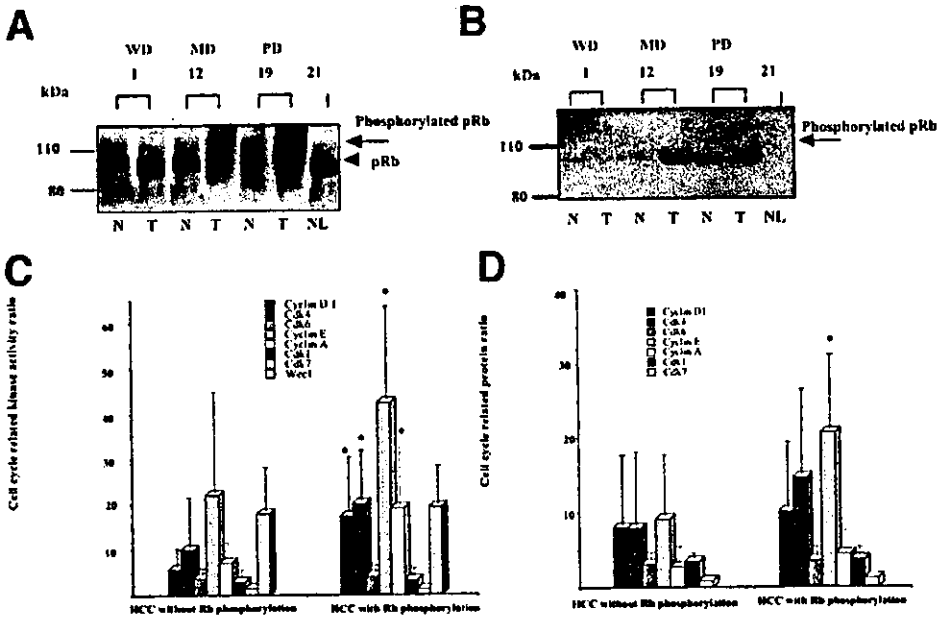

Fig. 4. (A) Representative Western blot of pRb using the antibody (C-15) recognizing both the hypophosphorylated and hyperphosphorylated forms of pRb in normal liver (NL), and nontumorous (N) and tumorous (T) tissues of HCC. Note that the pRb band was shifted in HCC (cases 12 and 19), suggesting that pRb was much more heavily phosphorylated in HCC. (B) Representative Western blot of pRb using antibody against phosphorylated Rb (Ser 780) in NL, N, and T tissues of HCC. The amount of phosphorylated pRb in HCC was higher than that in N tissues and NL. (C) Cell cycle–related kinase activity ratios in HCC with and without pRb phosphorylation. The level in normal livers was used as the reference level (= 1). Values shown represent the mean ± SEM of each cell cycle–related kinase activity for each group. $P < .05$ versus HCC without pRb phosphorylation. (D) Cell cycle–related protein ratios in HCC with and without pRb phosphorylation. The level in normal livers was used as the reference level (= 1). Values shown represent the mean ± SEM of each cell cycle–related protein for each group. $P < .05$ versus HCC without pRb phosphorylation.

cyclin D1/Cdk4, but not cyclin D1/Cdk6, may be associated with the process of transition from hepatitis C–induced cirrhosis to HCC.

The cyclin D1 gene has been mapped on the long arm of chromosome 11 close to the INT-2 gene,[34] and the chromosome 11q13 region is amplified in HCC carrying integrated HBV-DNA.[1] In this study, we showed the enhancement of cyclin D1–related kinase activity in HCC, suggesting that cyclin D1–related kinase activity may play a role in the tumorigenic transition of cirrhosis to HCC. Recently, the locus of a tumor suppressor gene, $p16^{INK4}$, coding for an inhibitor of cyclin D1/Cdk4, was found to

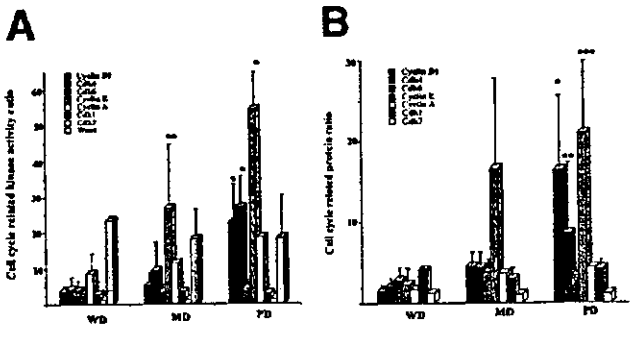

Fig. 5. Relationship between various cell cycle–related kinase activities (A) or cell cycle–related protein levels (B) and histopathologic differentiation of HCC. The values were expressed for well- (W), moderately (M), and poorly (P) differentiated HCC relative to value in normal livers (= 1). (A) Values shown represent the mean ± SEM of each cell cycle–related kinase activity for each group. $P < .01$ versus well-differentiated HCC and moderately differentiated HCC. $P < .05$ versus well-differentiated HCC. (B) Values shown represent the mean ± SEM of each cell cycle–related kinase activity for each group. $P < .005$ and $P < .001$ versus well-differentiated HCC and moderately differentiated HCC. $P < .05$ versus well-differentiated HCC.

