepatocellular Carcinoma

Formalin-fixed paraffin-embedded sections of 43 of HCC with (n=27) or without (n=16) cirrhosis were analyzed by immunohistochemistry. Immunoreactivity for cyclin D3 was found in both non-neoplasmic and neoplastic tissues. Heterogeneous nuclear staining was predominant, and in some cases, cytoplasmic staining was detected together with nuclear staining (Figure 1A). Cyclin D3 immunoreactivity was also observed in endothelial cells, bile duct epithelium, Kuffer cell, and hepatocyte in HCC.

Thirty-four tumors were positive for cyclin D3 (++/+++), while nine tumor cases were negative (0/+). The profile of immunoreactivity and other histological parameters are summarized in Table 1. Expression of cyclin D3 was more frequently observed in the tumors with high histologic grade, and the tumors with metastasis. Cyclin D3 immunoreactivity was found in twenty-two (51.1%) tumors and twenty-four (60%) non-neoplastic background. Cyclin D3 immunoreactivity was found in similar proportion of 16 samples with the cirrhotic background (61.5%, score =1.73) and 8 samples with the non-cirrhotic background (61.5%, score=1.65) (Table 1, Figure 1B). Although the values of scores between cirrhotic background and non-cirrhotic background are difference, there is no significantly difference ($P<0.91$). But, cyclin D3 was highly expressed in the 15 tumors with amplification of 6p21 region (88.2%) when compared with the 18 tumors without (69.3%). Especially, all the cirrhotic background of tumors with amplification of 6p21 region was cyclin D3 positive (Table 1, Figure 1C).

## The Relationship between Apoptosis and Cyclin D3 Protein Expression in HCC

For the analysis of the relationship between apoptosis and cyclin D3 expression, we carried out TUNEL assay. Because two of cases were failed, apoptotic index was analyzed in forty-one of tumor samples.

Apoptosis was more frequent in the tumors with grade II and III (Figure 2A). Twenty-five (60.9%) of forty-one tumors were 2+ or 3+, while fourteen (41%) of thirty-four backgrounds region of tumor samples were grade 2+ or 3+. The number of apoptotic cell was high in cirrhotic HCC (16.2%) than in non-cirrhotic HCC (7.94%) ($P<0.001$, Figure 2B). An apoptosis in the cyclin D3 positive tumors was found in eleven of twenty tumor samples, while apoptosis of the cyclin D3 negative tumor was found in fourteen of twenty one cases ($P<0.005$, Figure 2C). Apoptosis of the cyclin D3 positive and negative background region was found in ten out of twenty-one samples and four out of twelve samples, respectively.

**Table 1.** Summarized in expression of cyclin D3 in hepatocellular carcinoma.

| Groups | | Expression of cyclin D3 | | | |
|---|---|---|---|---|---|
| | Types | Negative | Low | Moderate | Strong |
| Size (cm) | < 3.0 (n=8) | 0 | 1 (12.5%) | 2 (25%) | 5 (62.5%) |
| | > 3.0 (n=30) | 1 (3.3%) | 7 (23.3%) | 10 (33.4%) | 12 (40%) |
| | Add (n=5) | | | | |
| Grade | I (n=2) | 0 | 0 | 2 (100%) | 0 |
| | II (n=13) | 1 (7.7%) | 4 (30.8%) | 2 (15.4%) | 6 (46.1%) |
| | III (n=23) | 0 | 4 (17.4%) | 8 (34.8%) | 11 (47.8%) |
| | Add (n=5) | | | | |
| Metastasis | 0 | | | | |
| | 1 (n=22) | 1 (4.5%) | 5 (22.7%) | 8 (36.4%) | 8 (36.4%) |
| | 2 (n=6) | 0 | 1 (16.7%) | 3 (50%) | 2 (33.3%) |
| | Unknown (n=10) | | | | |
| | Add (n=5) | | | | |
| Region | Tumor (n=43) | 6 (14%) | 15 (34.9%) | 8 (18.6%) | 14 (32.5%) |
| | Background (n=40) | 1 (2.5) | 15 (37.5%) | 20 (50%) | 4 (10%) |
| | Unknown (n=3) | | | | |
| Subtype of HCC background | Cirrhotic HCC (n=26) | 1 (3.8%) | 9 (34.7%) | 13 (50%) | 3 (11.5%) |
| | Unknown (n=1) | | | | |
| | Non-cirrhotic HCC (n=13) | 0 | 5 (38.5%) | 7 (53.9%) | 1 (7.6%) |
| | Unknown (n=3) | | | | |
| 6p | Normal (n=26) | 1 (3.8%) | 7 (26.9%) | 7 (26.9%) | 11 (42.4%) |
| | Amplification (n=17) | 0 | 2 (11.8%) | 8 (47.0%) | 7 (41.2%) |
| Combination | Cirrhotic (n=17) | 0 | 5 (29.4%) | 5 (29.4%) | 7 (41.2%) |
| | Cirrhotic/6p21+, (n=10) | 0 | 0 | 5 (50%) | 5 (50%) |
| | Non-cirrhotic (n=9) | 1 (11.1%) | 2 (22.2%) | 2 (22.2%) | 4 (44.5%) |
| | Non-cirrhotic/6p21+, (n=7) | 0 | 2 (28.6%) | 3 (42.9%) | 2 (28.6%) |

