PROGNOSTIC SIGNIFICANCE OF p21 AND p53 EXPRESSION IN HEPATOCELLULAR CARCINOMA

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Abstract : The cyclin-dependent kinase inhibitor p21Waf1/Cip1(p21), which can be transcriptionally activated by p53-dependent and -independent manners, functions to block cell cycle progression. In this study, we analyzed the expression of p21, p53 and proliferating cell nuclear antigen (PCNA) in 85 patients with hepatocellular carcinoma (HCC) by immunohistochemistry, and examined whether expression of these proteins was related to prognosis in patients with HCC. In HCC, p21 positive tumors significantly showed high PCNA LI and small size, compared with p21 negative tumors. No relationship between p21 and p53 expression was detected. A multivariate Cox model analysis revealed p21, p53 expression and PCNA LI as independent prognostic factors ($p=0.0059$, $p=0.0004$, and $p=0.0165$, respectively). Furthermore, p21 expression significantly correlated with low recurrence rate in the p53 negative cases ($p=0.020$) and related to a good outcome in the high PCNA LI cases ($p=0.056$). Accordingly, the analysis of p21, p53 and PCNA could play an important role in early detection of intrahepatic recurrence and might contribute to improvement of the prognostic characterization.

Key words: Hepatocellular carcinoma, p53, p21, PCNA

INTRODUCTION

Regulation of the cell cycle plays an important role in normal cell growth and differentiation, and in tumor progression. The sequential formation, activation, and subsequent inactivation of a series of cyclin-cyclin dependent kinase (cdk) complexes regulate the checkpoints that control the cell cycle progression[1,2]. The activation of cyclin-cdk complexes progresses the cell cycle while the cdk-inhibitory proteins, which bind to and inactivate cyclin-cdk complexes, block the cell cycle progression[3]. Disruption of the checkpoints in the cell cycle is one mechanism by which abnormal tumor cells can proliferate.

The p53 gene product can act as a transcription factor with an important role in controlling cell proliferation and differentiation[4]. Mutations of the p53 gene contribute to development of up to 50 % of all human cancers, including hepatocellular carcinoma (HCC)[5,6]. As a transcription factor, p53 mediates growth suppression by altering the expression of other key molecules[7]. One of such genes, p21Waf1/Cip1 (p21), has been cloned and shown to mediate some of the key functions of p53[8]. Interestingly, two other independent studies led to the discovery of the same p21 gene[9-11]. The p21 promoter contains p53 binding sites, and p21 transcription can be induced by the p53 protein. Therefore, p21 is thought to be a key downstream mediator of p53-induced cell cycle arrest and apoptosis[8]. The p21 protein plays

an important role in cellular differentiation and senescence[11,12]. p21 also directly inhibits the proliferating cell nuclear antigen (PCNA)-dependent DNA synthesis in the absence of cdks[13]. Accordingly, p21 is strongly suggested to make an important contribution to inhibiting the proliferative activity of cancer cells and consequently disease progression of malignant tumors. There are few reports about p21 expression in HCCs and Hui et al.[14] demonstrated that the expression of p21 messenger RNA was dependent upon normal p53 function in HCCs, but they did not refer to the correlation between p21 expression and the clinicopathological features. On the other hand, recent studies indicated that p53-independent pathways may also lead to increased p21 expression[15,16]. Furthermore, there are few reports about the correlation between p21 and PCNA expression, and this correlation is still controversial[17,18]. So, we investigated the expression of p21, p53 and PCNA proteins by immunohistochemical procedures, analyzed the relationship among p21, p53 and PCNA in HCC, and evaluated whether the expression of these proteins correlated with the clinicopathological features including postoperative recurrence.

MATERIAL AND METHODS

Patients

Eighty-five patients underwent hepatic resection at the First Department of Surgery, Nara Medical University Hospital. All cases were diagnosed histologically. The patients after hepatic resection were strictly followed up with monthly measurement of alpha-fetoprotein, ultrasonography and dynamic computed tomography every 3 months, and angiography when recurrence was strongly suspected. The median follow-up period for the total number of patients was 36.9 months, with a range of 4-88 months.