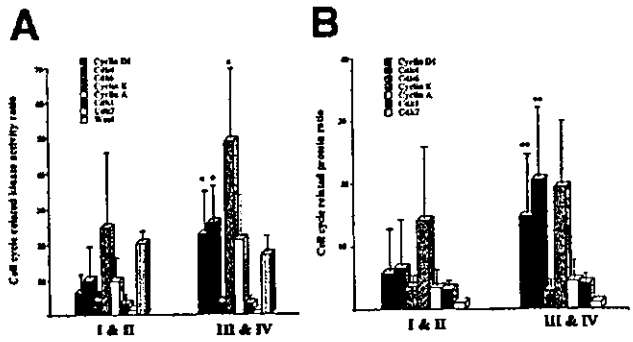

Fig. 6. Relationship between various cell-related kinase activities or (A) cell cycle–related proteins (B) and stage of HCC (HCC). Values were expressed for HCC in stages I and II, and stages III and IV relative to the activity of each cell cycle–related kinase in normal liver. (A) Values shown represent the mean ± SEM of each cell cycle–related kinase activity for each group. $P < .01$ compared with HCC in stages I and II. (B) Values shown represent the mean ± SEM of each cell cycle–related kinase activity for each group. $P < .05$ compared with HCC in stages I and II.

be frequently lost in various malignancies.[35] Inactivation of the $p16^{INK4}$ gene has been reported in various human cancers, including human HCC, and in half of HCC cell lines and 34% of HCC tissues, $p16^{INK4}$ protein is not detected by immunoblot,[36] suggesting that the level of $p16^{INK4}$ is down-regulated. In addition, Ray et al.[37] reported that the HCV core protein appears to act as a promoter of cell growth by repressing the transcription of another Cdk inhibitor (CKI), $p21^{WAF1}$. Thus, there is a possibility that the up-regulation of Cdk4 activity in a subset of HCCs is related not only to an increase of Cdk4 protein but also to the reduction of $p16^{INK4}$ and $p21^{WAF1}$, inhibitors of Cdk4. In addition, the Cdk4 gene has been mapped to chromosome 12 close to the proto-oncogene MDM2,[38] and thus the increased Cdk4 activity in HCC in the present study may be related to the fact that the chromosome 12q13 region is amplified in some human cancers.[39]

The findings of the present study are consistent with the data of Mate et al.,[40] suggesting that cyclin D1 overexpression plays an important role in the process of tumor differentiation. In fact, cyclin D1 and Cdk4 activities showed a tendency to be increased in poorly differentiated HCC as compared with those in well- and moderately differentiated HCC (Fig. 5A). Thus, the enhancement of cyclin D1 and Cdk4 activities may play an important role in the differentiation of HCC, although the basis of this relationship remains obscure. However, both *in vitro*[41] and *in vivo*[42] studies have shown that cyclin D1 overexpression can inhibit the differentiation of myoblasts and intestinal epithelial cells, thus raising the possibility that cyclin D1 overexpression plays a role in the inhibition of tumor cell differentiation in some cell types.

Cyclin E is one of the G1-related cyclins that play an important role in cell proliferation.[43] Although amplification and overexpression of cyclin E have been shown in several human solid tumors,[43,44] little is known about the involvement of cyclin E in HCC. In the present study, we found increased levels of cyclin E protein and its kinase activity in moderately and poorly differentiated HCC (Fig. 5A). In addition, we showed that cyclin E was more frequently elevated in advanced HCC (Fig. 6A), indicating that activation of cyclin E kinase may contribute to HCC progression. The protein level of cyclin E was higher in moderately and poorly differentiated HCC tissues than in surrounding nontumorous cirrhotic tissues (Fig. 2D), whereas the protein level of the cyclin E catalytic subunit, Cdk2, did not differ between nontumorous cirrhotic and tumorous tissues (Fig. 2E). These data suggest that the enhancement of cyclin E kinase activity in HCC may be caused by an increase of cyclin E protein rather than an increase of the catalytic subunit, Cdk2.

The human cyclin A gene has been identified as an integration site of HBV-DNA in HCC, and HBV-cyclin A fusion protein was shown to be resistant to ubiquitin-dependent degradation because the NH2-terminal region of cyclin A was replaced by a viral sequence.[1] In addition, enhanced expression of cyclin A messenger RNA was reported previously in 42% of studied HCCs, especially in poorly differentiated HCC.[6] In the present study, the cyclin A protein level and its kinase activity had a tendency to increase in poorly differentiated and advanced HCCs (Figs. 5A, 5B, 6A, and 6B) that developed from HCV infection. This may be consistent with a previous study showing that patients with HCC expressing high levels of cyclin A had a significantly shorter median survival time than those with lower levels of cyclin A.[6]

On the other hand, the kinase activity of Cdk1 and Cdk7 remained unchanged in the transition from cirrhosis to HCC development (Fig. 3I), suggesting that Cdk1 and Cdk7 kinase activities may not be involved in the transition from cirrhosis to HCC. Furthermore, cyclin B/Cdk1 complexes accumulate in an inactive state during S and G2 phases, and the kinase activity of these complexes is known to be inactivated by phosphorylation of Tyr-15 of Cdk1 by the Wee1 protein kinase. O'Connor et al.[46] reported that many tumor cell lines contained activated cyclin B/Cdk1 regardless of the state of the DNA and mitosis, suggesting that there may be a defect in the regulation of Tyr phosphorylation in cancer cells. However, in the present study, Wee1 kinase was shown to be increased in HCC (Fig. 3I), although the mechanism of the increase of Wee1 kinase is still unknown.

When the level in normal livers is used as the reference level (= 1), the ratio (HCC versus normal liver) of the protein level of cyclins D1, E, and A and for Cdk4 was smaller than that of the kinase activity level (Figs. 2K and 3I). It was shown previously that the activation of these cyclins and Cdks in HCC of the Long Evans Cinnamon rat is not always caused by the elevation of the respective proteins.[18] Therefore, it may speculated that the elevated kinase activities of cyclins and Cdks in a subset of HCC were caused by the inactivation of CKIs.

The CKI proteins are potent negative regulators of the cell cycle and are potentially tumor suppressor proteins, and their loss might play a role in the development of human cancers.[46] In fact, loss of INK4 family members, for instance, $p15^{INK4B}$, $p16^{INK4A}$, and $p18^{INK4C}$, by gene mutation, deletion, and/or methylation has been observed in a variety of human cancers.[35] Recently, we showed that loss of $p57^{KIP2}$ was detected in a subset of HCC, especially in poorly differentiated HCC.[47] In fact, cyclin E kinase activity was upregulated in HCC with loss of $p57^{KIP2}$ as compared with nontumorous liver portions.