In order to confirm the relationship between cyclin D3 and apoptosis, we carried out double immunofluorescence. Staining with the cyclin D3 showed mainly heterogeneous nuclear staining, and occasionally, cytoplasmic staining (Figure 3A). The staining pattern of SC-45 was cytoplasmic (Figure 3B). Nucleus was stained by DAPI and co-localization of cyclin D3 and SC-45 was found in both tumor and background region of HCC (Figure 3C, 3D).

## Discussion

Carcinogenesis of HCC was involved in various factors, which were viral infection, expose of carcinogene, disfuction of cell cycle control and DNA repair system, environmental factor, and multistage[3,10]. Although, until now, specific genetic changes have not been identified for these stages of carcinogenesis, the genetic changes little identified so far include amplification of proto-oncogene, gene mutation or deletion of tumor suppressor gene, and reactivation of telomerase activity.

Since previous studies were reported that the accumulation of various chromosomal aberrations were involved in carcinogenesis of HCC, many research are continued for identification of chromosomal aberration using various molecular tools such as FISH, CGH, LOH, and mutation analysis[3,4]. Especially, because the function of cell cycle related genes were very important factors in progression of cancer, the relationship between cyclins and CDKs including CDKi was studied in HCC as well as various cancers[11-13].

In previous study, we observed various chromosomal aberrations of cell cycle related genes. Interestingly, amplification of 6p21 containing cyclin D3 (CCND3) was frequently observed in seventeen (34%) of fifty cases of HCC[4]. Cyclin D3 shares considerable homology with cyclin D1 and D2 and are considered activating cyclin-dependent kinases (CDK) 4 and/or 6 to promote G1 to S phase transition by phosphorylating RB protein[8,14]. Most studies have focused on the role of cyclin D1 in tumor progression, and little is known about the contribution of cyclin D3. Especially, cyclin D1 and cyclin D2 were highly expressed in hyperplasia containing proliferating cells, but cyclin D3 was expressed in proliferating and differentiating

