Frequent loss of p19^{INK4D} expression in hepatocellular carcinoma: Relationship to tumor differentiation and patient survival

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Abstract. p19^{INK4D} belongs to the family of cyclin-dependent kinase inhibitors (CdkIs) that target the cyclin-dependent kinases and inhibit their catalytic activity. The role of p19^{INK4D} in cell cycle progression in hepatocellular carcinoma (HCC) is poorly characterized. The aim of this study was to examine the expression of p19^{INK4D} in various liver diseases including HCC and to assess its clinical significance in HCC. We examined the expression of p19^{INK4D} by immunohistochemistry in 81 cases of various liver diseases, including 51 HCCs. We analyzed the relationship among p19^{INK4D} expression in HCC in combination with histopathological stage, differentiation, several histopathological factors of possible prognostic value and patient survival. Immunohistochemical analysis revealed the frequent loss of p19^{INK4D} expression consistent with the differentiation of HCC. The loss of p19^{INK4D} expression was shown to be associated with a poor prognosis by analyzing clinicopathological features. In conclusion, we found that loss of p19^{INK4D} protein was frequent in HCC, especially in poorly differentiated HCC, suggesting that p19^{INK4D} may play a role in the differentiation of HCC. Furthermore, expression of p19^{INK4D} may be an effective predictor of clinical behavior in HCC, and therefore, a new prognostic marker for HCC.

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Abbreviations: Cdk, cyclin-dependent kinase; CdkI, cyclin-dependent kinase inhibitor, CH, chronic hepatitis; HCC, hepatocellular carcinoma; NL, normal liver

Key words: p19^{INK4D}, hepatocellular carcinoma, differentiation, Cdk

Introduction

Hepatocellular carcinoma (HCC) is one of the major causes of cancer-related death due to its high frequency and poor prognosis. In spite of the development of various treatments for HCCs, such as, surgical resection and transarterial chemoembolization (TACE), percutaneous ethanol injection (PEI) and percutaneous radiofrequency (RFA), the 5-year survival rate for all stages of HCC has remained less than 60% (1,2). In general, prognostic evaluation is mainly based on the clinical factors, such as, histopathological stage, clinical stage, tumor size, and tumor numbers. In addition, some studies (3,4) including our reports (5,6) have suggested that other factors, such as the molecular characteristics of the tumor, may offer novel approaches to the identification of groups of patients that could benefit from more aggressive treatment.

Cyclins, cyclin dependent kinase (Cdk) and Cdk inhibitors (CdkIs) are frequently altered in human cancers (7-9). In mammalian cells, to date, at least 2 distinct families of CdkIs are known. The inhibitor of Cdk4 (INK4) family consists of p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, p19^{INK4D}, which specifically inhibit cyclin D-related kinase activity by binding to Cdk4 or Cdk6 (10-14). The other known CdkI family, the p21 family, consists of p21$^{CIP/WAF1}$, p27^{KIP1,} and p57^{KIP2}, which are general inhibitors of the G1 to S transition in the cell cycle (13,15). At clinical involvement, the loss of the expression of KIP family members' p21^{CIP1}, and p27^{KIP1} has recently been shown to be associated with poor prognosis for various human cancers (16-21). p57^{KIP2} is also correlated with the differentiation and prognosis for patients with HCC (5).

p16^{INK4A} and p19^{INK4D}, members of the INK4 family, are the most extensively studied in various human cancers including HCC with respect to the clinical significance (3,4,12,14,22). However, very little data are available on the relationship between p19^{INK4D} and human malignancies. The clinical significance of p19^{INK4D} expression also remains unknown in human cancers, including HCC. Therefore, in this study, we examined the expression of p19^{INK4D} and analyzed the relation-

ship between its expression and clinicopathological features including the prognosis in HCC.