In the present study, the enhancement of cyclin E kinase activity in HCC was more marked than the increase of the cyclin E protein amount. These data suggest that the cause of the up-regulation of various cyclin and Cdk activities in a subset of HCC might be not only the increase of these proteins, but also inactivation of some CKIs.

Cyclin D/Cdk4, cyclin D/Cdk6, cyclin E/Cdk2, and cyclin A/Cdk2 phosphorylate pRb, and the transcriptional factors (collectively termed pRb) released due to the phosphorylation enhance cell cycle activity.[9] In the present study, the phosphorylation of pRb was detected in a subset of HCC. The kinase activities of cyclin D1, Cdk4, cyclin E, and cyclin A in HCC with pRb phosphorylation were significantly higher than those in HCC without pRb phosphorylation. These data suggest that pRb hyperphosphorylation occurs in HCC as a result of up-regulation of cell cycle–related kinase activities, especially of cyclin D1, Cdk4, cyclin E, and cyclin A kinase activities.

Finally, we want to emphasize the following points. Our results also showed that although the activity of various cell cycle–related kinases was very low in normal liver, these kinases already were activated in cirrhosis. Because the kinase activity of various cyclins (cyclin D1, cyclin E, and cyclin A) and Cdks (Cdk4, Cdk6, and Cdk1) tends to be already activated at the preneoplastic stage (cirrhosis) before the development of HCC, this activation appears to be an early event in hepatocarcinogenesis. These data suggest that hepatitis C virus-induced cirrhosis is a precancerous condition.

In conclusion, our findings indicate that activation of cyclin D1, Cdk4, cyclin E, cyclin A, and Wee1 may play important roles in the process of malignant transformation of cirrhosis to HCC. In addition, the up-regulation of cyclin D1, Cdk4, and cyclin E also was shown to be related to the differentiation and progression of HCC. Thus, the inhibition of cyclin D1, Cdk4, cyclin E, and cyclin A kinase activities may provide a novel strategy for the suppression of the development of HCC.

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Involvement of the vascular endothelial growth factor receptor-1 in murine hepatocellular carcinoma development

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***Background/Aims*: The role of the vascular endothelial growth factor receptor-1 (VEGFR-1) in hepatocellular carcinoma (HCC) development has not been elucidated yet. The aim of this study was to examine the role of VEGFR-1 in VEGF-mediated HCC development and angiogenesis as compared to that of VEGFR-2.**

***Methods*: We examined the effects of VEGFR-1, and VEGFR-2 neutralizing monoclonal antibodies (R-1mAb and R-2mAb, respectively) on VEGF-mediated HCC development both in an allograft and orthotopic models.**

***Results*: In the allograft model, both R-1mAb and R-2mAb significantly attenuated the VEGF-mediated tumor development in a dose dependent manner with associated reduction of angiogenesis in the tumor. The inhibitory effect of R-2mAb was more potent than that of R-1mAb, and the combination treatment with both mAbs almost completely attenuated VEGF-mediated HCC development. Immunohistochemical analysis revealed that apoptosis increased markedly in the tumor. Furthermore, these inhibitory effects with both mAbs were achieved even on established tumors and orthotopic transplantation.**

***Conclusions*: In addition to VEGFR-2, VEGFR-1 also lies on the signal transduction pathway by which VEGF augments HCC development and angiogenesis not only at the initial stage but also in the established tumor.**

***Keywords*: VEGF; flt-1 (VEGFR-1); KDR/Flk-1 (VEGFR-2); Hepatocellular carcinoma; Angiogenesis**

1. Introduction

The growth of any solid tumor, including hepatocellular carcinoma (HCC) is now widely recognized to depend on the process of neovascularization [1–3]. Among the identified pro-angiogenic factors, the vascular endothelial growth factor (VEGF) is the most intriguing factor with regard to the angiogenesis process [4,5]. In the human specimens, the increased expression of VEGF correlated with aggressive behavior and poor prognosis. In the animal experimental models, overexpression of VEGF enhanced tumor growth, whereas suppression of VEGF inhibited tumor growth [4–6]. Two tyrosine kinases, *fms*-like tyrosine kinase (flt-1: VEGFR-1) and the kinase insert domain-containing receptor/murine homologue, fetal liver kinase-1 (KDR/Flk-1: VEGFR-2), both of which are type III tyrosine kinase receptors, have been identified as the main VEGF receptors. Although VEGFR-1 shows affinity to VEGF at least 10-fold higher than VEGFR-2, it has been reported that VEGFR-2 is a major positive signal transducer through its strong tyrosine kinase activity as compared to VEGFR-1 both in vitro and in vivo [4,5,7–10]. However, recent studies have revealed that VEGFR-1 has a dual function in angiogenesis, acting in a positive or negative manner under different conditions [11]. In physiological angiogenesis, such as embryo development, VEGFR-1 exerts a negative regulatory function, probably via its strong VEGF-trapping activity [12]. It has been revealed that VEGFR-1 can act as a potent positive regulator of VEGF under pathological conditions, such as tumor angiogenesis [13]. Furthermore, it has been reported that VEGFR-1 is an important mediator in tumor angiogenesis,

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arthritis, and arteriosclerosis via bone marrow-derived hematopoietic stem-cell recruitment, and mobilization [14].