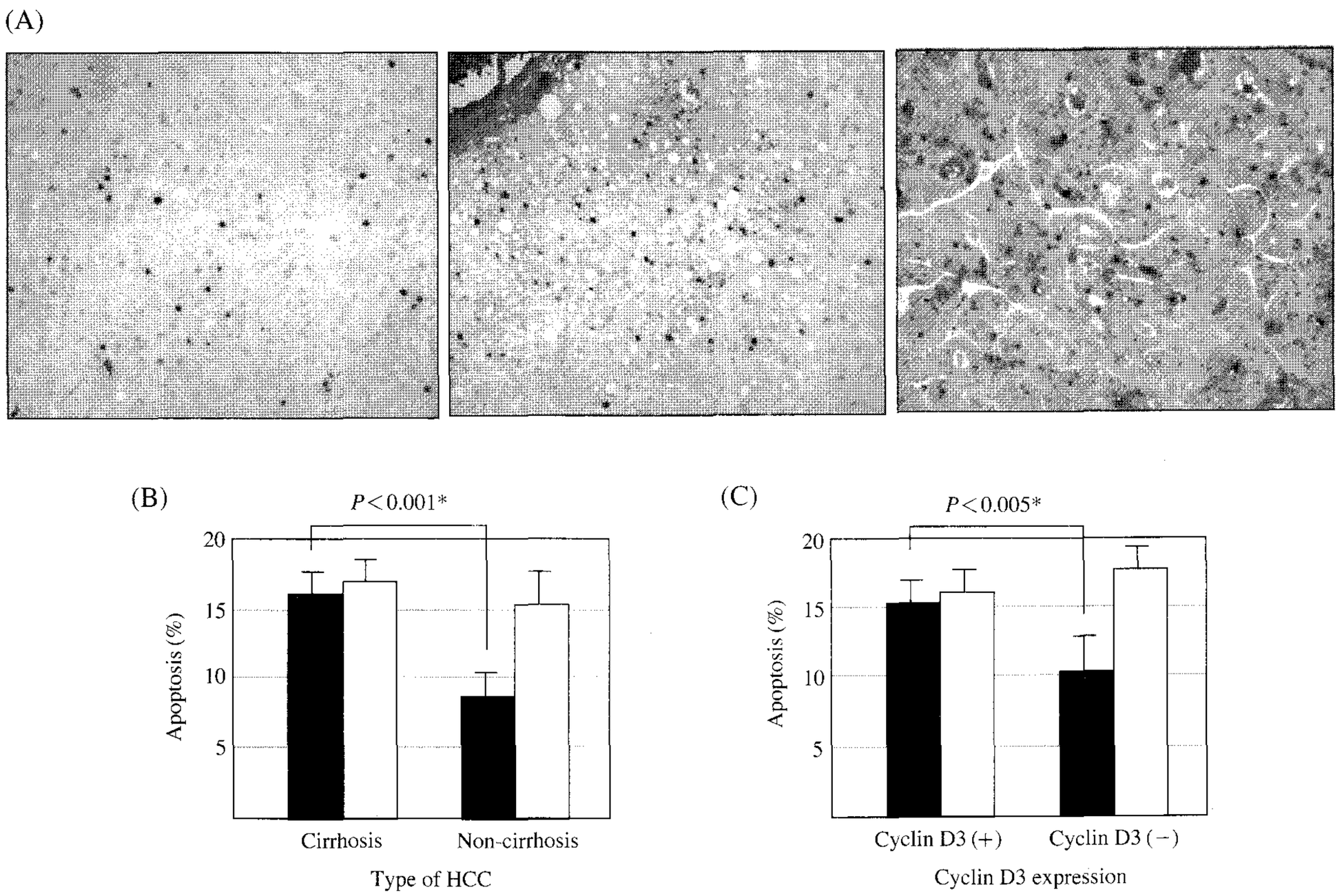


**Figure 2.** Apoptosis in hepatocellular carcinoma using TUNEL assay. Normal liver (left panel), non-cirrhotic background HCC (middle panel), and cirrhotic background (right panel) (A, arrow), the percentages of apoptosis between cirrhotic and non-cirrhotic background (Black), and tumor region (White) (B), the percentages of apoptosis between cyclin D3-positive group and cyclin D3-negative group in cirrhotic and non-cirrhotic background (Black), and tumor region (Red) (C). Original magnification 200X.

cells[9]. Furthermore, expression patterns of cyclin D1 and cyclin D3 were different during the development of tissues and tumors[15]. The expression patterns of cyclin D1 and various cell cycle regulators were reported in HCC, but expression of cyclin D3 in HCC has not been described yet[16].

Cyclin D3 was mainly expressed in the nuclei, and in some cases, cytoplasmic staining was detected together with nuclear staining. Wang *et al.* (1996) reported that the cellular localization of cyclin D3, which was nuclear protein, in mice tissues might be involved in the function of cyclin D3[17]. The underlying mechanism for regulation of many processes in the cell is the localization of protein complexes within specific subcellular compartments. Some cyclin-CDK complexes are subject to this mode of regulation[18,19]. Although cyclin D3 associate with protein kinases (Cdk4) or interact with other proteins located in the cytoplasm was reported, its functions was not clear[20,21].