Materials and methods

Patients. Between 1987 and 2006, liver biopsy specimens were obtained from 30 patients with chronic hepatitis (CH) and 51 patients with HCC at Kagawa Medical University, Japan. Of these 30 patients with CH, 27 patients were positive for HCV RNA, and 3 patients were positive for the hepatitis B antigen. Six patients were classified in F1, 7 patients in F2, 8 patients in F3, 8 patients in F4 according to Desmet's classification (23). Seven normal livers (NLs) were obtained by surgical resection of liver metastasis of colon cancer. Normal controls had no hepatitis virus-associated liver disease. HCC specimens were obtained from 6 patients by surgical resection and the others were obtained by needle biopsy before therapy. Thirty-seven patients were male, and 14 patients were female. The mean age of these patients was 62.9±7.2 (mean ± SD; range, 44-75 years). Forty-five patients with HCC were positive for HCV-RNA, and 6 patients with HCC were positive for HBsAg. According to the pathologic tumor-nodes-metastasis (pTNM) classification proposed by the International Union Against Cancer and the American Joint Committee on Cancer (UICC, 1997) (24), 9 patients were in stage I, 15 in stage II, 8 in stage III, and 19 in stage IV. Histological grade of HCC was determined as well differentiated, moderately differentiated or poorly differentiated according to the criteria of the International Working Party (24). The numbers of patients with well-, moderately- and poorly differentiated HCCs were 16, 30 and 5, respectively (Table I). Informed consent was obtained from each patient prior to participation.

Antibodies. We examined the expression of p19^{INK4D} in HCC by immunohistochemistry using a specific antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunohistochemistry was performed according to the method of our previous report (25), and the p19^{INK4D} was detected by the avidin-biotin-peroxidase complex (ABC) method using the Vectastain ABC Elite kit (Vector Laboratories, Inc., Burlingame, CA) and diaminobenzidine as the chromogen. In each case, the predominant staining pattern for p19^{INK4D} was classified as negative, cytoplasmic or nuclear, depending on the specificity of the immunoreactivity.

Immunohistochemistry. Sections (2 μm thick) were cut from formalin-fixed paraffin-embedded tissue blocks, deparaffinized in xylene, rehydrated in graded series of ethanol solutions, and then mixed with a solution containing 0.5% hydrogen peroxidase to block the endogenous peroxidase activity. The sections were placed in a 10 nmol/l citrate buffer (pH 6.0) and processed at 500 W at 95˚C for 10 min in a microwave oven (MR-M201 Microwave Processor; Hitachi, Tokyo, Japan). After washing with phosphate-buffered saline (PBS), the sections were processed for immunostaining by the ABC method outlined above. Primary incubation was performed overnight at 4˚C with monoclonal antibody against p19^{INK4D}. As a negative control, non-immune mouse IgG was substituted for the primary antibody. For signal amplification, the Renaissance TSA amplification kit (NEN™ Life Science Products, Boston, USA) was used (26). Diaminobenzidine was used as the chromogen. All slides were examined and scored independently by two pathologists (T.M., S.W.) who were blinded to the pathological and clinical data assessed by a third observer (A.M.). For assessment of the expression of p19^{INK4D}, we categorized samples into two groups on the basis of the percentage of HCC cells demonstrating p19^{INK4D} immunoreactivity, p19^{INK4D} expression negative (<50%) or p19^{INK4D} expression positive (>50%). The patterns of expression were classified into three groups (nuclear positive, cytoplasmic positive and negative staining pattern). Necrotic areas and edges of the tissue sections were not included in the counting to avoid possible immunohistochemical false-positives.

Statistical analysis. We performed statistical analysis of the relationship between the p19^{INK4D} expression and clinicopathological parameters by means of the Fisher's t-test. The survival curves were plotted using the Kaplan-Meier method, and differences were evaluated by a log-rank test. In addition, to identify independent predictors of patients' prognosis, we performed multivariate analysis, using the Cox proportional hazards model. Differences were considered significant when $P<0.05$. All statistical analyses were performed using the computer-assisted StatView program (SAS Institute, Cary, NC, USA).