HCC is one of the most prevalent malignancies worldwide and causes more than one million deaths annually, and its incidence continues to increase not only in Asia but also in the United States [15,16]. One of the representative characteristic features of HCC in the clinical practice is hypervascularity. Several studies showed that angiogenesis was implicated in survival and growth of HCC. It has been reported that VEGF expression was up-regulated in the tumors of HCC as compared to the non-cancerous lesions, and that overexpression of VEGF exerted a marked increase in HCC development accompanied by augmentation of neovascularization [17–21]. We have reported that inhibition of VEGFR-2 with the neutralizing monoclonal antibody (R-2mAb) significantly suppressed the HCC development associated with suppression of angiogenesis [22]. However, it is still unknown whether VEGFR-1 plays some biological roles and coordinates with VEGFR-2 in HCC development and angiogenesis.

The tetracycline-controlled transactivator-responsive promoter (Tet-system) is a novel drug-regulated gene expressing system, because it can manipulate the gene of interest in an 'on and off' manner in vivo [23]. We used a modified retrovirus-mediated vector (Retro-Tet) system to achieve tighter regulation and less leaky background basal expression than the original Tet system as described previously [21,24–26]. With this modified Retro-Tet system, it is possible to observe the tumor kinetics that can be achieved by VEGF gene expression.

In our present study, we examined the roles of VEGFR-1 and VEGFR-2 in HCC development and angiogenesis at different stages by means of combination of the Retro-Tet system, specific neutralizing monoclonal antibodies of VEGFR-1, and VEGFR-2 (R-1mAb and R-2mAb, respectively). Furthermore, since the orthotopic transplantation has been reported to show a preferential site of growth of the transplanted tumor cells [27,28], we also examined the inhibitory effects of R-1mAb and R-2mAb on the development of HCC in the liver.

2. Methods

2.1. Construction of the vector, cell culture, and compounds

A complete description of the construction of the Retro-Tet vector, in which the human VEGF cDNA was inserted (Tet-VEGF), and stable Tet-VEGF-expressing BNL.1ME A 7R. 1 HCC (Tet-VEGF-HCC) has been reported previously [21]. This BNL-HCC cell line expressed neither VEGFR-1 nor VEGFR-2 as described previously [21,22]. The cells were grown in media recommended by the suppliers. The R-1mAb and R-2mAb were generated as described previously [22,29–31], and the specific neutralization of their respective receptors were reported elsewhere [14,32].

2.2. Animal treatment

In an allograft model, 1×10^6 Tet-VEGF-cells or *lacZ*-HCC cells were syngenetically inoculated into the flank of BALB/c mice. At first, we performed a dose-dependent study. Either R-1mAb or R-2mAb was intraperitoneally (i.p.) injected twice a week at doses of 400 and 800 μg/mouse, respectively. As a negative control, the same amount of immunoglobulin G (IgG) was injected as described previously [22,31]. To examine the combination effect of both mAbs, 800 μg/mouse of R-1mAb and R-2mAb were administered simultaneously. The next experiment was conducted to examine the effects of mAbs on the fully established tumor growth. In this experiment, either R-1mAb or R-2mAb administration was started on day 24 (the mean tumor volume was 827 mm^3). To suppress the VEGF expression in the Tet-VEGF tumor, the mice were given Tet-containing water (1 mg/ml) through the experiment. The tumor volume was measured twice a week, and the mice were killed 48 days after the tumor cell implantation under anesthesia. In the allograft studies, each experimental group consisted of eight mice. For the direct liver injection experiment, 1×10^6 of Tet-VEGF or *LacZ*- transduced BNL-HCC cells were directly injected into the liver ($n = 7$ for each group). The cells were suspended in 10 μl of PBS and implanted directly under the capsule of the left-lateral hepatic lobe under direct visualization, by means of a 10-μl Hamilton syringe with a 26-gauge needle over a period of 2–3 min. Treatments with R-1mAb and R-2mAb were the same as in the xenograft experiments. Fourteen days after the injection, the mice were killed and examined macro and microscopically for HCC development in the liver. All animal procedures were performed according to approved protocols and in accordance with the standard recommendations for the proper care and use of laboratory animals.

2.3. Immunohistochemical examination

For determination of the in vivo angiogenesis, we employed immunohistochemical detection of CD31 in frozen sections of tumors having the same size to avoid the necrotic effect of hypoxia with a primary rat anti-mouse CD31 antibody (Pharmingen, San-Diego, CA, USA) as described previously [22,26,33]. These immunopositive vessels were evaluated with Adobe Photoshop and NIH image software as described previously [22,26,33]. Apoptosis was detected with the DNA fragmentation products that were stained by in situ 3′ end labeling (terminal deoxynucleotidyl transferase-mediated dUTP nick labeling) [TUNEL] with paraffin-embedded sections as described previously [31,34,35]. For each tumor, the positive cells in 30 high-power fields at a magnification of × 400 were examined.

2.4. Immunoprecipitation and Western blotting

To examine the effect of R-1mAb and R-2mAb on autophosphorylation and protein expression of the respective receptors in the tumor, immunoprecipitation (IP) and Western blotting (WB) were performed as previously described [22,31]. The tumor pool lysate solution was concentrated and used for IP and WB. To conduct IP, the tumor lysates were immunoprecipitated with anti-phosphotyrosine before conducting SDS-PAGE. Anti-tyrosine (4G10) was purchased from Upstate Biotechnology (NY, USA) and anti-VEGFR-2 (C-1158), VEGFR-1 (C-17) were obtained from Santa-Cruz (CA, USA).

2.5. Statistical analysis

To assess the statistical significance of the inter-group differences in the quantitative data, the Mann–Whitney *U* test was used to compare the mean values between two groups. The Kruskal–Wallis test was used to compare the mean values between more than two groups.

3. Results

3.1. Tumor growth kinetics in VEGF-mediated HCC development

When VEGF expression in the tumor was up-regulated with Tet-free normal water, the tumor growth rate was significantly augmented as compared with the *lacZ*-transduced

control group. On the contrary, when the VEGF expression was shut down by administering Tet-containing water (1 mg/ml) to the mice, the tumor growth was significantly decreased to almost the same level as the control (Fig. 1). These results indicated that the increased tumor growth kinetics in this study were exclusively due to VEGF overexpression.