Expression of cyclin D3 positively correlated with high histologic of grade and metastasis. These data are similar to these of previous reports[22,23]. Expression of cyclin D3 was increased in the tumors with amplification of 6p21 region (88.2%) when compared with the tumors without amplification of 6p21 region (69.3%). Especially, all the cirrhotic background of tumor with amplification of 6p21 region was cyclin D3 positive. Although there are many genes on chromosome 6p21 region, only cyclin D3 was cell cycle regulator. In general, staining intensity correlated with the amplification of 6p21, but many tumors over-expressing cyclin D3 showed only marginal or undetectable increases in copy number, implying that other mechanisms can lead to deregulated expression. These data suggested that cyclin D3 (*CCND3*) gene was amplified in cirrhotic background of HCCs, thereby play a significant role in progression of cancer.

Several reports described that apoptotic index is, in general, increased in tumors and an increase of apoptosis are related to the activation of oncogenes or tumor suppressors during carcinogenesis[24,25]. Recent studies reported that cyclin D3 activates caspase 2, and then expression of cyclin D3 is inducing apoptosis in yeast[26]. During the stage of tumor progression,

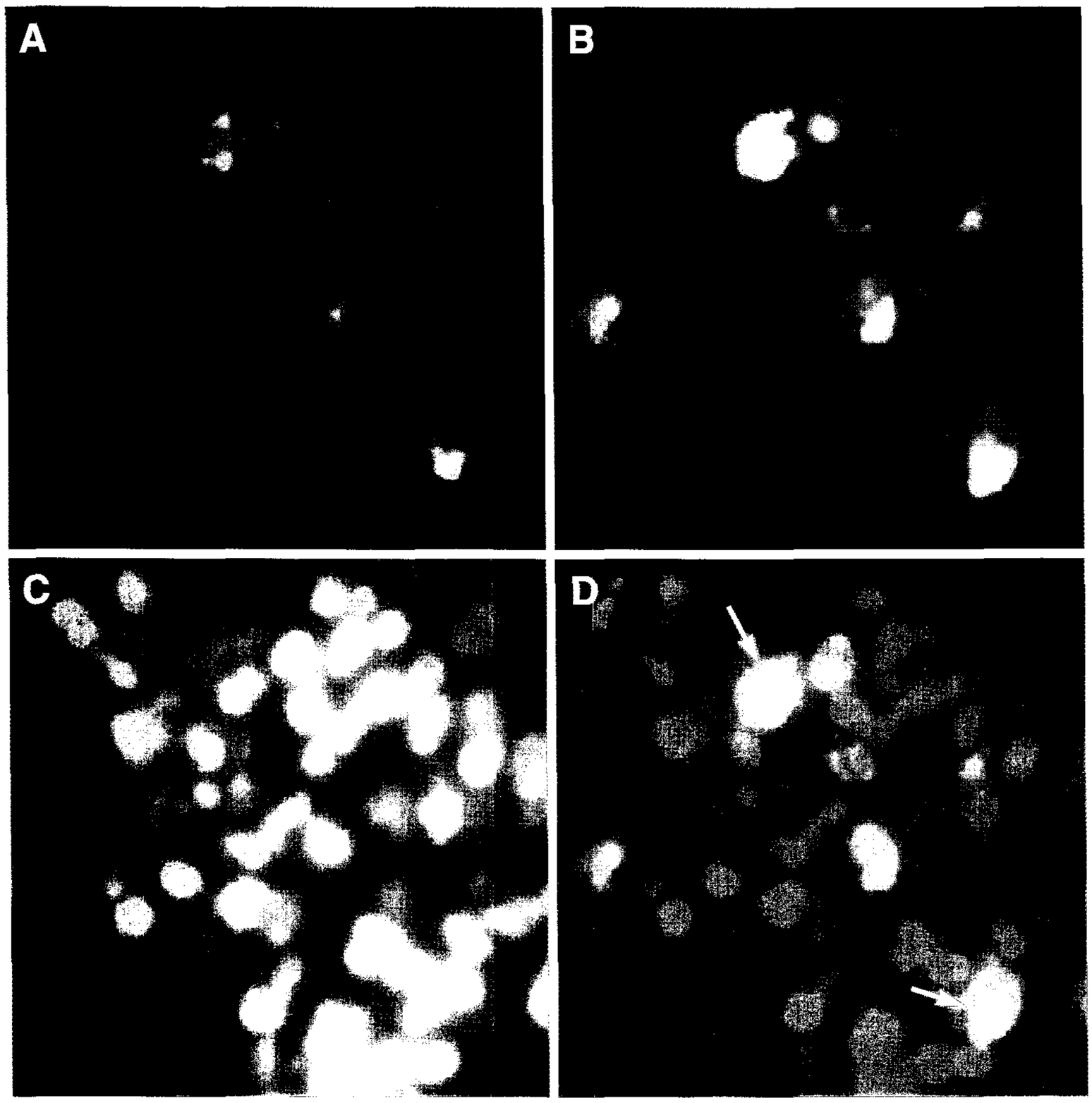


**Figure 3.** Co-localization of cyclin D3 and SC-45 in hepatocellular carcinoma using double immunofluorescence. cyclin D3 positive cell (Green, A), SC-45 positive cell (Red, B), Nucleus staining (Blue, C), cyclin D3 and SC-45 positive cell (D, arrow). Original magnification 200X.

the rate of apoptosis appears to increase with increasing cell proliferation[27,28]. So, the relationship between expression of cyclin D3 and apoptosis in HCC was evaluated using TUNEL assay. Also, co-localization of cyclin D3 and SC-45 was found in HCC using double immunofluorescence. The results strongly suggest that cyclin D3 may play a role in apoptosis as well as proliferation and differentiation in cancer.