Immunohistochemical Staining

The resected specimens were fixed in 10% phosphate buffered formalin and embedded in paraffin. For each case, all available hematoxylin eosin stained sections were reviewed, and a representative block was selected for additional studies. Four-micron sections were cut from the paraffin blocks. In brief, the deparaffined sections were treated in a microwave oven for 10 minutes for antigen retrieval and the endogenous peroxidase activity was blocked with methanol containing 3% hydrogen peroxidase for 20 minutes. The sections were incubated overnight at 4 ℃ with mouse monoclonal antibodies against p53 (DO-7 : Dako Co., Glostrup, Denmark ; 1 : 60 dilution), p21 (WAF1 : Oncogene Research Products, Gaithersburg, USA ; 1 : 40 dilution) and PCNA (PC-10 : Dako Co. ; 1 : 100 dilution) after incubation with normal goat serum to reduce nonspecific binding. Immunostaining was performed by the streptavidin-biotin (SAB) method using a Histofine SAB-PO(M) kit (Nichirei Co., Tokyo, Japan). The staining was visualized with diaminobenzidine-tetrachloride. The sections were finally counterstained with hematoxylin. A negative control slide was already included in each immunostaining, in which the first antibody was replaced by normal serum. The sections were assessed independently by two investigators not informed about the patients' outcomes.

p53 expression was evaluated as positive if the stained cells were distributed in more than

20 % of the cancer cells, because some authors have indicated that a diffuse pattern of p53 positive cells could be categorized as overexpression of p53 protein and a marker for carcinoma[19]. The cases with more than 20 % of p53 positive carcinoma cells were considered to be a diffuse pattern. Classification of p21 positivity was followed by that of p53 positivity.

PCNA labeling index

As there was a variation in the number of PCNA-positive cells among the fields, the sections were scanned under low power to determine the areas that were most evenly and heavily labeled. The PCNA labeling index (PCNA LI) was determined by observing 1000 nuclei in the selected areas, and used for the analysis. To reduce any interobserver bias, the mean of the two counts of each observer was considered to represent the PCNA LI.

Statistical analysis

The relationships among the parameters were analyzed with the chi-square test or Mann-Whitney U test. The disease-free survival curves were drawn by Kaplan-Meier method, and comparisons were made by the log-rank test. The Cox proportional hazards model was used to determine the factors in tumor characteristics most significantly related to recurrence. Each variable was transformed into categorical data consisting of two, simple ordinary numbers for multivariate analysis.

RESULTS

Expression of p21, p53 and PCNA protein in HCC

p53 expression was not detected in the non-cancerous liver cells. However, p21 and PCNA positive non-cancerous liver cells were frequently found in the pseudolobules and periportal areas. In HCCs, p21 and p53 expression were positive in 10 (11.8 %) and 20 (23.5 %) of 85 cases (Fig. 1).

Relationship between p21 and clinicopathological features

Table 1 shows the correlation between p21 status and clinicopathological features. The p21 positive cases correlated significantly with small tumor size ($p=0.024$) and p21 positivity was more frequently detected in females than in males ($p=0.051$). Furthermore the p21 positive cases showed high PCNA LI compared with the p21 negative cases ($p=0.083$). The other parameters commonly analyzed in HCCs did not correlate with p21 expression. There was no significant correlation between p21 and p53 expression.

The disease-free survival rate of the p21 positive cases was statistically higher than that of the p21 negative cases (Fig. 2A). Conversely, the p53 positive cases had lower disease-free survival rate than the p53 negative cases ($p=0.009$). Furthermore p21 expression significantly related to a good outcome in the p53 negative cases (Fig. 2B). The mean PCNA LI in 85 HCC cases was 25.6 % , and the patients were divided into two groups as follows : (1) a high PCNA LI group ($\geq$ 25.6 % ; n=37) ; (2) a low PCNA LI group (<25.6 % ; n=48). No difference in the disease-free survival rate was observed between these two groups ($p=0.142$). However, the p21 positive cases tended to have a higher disease-free survival rate than the p21 negative cases in the group of high PCNA LI (Fig. 2C). Table 2 shows the relationship