To examine the roles of VEGFR-1 and VEGFR-2 in VEGF-induced HCC tumor development, the respective mAb was injected i.p. into mice bearing Tet-VEGF–HCC cells. At first, we examined the effect of R-1 mAb on the primary tumor growth in the allograft model. As shown in Fig. 1, the tumor development in the R-1mAb-treated groups at doses of 400 and 800 μg/mouse was significantly suppressed as compared to the IgG-treated group ($P < 0.05$ and $P < 0.01$, respectively). The treatment at 800 μg/mouse exerted a further inhibitory effect as compared with that at a dose of 400 μg/mouse ($P < 0.05$). We next performed the same experiment with R-2mAb. In accordance with a previous report [22], R-2mAb treatment significantly attenuated the VEGF-mediated HCC development. Similar to R-1mAb, R-2mAb treatment at a dose of 800 μg/mouse showed a more potent inhibitory effect than at 400 μg/ mouse in a dose ranging study ($P < 0.05$) (Fig. 2). At a dose of 800 μg/mouse, the inhibitory impact was more potent with R-2mAb than that of R-1mAb ($P < 0.05$). The combination treatment with both mAbs revealed further inhibition as compared with that of R-2mAb alone ($P < 0.05$). This combination treatment almost completely attenuated the VEGF-induced HCC development (Fig. 3).

We also examined whether the inhibitory effects of these mAbs could be found even after the tumor was established.

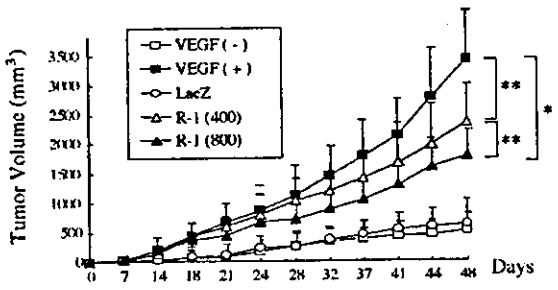

Fig. 1. The effect of R-1mAb-mediated suppression on the VEGF-induced HCC development. To maintain VEGF overexpression or suppression, the mice were given Tet-free normal and Tet-containing drinking water, respectively. The *lacZ* HCC-injected mice served as a negative control. These groups were injected with control IgG. The R-1mAb was i.p. administered twice a week. The R-1mAb treatment significantly attenuated the VEGF-mediated HCC development in a dose dependent manner. The tumor volume was determined by three-dimensional calipers at the indicated time points. Each point represents the mean ± SD ($n = 8$). *, **: Statistically significant differences between the indicated groups ($P < 0.01$ and $P < 0.05$, respectively). VEGF (+) and VEGF (−): VEGF overexpression and suppression in the tumor, respectively. R-1 (400) and R-1 (800): The mice with VEGF over-expressing tumor were treated with R-1mAb at doses of 400 and 800 μg/mouse, respectively.

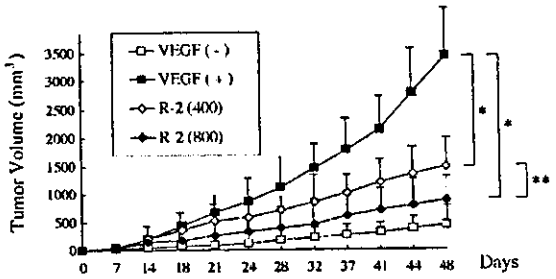

Fig. 2. The effect of R-2mAb-mediated suppression on the VEGF-induced HCC development. The R-2mAb was i.p. administered twice a week from the beginning of the experiment. The R-2mAb treatment also significantly attenuated the VEGF-mediated HCC development in a dose dependent manner. The tumor volume was determined by three-dimensional calipers at the indicated time points. Each point represents the mean ± SD ($n = 8$). *, **: Statistically significant differences between the indicated groups ($P < 0.01$ and $P < 0.05$, respectively). VEGF (+) and VEGF (−): VEGF overexpression and suppression in the tumor, respectively. R-2 (400) and R-2 (800): the mice with VEGF over-expressing tumor were treated with R-2mAb at doses of 400 and 800 μg/mouse, respectively.

When the mean tumor volume reached about 845 mm^3, either R-1mAb or R-2mAb treatment at a dose of 800 μg/mouse was started twice a week. As shown in Fig. 4, the R-1mAb and R-2mAb treatments exerted significant inhibitory effects as compared with the control group even after the tumor was fully established ($P < 0.05$ and $P < 0.01$, respectively). Noteworthy is the fact that the combination treatment with R-1mAb and R-2mAb almost achieved a dormancy status of the established tumor.

We next examined the effects of R-1mAb and R-2mAb on HCC development in the liver direct injection experiment. Similar to the results of allograft studies, both R-1mAb and R-2 mAb significantly suppressed the number and size of HCC development in the liver (Table 1). Neither tumor invasion into other organs nor metastasis was

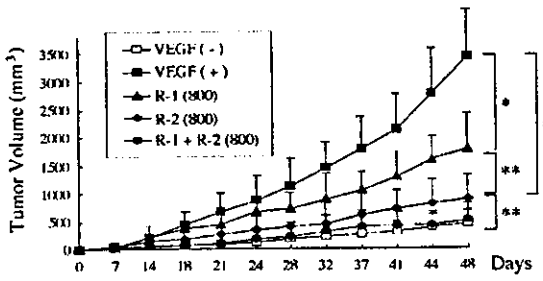

Fig. 3. The effect of combination treatment of R-1mAb and R-2mAb on the VEGF-induced HCC development. R-1mAb and R-2mAb were i.p. administered twice a week from the beginning of the experiment. The R-1mAb and R-2mAb combination treatment almost completely attenuated the VEGF-induced HCC development. The tumor volume was determined by three-dimensional calipers at the indicated time points. R-1 (800), R-2 (800): R-1mAb and R-2mAb-treated group at a dose of 800 μg/mouse, respectively. R-1 + R-2 (800): R-1mAb and R-2mAb combination-treated group at a dose of 800 μg/mouse. Each point represents the mean ± SD ($n = 8$). *, **: Statistically significant differences between the indicated groups ($P < 0.01$ and $P < 0.05$, respectively).