Results

p19^{INK4D} expression in NL, CH and liver cirrhosis. Representative immunostaining of p19^{INK4D} in CH are shown in Fig. 1A and B and of liver cirrhosis in Fig. 1C and D. Fibrotic stages of Fig. 1A and B were F2, and of Fig. 1C and D were F4 according to Desmet's classification. The localization of p19^{INK4D} in F2 (Fig. 1A and B) stages of fibrosis was observed in the hepatocyte nuclei (arrows). Interestingly, p19^{INK4D} was detected in all 30 patients with CH or cirrhosis in hepatocytes (Table I). In liver cirrhosis, the expression of p19^{INK4D} was observed in 7 of 9 cases with liver cirrhosis and was detected in the cytoplasm of hepatocytes, but not in the nucleus (F4; Fig. 1C and D). On the other hand, p19^{INK4D} was expressed not only in the nucleus, but also in the cytoplasm in 2 of 9 cases of liver cirrhosis (data not shown). The expression of p19^{INK4D} was also detected in the nucleus of hepatocytes in all NLs examined in this study (data not shown).

p19^{INK4D} expression in malignant liver tissues. As shown in Table II, the immunohistological study of p19^{INK4D} identified no staining in 19 of 51 HCCs (37.3%) and 32 cases expressed p19^{INK4D} (62.7%). In well-differentiated HCCs, 13 of 16 tumor samples stained positive for nuclear p19^{INK4D} (81.3%; Fig. 2A-C), and the remaining cases were negative for staining (18.7%). In moderately differentiated HCCs, the expression of p19^{INK4D} was detected in 17 of 30 cases (56.7%), while the remaining 13 cases (43.3%) were p19^{INK4D}-negative (Fig. 2D-F). Among the 5 poorly differentiated HCCs, p19^{INK4D} was not detected in any cases (0%). p19^{INK4D} expression and clinicopathological variables were correlated by univariate analysis (Table II). Loss of p19^{INK4D} expression was significantly associated with histological grade and advanced TNM stage. However, no significant relationship was seen between p19^{INK4D} expression and gender, age, infection with hepatitis B or C virus.

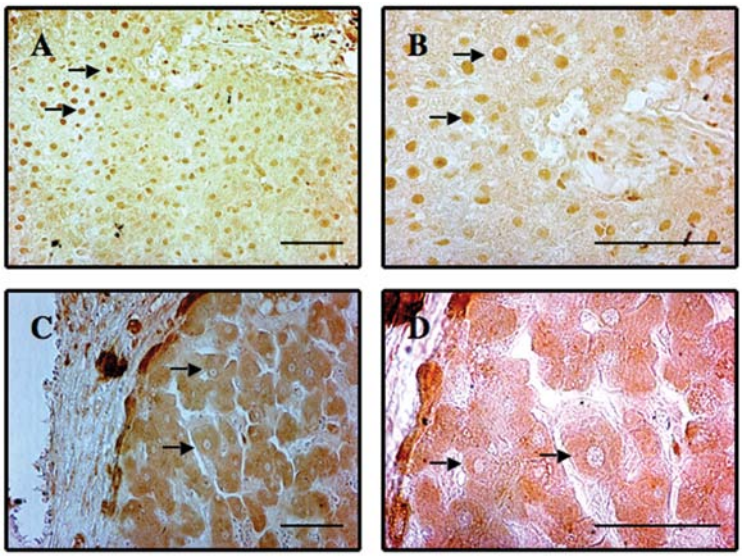

Figure 1. Immunohistochemistry of p19^{INK4D} in chronic hepatitis (A and B) and liver cirrhosis (C and D). Fibrosis stages indicated in (A, B) and (C, D) were grades F2 and F4 according to Desmet's classification, respectively. Staining for p19^{INK4D} in chronic hepatitis was seen in the hepatocellular nucleus (arrows in A and B). Expression of p19^{INK4D} in liver cirrhosis was positive for p19^{INK4D} staining in the hepatocellular cytoplasm but not in the hepatocellular nucleus (arrows in C and D). The original magnifications are x100 in (A and C) and x200 in (B and D). Bars on the lower right of pictures, 50 μm.