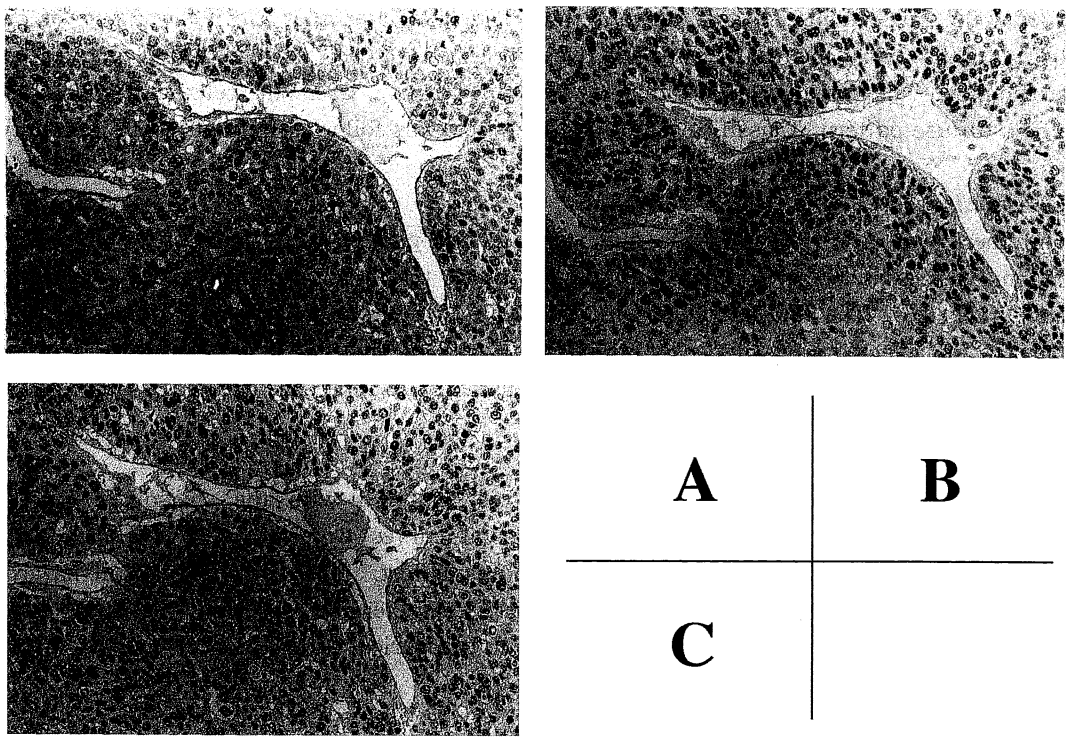

Fig. 1. Nuclear staining for p21 (A), p53 (B) and PCNA (C) in consecutive secitons of moderately differentiated hepatocellular carcinoma (original magnification × 100). High level of p21 p53 and PCNA expression in cancer cells.

Table 1. Clinicopathological features of hepatocellular carcinoma with regard to p21 expression

Factors	p21 positive (n = 10)	p21 negative (n = 75)	p value
Gender (Male/Feemale)	5/5	60/15	0.051[3]
Age (median)	58.5 yr	62.5 yr	N.S.[4]
Hepatitis viral infection (B/C/NBNC)[1]	2/9/1	12/55/9	N.S.[3]
AFP (median)	25.8 ng/ml	36.4 ng/ml	N.S.[4]
Tumor size (median)[2]	2.0 cm	3.0 cm	0.024[4]
PCNA LI (median)	32.1	21.2	0.020[4]
p53 positive cases	40% (4/10)	21.3% (16/75)	N.S.[3]
Cases with multiple tumors	10% (1/10)	20% (15/75)	N.S.[3]
Histology (well/moderately/poorly)[2]	1/4/5	6/49/19	N.S.[3]
Cases with portal invasion	30% (3/10)	60% (45/75)	N.S.[3]
Cases with histological metastatic foci	0%	12% (9/75)	N.S.[3]
Stage Ⅰ/Ⅱ/Ⅲ/Ⅳ	3/4/3/0	12/38/21/4	N.S.[4]

N. S., not statistically significantly

[1] B, positive for hepatitis B surface antigen; C, positive antibody against hepatitis C virus and NBNC, negative B antigen nor C antibodies.

[2] In case of multiple tumors, tumor size and histology are expressed by examining the largest tumor.

[3] chi square test or Fisher exact test.

[4] Mann-Whitney U test.