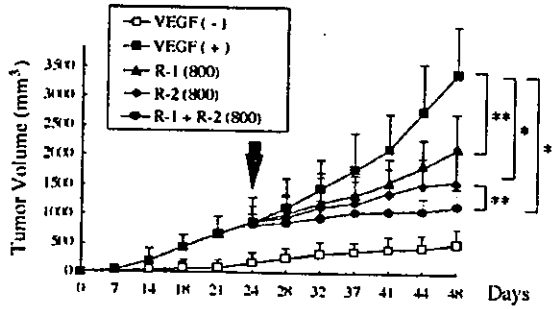

Fig. 4. The inhibitory effects of R-1mAb and R-2mAb against established tumors. The mice received either R-1mAb or R-2mAb at a dose of 800 μg/ml from day 24 (the mean tumor volume was 845 mm³). The R-1mAb and R-2mAb treatments exerted significant inhibitory effects as compared with the control group even after the tumor was established. The arrows indicate the time points of administration of the respective mAb. Each point represents the mean ± SD (each group consisted of eight mice). *, **: Statistically significant differences between the indicated groups ($P < 0.01$ and $P < 0.05$, respectively).

observed at the sacrifice time both in the allograft and orthotopic studies, and the systemic administration of neither R-1mAb nor R-2mAb had any apparent effect on the state of health of the mice (data not shown).

3.2. Tumor neovascularization

To determine whether the inhibitory effects of R-1mAb and R-2mAb on the tumor development were accompanied by suppression of neovascularization, we examined the expression level of CD31 in the tumor. As shown in Fig. 5, similar to the result of HCC development, the CD31-positive vessels in the tumors of either R-1mAb or R-2mAb-treated groups (800 μg/mouse) were significantly fewer

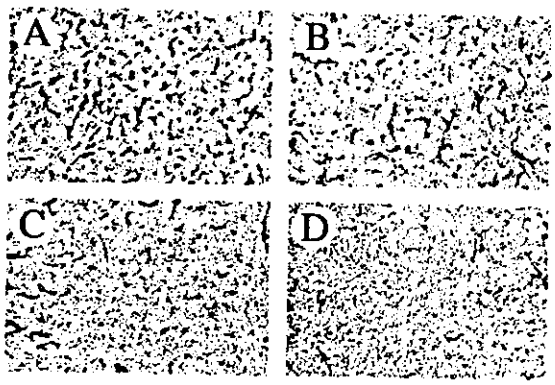

Fig. 5. The effects of R-1mAb and R-2mAb on CD31 expression in the tumors. The tumor vascularization was visualized by immunostaining of the CD31 vascular endothelial adhesion protein. (A) Tet-VEGF-HCC control group; (B) R-1mAb-treated group; (C) R-2mAb-treated group; (D) R-1mAb and R-2mAb combination-treated group. The description of each group is shown in Section 2. Original magnification was × 200.

Table 1
Effect of R-1 mAb and R-2 mAb on HCC development in the liver

	Number of tumors in the liver/mouse				Incidence of large tumor in the liver[a]
VEGF	29 ± 9[b]	**	*	*	7/7 (100%)
R-1 mAb	20 ± 6	*			7/5 (71%)
R-2 mAb	7 ± 4	*			7/2 (29%)
R-1 + R-2 mAbs	2 ± 1				7/0 (0%)

*$P < 0.01$ and **$P < 0.05$ between the indicated groups.
[a] A large tumor is over 5 mm in diameter.
[b] Data are expressed as mean ± SD ($n = 7$).

than those in the control (IgG-treated) group. The combination treatment with R-1mAb and R-2mAb almost attenuated the CD31-positive vessels in the tumor. A semi-quantitative analysis of the CD31-positive vessels in R-1mAb or R-2mAb revealed a statistically significant suppression as compared to the control group ($P < 0.01$). Similar to the tumor growth suppression, the inhibitory effect of R-2mAb was more potent than that of R-1mAb ($P < 0.05$). The combination treatment with R-1mAb and R-2mAb exerted a much stronger inhibition of the CD31-positive vessels in the tumor as compared to the treatment with R-2mAb alone ($P < 0.05$) (Fig. 6A). Neither R-1mAb nor R-2mAb altered the vascularization in the other normal organs, such as the lung and heart (data not shown).

3.3. Effect of R-1mAb and R-2mAb on apoptosis in vivo

We next examined whether the tumor inhibition by R-1mAb and R-2mAb was reflected by apoptosis in the tumor. As shown in Fig. 6B, the TUNEL-positive apoptotic cells more markedly increased with R-1mAb or R-2mAb treatment (800 μg/mouse) than in the control group ($P < 0.01$). The number of apoptotic cells with R-2mAb treatment significantly increased as compared to that with R-1mAb treatment ($P < 0.05$), and the combination treatment with R-1mAb and R-2mAb revealed much more TUNEL-positive cells in the tumor than with R-2mAb treatment alone ($P < 0.05$). The incidence of apoptosis in the tumor almost corresponded to the effect of tumor development inhibition. On the contrary, the PCNA-positive cells in the tumor did not show any difference between these mAbs-treated groups and the control group (data not shown).