From these data, over-expression of cyclin D3 in HCC with cirrhotic background induces apoptosis through more rapid turnover of cell cycle and may be involved in the pathway of tumor progression through the regulation of cell cycle and the balance between proliferation and apoptosis.

## Methods

### Tumor Specimens

A total of 43 HCC specimens including 27 of cirrhotic HCC and 16 of non-cirrhotic HCC were obtained from the files of Department of Pathology Korea University College of Medicine, and the Department of Pathology, University of Hallym College of Medicine between 1995 and 2001. Paraffin sections 5 μm thick were cut, mounted on poly-L-lysine-coated glass slides and then heated for 1 hour at 58°C.

### Immunohistochemistry

Immunohistochemical staining was performed using the VECTARSTAIN ABC system (Vector laboratories, Burlingame, CA, USA) and the DAB substrate kit according to the manufactures' instructions with slight modifications. Briefly, deparaffinzed tissues sections were first treated with fresh 3% $H_2O_2$ in methanol followed by incubation with blocking serum for 30 min. The sections were then reacted with either 1 : 100 diluted primary antibody against cyclin D3 (Oncogene, Boston, MA, USA, CC13) or with buffer alone at 4°C for overnight in a humidified chamber. After further incubation with biotinylated linked antibody and horseradish peroxidase conjugated streptavidin-biotin complex, the staining was developed by reaction with diaminbenzine (DAB) substrate-chro-

mogen solution followed by counterstaining with Mayer hematoxylin. Antigen retrieval was performed using heat-pressure cooker for 7 minutes in 10 mM citrate buffer (pH 6.0). Positive reaction with DAB were identified as a dark brown reaction product on the cell and the specimens were graded as negative, - (<5%); weakly, + (5-15%); intermediate, ++ (15-50%); strong positive, +++ (>50%) based on both the percentage of stained cells. The specimens with intermediate or strong IHC positive were considered to have cyclin D3 expression. Immunoreactivity of cyclin D3 was semi-quantitatively analyzed using an immunostaining score. The immunostaining score was calculated by multiplying the intensity score (negative: 0, weakly positive: 1, strong positive: 2) by the fraction (%) of immunopositive cells.