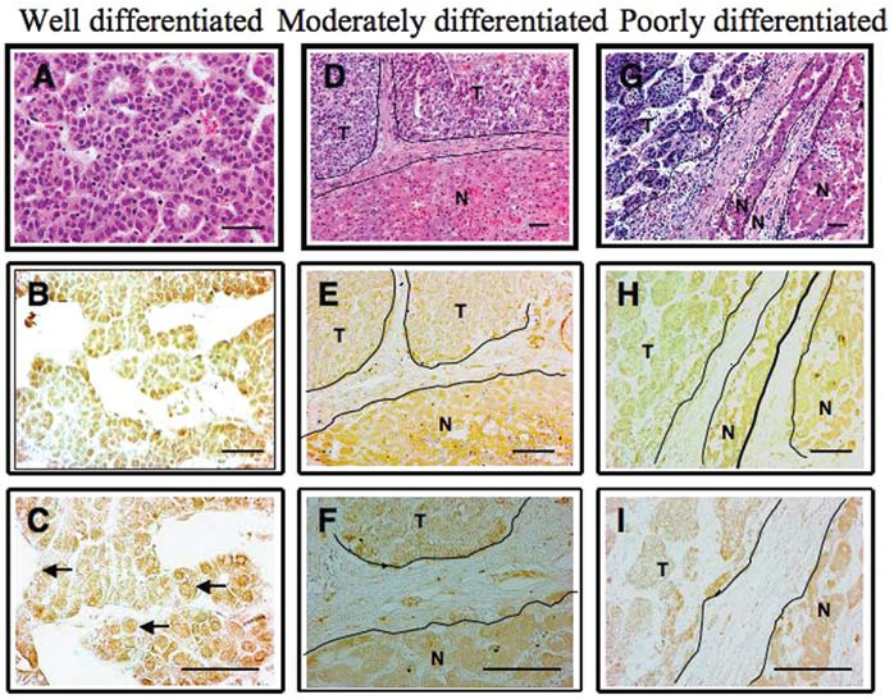

Figure 2. Immunohistochemistry of p19^{INK4D} in well- (B and C), moderately- (E and F), and poorly-differentiated (H and I) HCCs. (A, D and G) show hematoxylin and eosin staining of the section adjacent to (B, E, and H), respectively. (C, F, and I) represent the higher magnification of the sections of (B, E, and H), respectively. T and N indicate HCC and non-tumorous cirrhotic tissues, respectively. The expression of p19^{INK4D} in most well-differentiated HCCs was localized in the nuclei of cancer cells (arrows). Conversely, its expression in most moderately- and poorly-differentiated HCCs was not detected. The scar indicates the boundary between N and T tissues. The original magnifications in (A, B, E and H), (D and G), and (C, F and I) are x100, x0 and x200, respectively. Bars on the lower right of pictures, 50 μm.

Prognostic significance of p19^{INK4D}. In addition, we analyzed the prognostic significance of p19^{INK4D} on patient survival. Survival analysis in HCC was performed by using the Kaplan-Meier method, and the differences were evaluated by the long-rank test. The tumors were divided according to p19^{INK4D} expression into the p19^{INK4D}-positive (n=32) and p19^{INK4D}-negative groups (n=19). Patients with p19^{INK4D}-negative HCC had a significantly worse prognosis than those with p19^{INK4D}-positive HCC (P=0.0179; Fig. 3).

Multivariate analysis using the Cox proportional hazards models. Among age, gender, infection, histological grade,

Table I. Relationship between expression of p19^{INK4D} in various liver diseases and histological features.

Histology	n	p19^{INK4D} staining pattern			P-value
		Negative (%)	Nuclear (%)	Cytoplasmic (%)	
NL	7	0 (0)	7 (100)	0 (0)	
CH[a]	30	0 (0)	23 (76.7)	7 (23.3)	
F1	6	0 (0)	6 (100)	0 (0)	<0.0001[b]
F2	7	0 (0)	7 (100)	0 (0)	
F3	8	0 (0)	8 (100)	0 (0)	
F4	9	0 (0)	2 (22)	7 (78)	0.0009[c]

NL, normal liver; CH, chronic hepatitis. [a]Classification of CH according to the criteria of Desmet. P-values were considered statistically significant if P<0.05. [b]P-value was three-sided (Kruskal-Wallis test). [c]P-value for F1/2/3 vs. F4 was two-sided (Mann-Whitney U-test).