3.4. Receptors activation in situ

To confirm that R-1mAb and R-2mAb at a dose of 800 μg/mouse actually inhibited autophosphorylation in the tumor, we investigated tyrosine-phosphorylated VEGFR-1 and VEGFR-2 in the tumor after i.p. injection of R-1mAb and R-2mAb. As shown in Fig. 7, the R-1mAb and R-2mAb significantly inhibited tyrosine-phosphorylation of the

respective receptors, and the combination treatment with R-1mAb and R-2mAb almost completely abolished the phosphorylation of both receptors in the tumor. We found co-expression of several bands of phosphorylated VEGFR in the tumor. These bands may reflect the process of additional glycosylations of VEGFR as reported previously [36]. Neither the activation of VEGFR-1 nor that of VEGFR-2 was altered by the administration of R-2mAb and R-1mAb, respectively. The R-1mAb and R-2mAb did not affect the protein expression of their respective receptors, either.

4. Discussion

Using the combination of Retro-Tet system, and specific neutralizing monoclonal antibodies against VEGFR-1 and VEGFR-2, we assessed the role of VEGF and receptor interaction in HCC development. Our data indicate that VEGF and receptor interaction plays a pivotal role in HCC development in a dose dependent manner in not only the initial stage but also when the tumor is fully established in association with angiogenesis suppression. Treatment with either R-1mAb or R-2mAb significantly suppressed the VEGF-induced HCC development, but neither single mAb treatment completely inhibited the tumor development. It has been reported that targeting of either VEGFR-1 or VEGFR-2 alone only partially blocks the growth of Lewis lung cell carcinoma (LLC) tumor, and inhibition of both VEGFR-1 and VEGFR-2 was necessary to completely ablate tumor growth [29]. We also observed that the combination treatment with both mAbs was required to achieve a complete attenuation of HCC tumor development and angiogenesis. These results suggested that, in addition to VEGFR-2, VEGFR-1 exerted a positive role in HCC development and angiogenesis. On the other hand, another report has shown that the LLC cells overexpressing VEGF showed no clear difference in the tumor growth rate between the wild type mice and the VEGFR-1 tyrosine kinase domain-deficient mice [13]. It has recently been reported that recruitment of the bone marrow-derived hematopoietic cells exerts some roles in tumor angiogenesis, and that VEGFR-1 plays an important role in the process [29]. It would be possible that recruitment of bone marrow-derived hematopoietic cells by VEGFR-1 was involved in HCC development in the current study, whereas it was not in the VEGFR-1 tyrosine kinase domain-deficient mice or in the LLC cells.

It is very important to examine whether the tumor shows re-growth or not in long-term study in spite of the continuous treatment with R-1mAb and R-2mAb. Since we killed all animals at the end of experiment (day 48), we unfortunately do not have an exact answer in the current study. However, it has been reported that no relapse of the

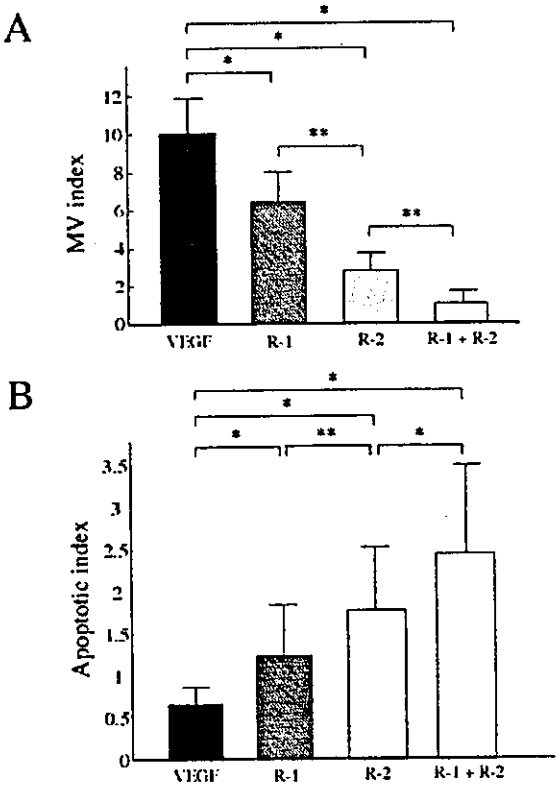

Fig. 6. Semiquantitative analysis of the CD31-immunopositive vessels (A), and the apoptosis (B) in the tumor. The description of each group is shown in Section 2. A semi-quantitative analysis of the CD31-positive vessels in R-1mAb or R-2mAb revealed a statistically significant suppression as compared to the control group. The inhibitory effect of R-2mAb was more potent than that of R-1mAb, and the combination treatment with R-1mAb and R-2mAb exerted a much stronger inhibition of the CD31-positive vessels in the tumor as compared to the treatment with R-2mAb alone. On the contrary, the incidence of apoptosis in the tumor almost corresponded to the effect of tumor development inhibition. R-1, R-2: R-1mAb and R-2mAb-treated group, respectively. R-1 + R-2: R-1mAb and R-2mAb combination-treated group. The data represent the mean ± SD ($n = 5$). *, **: Statistically significant differences between the indicated groups ($P < 0.01$ and $P < 0.05$, respectively). MV: microvessel.