### TUNEL Assay

Apoptotic cells were detected by the TUNEL technique using a TUNEL detection kit and NBT and BCIP according to manufacturer's instructions. In brief, paraffin sections were dewaxed, rehydrated in graded alcohol, and washed twice with D.W. The sections were incubated in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate) for 8 minutes. The slides were then treated with 50 μL of TUNEL reaction mixture with terminal deoxytransferase containing dUTP-FITC for 30 minutes at 37°C in a humidified chamber. The detection began by adding anti-fluorescein antibody conjugated with alkaline phosphatase (AP) solution on the slides to signal inversion. The slides were incubated for 10 minutes at room temperature. NBT/BCIP substrate solution was used for chromogenic reaction and counterstaining was done with Nuclear Fast Red solution for 2 minutes. The sections of lymph node treated with DNase 1 for 30 minutes at room temperature were used for positive control, and the TdT enzyme was replaced with PBS buffer for negative control. The number of TUNEL-positive cells in tumor and adjacent background cells was obtained by evaluating four fields under a microscope at X200 magnification. The apoptotic index was presented as the average percentage of apoptotic cells per field.

### Double Immunofluorescence

Five μm sections were deparaffinized with xylene and rehydrated with graded ethanol. Sections were placed in 0.01 M citrated buffer (pH 6.0) and then were boiled for 7 minutes. Sections were incubated in 5% horse normal blocking serum solution with 0.2% TritonX-100 at room temperature for 30 minutes in humidified chamber and then subsequently incubated together with cocktail solution of 1 : 100 diluted monoclonal anti-cyclin D3 (CC13, Oncogene, USA) and 1 : 500 diluted polyclonal SC-45 (Santa Cruze, USA), which as known recognized caspase-3 substrate, at 4°C overnight in a humidified chamber. Following three rinses with PBS, the sections were incubated with secondary antibodies solution containing 1 : 500 diluted goat Alexa 488-conjugated anti-mouse IgG (Molecular probes) and Cy3-conjugated anti-rabbit IgG (Jaxon), respectively, at room temperature for 30 minutes. Cell nucleus was counterstained with Hoechst 33342 (1 μg/mL, Molecular probes, Eugene, OR). Finally, the slides were washed three times with PBS, mounted with aqueous mount, and observed by Axiovert 2000 microscope (ZEISS, Germany).

### Statistics

Immunohistochemical scoring for cyclin D3 expression analyzed using Chi-square test and Mann-Whitney U test. Results were considered to be significant when $P$ was $<0.05$.

## Acknowledgements

This work was supported by a grant from Medical Science Research Center Korea University and the Brain Korea 21 Project. We also thank Dr. Seong-Jin Cho (Department of Pathology, University of Hallym College of Medicine) for his pathological comments.

## References

1. Llovet, J. M., Burroughs, A. & Bruix, J. Hepatocellular carcinoma. *Lancet* **362**:1907-1917 (2003).
2. Suh, S. I., Pyun, H. Y. & Carson, D. A. 5-Aza-2′-deoxycytidine leads to down-regulation of aberrant p16INK4A RNA transcripts and restores the functional retinoblastoma protein pathway in hepatocellular carcinoma cell lines. *Cancer Lett* **160**:81-88 (2000).
3. Tannapfel, A. & Wittekind, C. Genes involved in hepatocellular carcinoma: deregulation in cell cycling and apoptosis. *Virchows Arch* **440**:345-352 (2002).
4. Kim, G. J. *et al.* Genomic imbalances in Korean hepatocellular carcinoma. *Cancer Genet Cytogenet* **142**: 129-133 (2003).
5. Hwang, H. J. *et al.* A comprehensive karyotypic analysis on Korean hepatocellular carcinoma cell lines by cross-species color banding and comparative genomic hybridization. *Cancer Genet Cytogenet* **141**:128-137 (2003).
6. Guan, X. Y. *et al.* Recurrent chromosome alterations in hepatocellular carcinoma detected by comparative genomic hybridization. *Genes Chromosomes Cancer* **29**:110-116 (2000).
7. Rougier, P., Mitry, E. & Taieb, J. Hepatocellular car-