Table II. Relationship between p19^{INK4D} immunoreactivity in HCC and clinicopathological features.

	n	p19 staining status (%)			P-value
		Positive (+N/+C)	Negative	Negative rate (%)	
Gender					
Male	37	24 (18/6)	13	35.1	
Female	14	8 (7/1)	6	42.9	0.7477
Age (years)					
<65	27	19 (14/5)	8	29.6	
≥65	24	13 (11/2)	11	45.8	0.2607
Infection					
HCV positive	45	30 (23/7)	15	33.3	
HBsAg positive	6	2 (1/1)	4	66.7	0.1792
Histological grade[a]					
WD/MD	46	30 (22/8)	16	34.8	
PD	5	0	5	100	0.005
Tumor stage[a]					
I/II	24	19 (15/4)	5	21	
III/IV	27	13 (10/3)	14	52	0.041

[a]Histological grade and tumor stage according to the classification of the Liver Cancer Study Group of Japan. WD, well-differentiated HCC; MD, moderately-differentiated HCC; PD, poorly-differentiated HCC. +N, nuclear staining; +C, cytoplasmic staining. The P-values were considered statistically significant if P<0.05.

tumor stage and p19^{INK4D} expression, p19^{INK4D} expression was only independent prognostic factors for patients' overall survival (P=0.0201; Table III).

Discussion

Deregulated cell cycle progression is one of the most significant alterations in cancer cells. The G1 to S phase transition is thought to be a key target of tumorigenesis, which is in part, negatively regulated by CdkIs. CdkIs regulate the progression of cell cycle by modulating the activity of Cdks (10-15). Inactivation of CdkI has been associated with neoplastic transformation in a large number of human epithelial tissues (10,27).

To date, there is limited information about the relationship between p19^{INK4D} and human malignancies. Bartkova *et al* reported that p19^{INK4D} protein is abundant in spermatocytes of normal human adult testes, whereas virtually no p19^{INK4D} is detectable in testicular cancer, including the preinvasive carcinoma *in situ* stage (28). However, the expression of p19^{INK4D} protein in HCC is not yet known. In the present study, therefore, we evaluated the expression of p19^{INK4D} in various liver diseases including HCC.

The major finding of this study was the loss of p19^{INK4D} expression in a subset of HCCs, especially in poorly differentiated HCCs. Interestingly, p19^{INK4D} was expressed in the nucleus of all cases of NL, CH and in the cytoplasm of liver cirrhosis,

Table III. Multivariate Cox model analysis of overall survival.

	Overall suvival		
	Hazard ratio	95% CI[a]	P-value
Age (≥65/<65)	1.289	0.617-2.692	0.5
Gender (female/male)	1.149	0.502-2.631	0.7429
Histology (PD/WD and MD)	1.596	0.462-5.519	0.4601
Infection (HBV/HCV)	1.41	0.542-3.670	0.4812
Tumor stage (III-IV/I-II)	1.466	0.633-3.393	0.3717
$p19^{INK4D}$ (negative/positive)	2.56	1.159-5.656	0.0201

[a]Confidence interval. WD, well-differentiated hepatocellular carcinoma; MD, moderetely-differentiated hepatocellular carcinoma; PD; poorly-differentiated hepatocellular carcinoma.

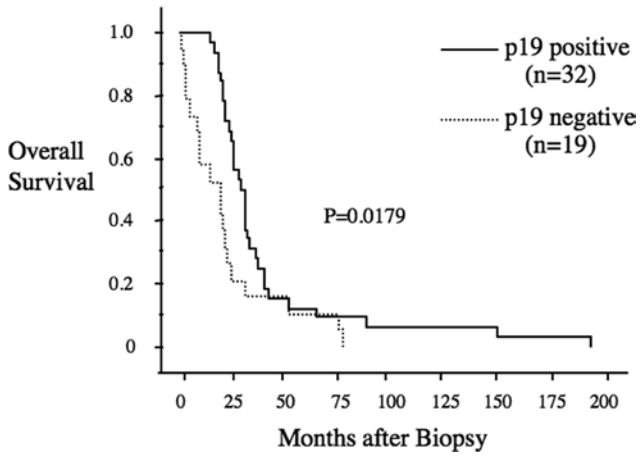

Figure 3. Kaplan-Meier curves of overall survival according to $p19^{INK4D}$ expression indicating positive vs. negative staining in 51 patients with HCC. Time to death was significantly shorter in patients with $p19^{INK4D}$-negative HCC than in those with $p19^{INK4D}$-positive HCC (P=0.0179).