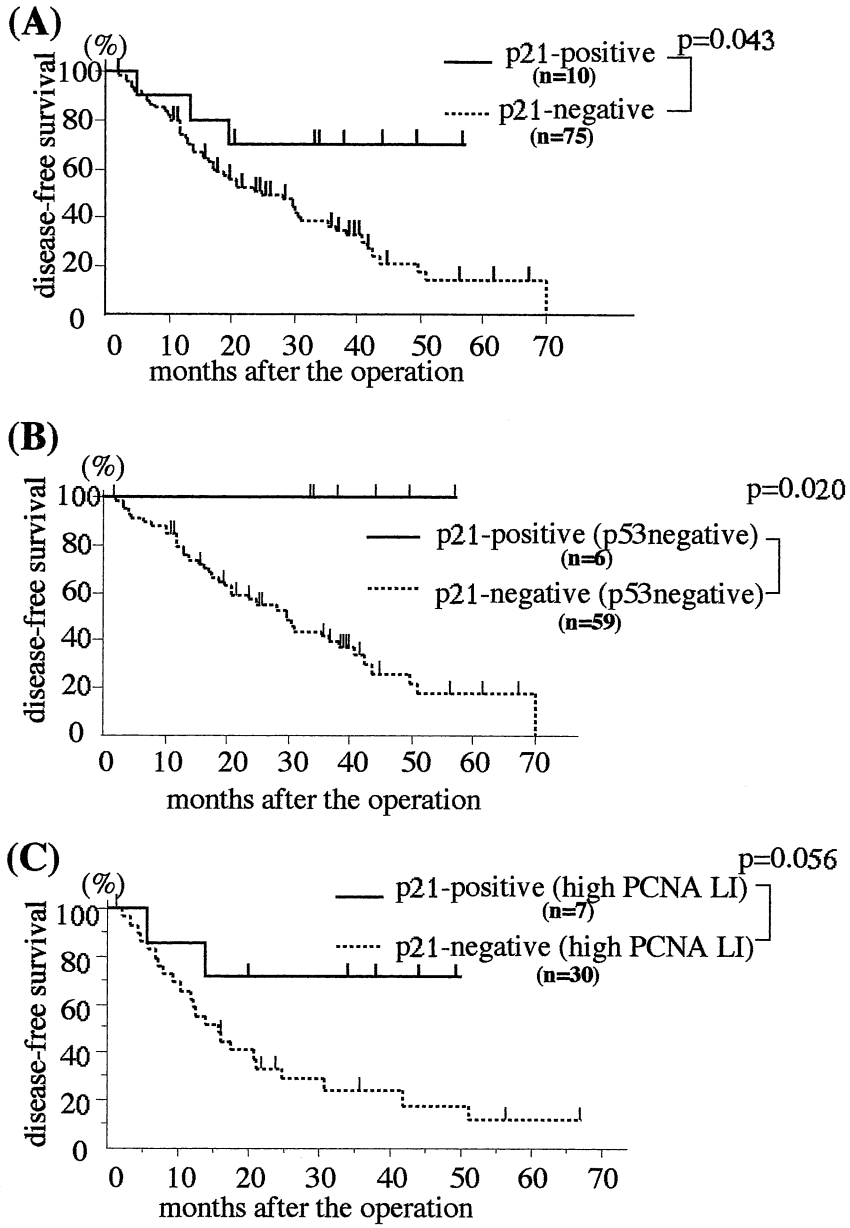

Fig. 2.

(A): Disease-free survial curves for p21-positive and -negative patients, respectively. The disease-free survival rate of p21-positive patients was significantly higher than that of p21-negative patients (p=0.043). Tick marks, censored observation.

(B): Disease-free survival curves for p21-positive and -negative patients in the p53 negative patients. The disease-free survival rate of p21-positive patients was significantly higher than that of p21-negative patients (p=0.020).

(C): Disease-free survival curves for p21-positive and -negative patients in the high PCNA LI patients. p21 positive patients tended to have a higher disease-free survival rate than the p21 negative cases in the high PCNA LI patients (p=0.056).

Table 2. Results of multivariate analysis for predictive factors associated with intrahepatic recurrence

Variables		Relative risk	p value[1]
p21	positive vs. negative	0.129	0.0059
p53	positive vs. negative	3.756	0.0004
PCNALI	high LI vs. low LI	2.306	0.0165
intrahepatic metastasis	positive vs. negative	2.660	0.0374
portal vein invasion	positive vs. negative	2.421	0.0135
tumor size	$\leq$ 2.0 cm vs. $>$ 2.0 cm	0.971	0.9463
AFP	$\leq$ 2.0 ng/ml vs. $>$ 2.0 ng/ml	0.969	0.9264
multiplicity	solitary vs. multiple	0.538	0.1459
histology	well, moderately vs. poorly	0.819	0.6091
stage	I + II vs. III + IV	0.755	0.4496

[1] Cox proportional regression model

between the clinicopathological findings and disease-free survival. p21 status, p53 status, PCNA LI, intrahepatic metastasis, and portal vein invasion were identified as significant factors indicative of a intrahepatic recurrence rate in the multivariate analysis.