cinoma (HCC): an update. *Semin Oncol* **34**:S12-20 (2007).
8. Motokura, T., Keyomarsi, K. & Arnold, A. Cloning and characterization of human cyclin D3, a cDNA closely related in sequence to the PRAD1/cyclin D1 proto-oncogene. *J Biol Chem* **267**:20412-20415 (1992).
9. Bartkova, J., Lukas, J. & Bartek J. Cyclin D3: requirement for G1/S transition and high abundance in quiescent tissue suggest a dual role in proliferation and differentiation. *Oncogene* **17**:1027-1037 (1998).
10. Feitelson, M. A. *et al.* Genetic mechanisms of hepatocarcinogenesis. *Oncogene* **21**:2593-2604 (2002).
11. Hui, A. M., Makuuchi, M. & Li, X. Cell cycle regulators and human hepatocarcinogenesis. *Hepatogastroenterology* **45**:1635-1642 (1998).
12. Li, K. K. *et al.* Activation of cyclin-dependent kinases CDC2 and CDK2 in hepatocellular carcinoma. *Liver* **22**:259-268 (2002).
13. Ortega, S., Malumbves, M. & Bavbacid, M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim Biophys Acta* **1602**:73-87 (2002).
14. Inaba, T. *et al.* Genomic organization, chromosomal localization, and independent expression of human cyclin D genes. *Genomics* **13**:565-574 (1992).
15. Doglioni, C. *et al.* Cyclin D3 expression in normal, reactive and neoplastic tissues. *J Pathol* **185**:159-166 (1998).
16. Wong, S. C., Chan, J. K. & Hsiao, W. Differnetial expression of p16/p21/p27 and cyclin D1/D3, and their relationships to cell proliferation, apoptosis, and tumour progression in invasive ductal carcinoma of the breast. *J Pathology* **194**:35-42 (2001).
17. Wang, A., Sicinski, P. & Ravid, K. Characterization of the mouse cyclin D3 gene: exon/intron organization and promoter activity. *Genomics* **3**:156-163 (1996).
18. Pines, J. & Hunter, T. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle -dependent nuclear transport. *J Cell Biol* **115**:1-17 (1991).
19. Baldin, V. & Ducommun, B. Subcellular localisation of human wee1 kinase is regulated during the cell cycle. *J Cell Sci* **108**:2425-2432 (1995).
20. Kang, M. J. *et al.* Cytoplasmic localization of cyclin D3 in seminiferous tubules during testicular development. *Exp Cell Res* **234**:27-36 (1997).
21. Lazaro, J. B., Bailey, P. J. & Lassar, A. B. Cyclin D-cdk4 activity modulates the subnuclear localization and interaction of MEF2 with SRC-family coactivators during skeletal muscle differentiation. *Genes Dev* **16**:1792-1805 (2002).
22. Hedberg, Y., Roos, G. & Landberg, G. Cyclin D3 protein content in human renal cell carcinoma in relation to cyclin D1 and clinico-pathological parameters. *Acta Oncol* **41**:75-181 (2002).
23. Ito, Y., Takeda, T. & Matsuura, N. Expression and possible role of cyclin D3 in human pancreatic adenocarcinoma. *Anticancer Res* **21**:1043-1048 (2001).
24. Brown, C., Sauvageot, J. & Epstein, J. I. Cell proliferation and apoptosis in prostate cancer-correlation with pathologic stage? *Mod Pathol* **9**:205-209 (1996).
25. Soini, Y., Paakko, P. & Lohto, V. P. Histopathologic evaluation of apoptosis in cancer. *Am J Pathol* **153**: 1041-1053 (1998).
26. Mendelsohn, A. R., Hamer, J. D. & Brent, R. Cyclin D3 avtivates caspase 2, connecting cell proliferation with cell death. *PNAS* **99**:6871-6876 (2002).
27. Grasl-Kraupp, B. *et al.* Inherent increase of apoptosis in liver tumors: implications for carcinogenesis and tumor regression. *Hepatology* **25**:906-912 (1997).
28. Hino, N., Higashi, T. & Tsuji, T. Apoptosis and proliferation of human hepatocellular carcinoma. *Liver* **16**:123-129 (1996).