but the loss of $p19^{INK4D}$ was detected in about 40% of HCCs, suggesting that it may be involved in hepatocarcinogenesis. In addition, the loss of $p19^{INK4D}$ in poorly differentiated HCCs was expressed at high frequencies compared to well- and moderately-differentiated HCCs. These results suggest that the down-regulation of $p19^{INK4D}$ is a common event in poorly differentiated HCCs. Therefore, the expression of $p19^{INK4D}$ represents a useful prognostic marker for survival in HCC patients.

The $p19^{INK4D}$ gene has been mapped to chromosome 19p13.2 (29). The results of previous studies indicating that 19p13.2 is deleted along with the frequent loss of heterozygosity (LOH) in various human cancers (29,30) may help explain the loss of $p19^{INK4D}$ expression found in a subset of HCC in this study. In addition, a marked reduction of $p19^{INK4D}$ has been detected in various cancers, such as oral cancer, breast carcinoma and testicular cancer (29,30). These previous reports support the importance of the finding in this study that reduced $p19^{INK4D}$ expression in HCC may be a critical step in patient survival.

The expression of $p19^{INK4D}$ in HCV- or HBV-induced CH and cirrhosis was detected in all cases. $p19^{INK4D}$ expression was not detected in about 40% cases of HCCs. The data suggest that the changes in $p19^{INK4D}$ expression are not influenced by hepatitis viral infection in the process of HCC from CH, but its changes are influenced by the malignant process.

To date, $p19^{INK4D}$ has been reported to play an important role in the regulation of cell differentiation (31). To investigate the possible involvement of this protein in the differentiation of HCC, we analyzed the relationship between the level of $p19^{INK4D}$ expression and the histological grade of HCC. $p19^{INK4D}$ expression was reduced in poorly differentiated HCC, compared to well- and moderately-differentiated HCC. Utilizing immunohistochemical analysis of $p19^{INK4D}$, we observed an inverse correlation between $p19^{INK4D}$ expression and tumour aggressiveness as indicated by the TNM classification. Our results demonstrate that loss of $p19^{INK4D}$ was particularly evident in advanced HCC (stages III and IV), suggesting that decreased $p19^{INK4D}$ may promote progression of HCC.

In order to facilitate treatment selection for patients and provide important information for predicting their prognosis, identification of the grade of tumor malignancy is quite important (32). In HCC, clinicopathological prognostic factors, such as tumor size, the number of tumor nodules, capsule formation, capsule invasion, and vascular invasion, have been extensively studied (32). Cell cycle-related molecules, such as proliferating nuclear antigen (PCNA), $p21^{CIP1}$, $p27^{KIP2}$, and p73, have been found to be prognostic biomarkers in various types of human cancer including HCC (33-36). However, there have been no reports on the relationship between $p19^{INK4D}$ expression and the prognosis in HCC. In this study, with regard to prognosis, the survival analysis by the Kaplan-Meier method revealed that $p19^{INK4D}$ expression was associated with the overall survival of patients with HCC. It is clinically important that the loss of $p19^{INK4D}$ expression was significantly associated with the short survival of patients with HCC. According to the multivariate analysis, $p19^{INK4D}$ and tumor stage were independent prognostic factors for overall survival. These results suggest that loss of $p19^{INK4D}$ in HCC may serve as an indicator of poor prognosis.

In conclusion, loss of $p19^{INK4D}$ expression may play an important role in the process of the differentiation and development of HCC. Furthermore, expression of $p19^{INK4D}$ may be an effective predictor of clinical behavior in HCC. In the future, novel therapeutic strategies targeting $p19^{INK4D}$ may be useful for preventing the development of HCC.

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