DISCUSSION

This study revealed the following : (1) significant correlation between p21 expression and tumor size instead of high PCNA LI. ; (2) no relationship between p21 and p53 expression ; (3) p21, p53 and PCNA LI were independent predictive factors for postoperative recurrence.

p21 has emerged as a key player in regulation of the cell cycle, involved in modulation of G1-S transition due to binding to and inhibiting a wide variety of cyclin-cdk complexes, cyclin D-cdk 4 and cyclin E-cdk 2[8-10]. Furthermore, recent studies have demonstrated that p21 retards not only G1-S transition but also S phase progression in vitro and in vivo[13, 20, 21]. The p21 inhibitory effect of S phase progression may result from inhibition of cyclinE-cdk2, cyclinA-cdk2, and PCNA. PCNA, which is used for the assessment of the proliferating activity, is synthesized in the late G1 and in the S phase of the cell cycle and it has been identified as an auxiliary protein of DNA polymerase-delta[22, 23]. So, we investigated the correlation between p21 expression and PCNA LI in HCC. Although co-expression of p21 and PCNA was not always detected in HCC, the correlation between p21 expression and high PCNA LI in this study may imply that p21 inhibits S phase progression as well as G1-S transition in HCC. We supposed that the other reason for the correlation between p21 and PCNA LI was that p21 overexpression might result from a feedback mechanism to reduce proliferation. Similarly to our results, Jung et al. found that p21 expression was low in normal brain tissue and high in the majority of gliomas[24]. They proposed that expression of high levels of p21 in gliomas may be a result of a feedback mechanism designed to halt proliferation. Albrecht et al. demonstrated that p21 mRNA upregulated during G1 phase and following S phase in regenerating the rodent liver after 70% hepatectomy, and that p21 expression increased in the human liver disease expressing cyclin D1[25]. Therefore, the cell cycle progression of cancer cells may induce p21 overexpression if the normal function regulating the cell cycle is preserved in HCC. Consequently, p21 positive tumors

significantly correlated with the small size in spite of high PCNA LI. It could support this hypothesis that p21 expression related to a good outcome in the high PCNA LI group. Studies are in progress to investigate whether p21 retards both G1-S transition and S phase progression by means of analyzing expression of cyclin A, D1 and E in HCC.

This study showed no relationship between p53 and p21. It is reported that the absence of p21 expression did not correlate with p53 mutation in ovarian cancers, pancreatic cancers and gliomas[24,26,27] and that p21 expression may be regulated not only by p53 protein but also by other various factors in vivo[15,16]. However, Hui et al. have reported that the reduced expression of p21 correlated with p53 mutation in HCC[14]. Some reasons explaining the discrepancy in results are proposed. First, we defined the case as positive if p21 positive cancer cells accounted for more than 20 %, because p21 may serve as an assembly factor for active cyclin/cdk complex at low stoichiometric concentration[28]. Hui et al. analyzed p21 mRNA expression in HCC compared with that in the non-cancerous liver tissue, but we observed the heterogeneity of p21 expression and the p21 positive rate of 0 − 10 % in the non-cancerous liver tissues (data not shown). Arbrecht et al. showed that p21 mRNA was upregulated in human liver diseases, and that the level of p21 expression varied significantly between specimens[25]. They have also reported that p21 mRNA expression was regulated by p53-dependent and -independent pathways in regenerating the rodent liver. Second, it is believed that p53 expression is not necessarily consistent with p53 mutation. Silent p53 mutations, such as frame shift and stop codon mutation, do not produce p53 mutated protein[29]. The analysis of p21 protein expression and p53 gene mutation will be required to determine whether p21 expression depends on the normal p53 function in HCC, although p21 expression is supposed to be regulated by both p53-dependent and-independent manners in HCC.

Intrahepatic recurrence after initial treatment is frequent and is a major cause of a poor outcome in HCC. It is generally accepted that the presence of portal invasion and histological metastatic foci are important risk factors for recurrence[30]. Recent studies have demonstrated that p53 status and PCNA LI are predictive factors of intrahepatic recurrence[31]. The results of the present study imply that p21 can also be a predictive factor of intrahepatic recurrence. The proportional hazards regression analysis confirmed that p21, p53 and PCNA had independent prognostic values in a model with multiple predictors.

In conclusion, the analysis of p21, p53 and PCNA, together with intrahepatic metastasis and portal vein invasion, could play an important role in early detection of intrahepatic recurrence and might contribute to improvement of the prognostic characterization.

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