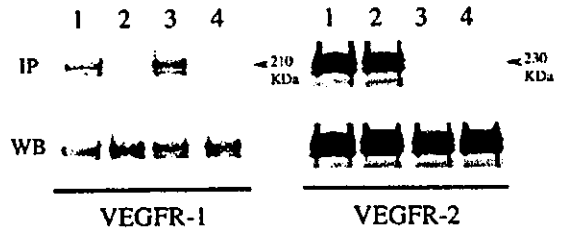

Fig. 7. The effects of R-1mAb and R-2mAb on the activation and protein expression of VEGFR-1 and VEGFR-2 receptors. The R-1mAb and R-2mAb significantly inhibited tyrosine-phosphorylation of the respective receptors. Neither the activation of VEGFR-1 nor that of VEGFR-2 was altered by the administration of R-2mAb and R-1mAb, respectively. The R-1mAb and R-2mAb did not also affect the protein expression of the respective receptors. IP, immunoprecipitation; WB, Western blotting. Lane 1: IgG-treated control group; lane 2: R-1mAb-treated group; lane 3: R-2mAb-treated group; lane 4: R-1mAb and R-2mAb combination-treated group.

tumor was observed with continued administration of R-2mAb at a dose of 400 μg/mouse for up to 120 days in LLC tumor-xenograft model. In addition, withdrawal of R-2mAb treatment resulted in re-growth of the tumors with kinetics similar to those of the control group [32]. Since we used the same antibody at higher dose in the current study, we assume that a similar phenomenon could be observed.

It has been shown that treatment with antiangiogenic agents induces a marked increase of apoptosis in the tumors, whereas it does not alter the tumor cell proliferation [37]. In this study, we noticed similar findings with R-1mAb and R-2mAb. It was an important point to determine whether apoptosis was observed mainly in the endothelial cells (EC) or in the HCC cells. We do not have an exact answer at this time. Although we performed a double immunohistochemical analysis with CD31 and TUNEL in a couple of times, we failed to obtain a good result. The background was very intense, and the interpretation was very difficult (data not shown). It has been suggested that apoptosis of EC occurred first, which led to secondary apoptosis of the tumor cells with antiangiogenic agents [38,39]. We previously found that VEGF did not affect the in vitro proliferation of the BNL-HCC cells, and neither VEGFR-1 nor VEGFR-2 expressed on the HCC cells [21,22]. It has been also reported that VEGFR-1 and VEGFR-2 up-regulated on EC during the tumor angiogenesis, and that VEGF was a survival factor for the tumor EC [4,11,40]. These findings, taken together, suggest that R-1mAb and R-2mAb also first induced the EC apoptosis, and this might induce the secondary apoptosis of the tumor cells. We also found that the R-1mAb and R-2mAb-treated tumors exhibited extensive necrosis accompanied with poor vascularization and increased tumor-cell apoptosis as compared with the large, vascularized control tumors. It has been reported that EC detachment and hemorrhage foci were detected as early as 24 h from VEGF withdrawal in the tumor. In addition at later points, extensive areas of the tumor underwent necrosis [41]. Similar observations were also reported with the R-1mAb-treated human epidermoid tumor in the nude mice [14]. Further studies are required to elucidate the exact mechanism in the future.

In summary, we found that both R-1mAb and R-2mAb significantly attenuated the HCC development in a dose dependent manner along with reduction of angiogenesis in the tumor. The inhibitory effect of R-2mAb was more potent than that of R-1mAb, and the combination treatment with both mAbs almost completely attenuated the VEGF-induced HCC development. Furthermore, these inhibitory effects with both mAbs could be achieved even on the established tumors, and in orthotopic transplantation. These results suggest that, in addition to VEGFR-2, VEGFR-1 also lies on the signal transduction pathway by which VEGF augments HCC development and angiogenesis not only at the initial stage but also after the tumor is fully established.

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Reduced Expression of Cell Cycle Regulator $p18^{INK4C}$ in Human Hepatocellular Carcinoma

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Cyclins, cyclin-dependent kinases (Cdks), and Cdk inhibitors (CdkIs) are frequently altered in human cancer. $p18^{INK4C}$, a member of the INK4 family of CdkIs, is a potential tumor-suppressor gene product. However, the expression of $p18^{INK4C}$ in hepatocellular carcinoma (HCC) remains unknown. The aim of this study was to examine the expression of $p18^{INK4C}$ in various liver diseases including HCC and to assess its clinical significance in HCC. To that end, we examined the expression of $p18^{INK4C}$ by immunohistochemistry in various liver diseases, including 51 HCCs, and also studied the relationship between $p18^{INK4C}$ expression, the phosphorylation of retinoblastoma protein (pRb), and the activity level of Cdk4 and Cdk6. Immunohistochemical analysis revealed the frequent loss of $p18^{INK4C}$ expression in HCC, especially in poorly differentiated HCC. The loss of $p18^{INK4C}$ expression was shown to be associated with a poor prognosis compared with that associated with $p18^{INK4C}$- positivity. Further, the kinase activity of Cdk4 was found to be higher in $p18^{INK4C}$-negative HCCs than in $p18^{INK4C}$- positive HCCs. However, the level of Cdk6 activity was similar in the 2 groups of HCCs. In $p18^{INK4C}$- positive HCCs, $p18^{INK4C}$ dominantly interacted with Cdk4 rather than with Cdk6. pRb phosphorylated at serine(Ser) 780 was detected more frequently in $p18^{INK4C}$ - negative than in $p18^{INK4C}$ - positive HCCs. In conclusion, the loss of $p18^{INK4C}$ expression may play a role in the differentiation and development of HCC through the up-regulation of Cdk4 activity. (HEPATOLOGY 2004;40:677–686.)

Recently, we have revealed that the aberrant expression of cell cycle-related proteins is one of the major factors contributing to the development of hepatocellular carcinoma (HCC).[1–3] The cell cycle is mainly governed by various cyclin-dependent kinases (Cdks); the activity is regulated positively by cyclins, and negatively by Cdk inhibitors (CdkIs). It is also regulated by phosphorylation and dephosphorylation events.[4–6] In mammalian cells to date, at least 2 distinct families of CdkIs are known. One, the p21 family, consists of $p21^{CIP1/WAF1}$, $p27^{KIP1}$, and $p57^{KIP2}$, which are general inhibitors of G1 to S in the cell cycle. The other known CdkI family, the inhibitor of Cdk4 (INK4) family, consists of $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$, which specifically inhibit cyclin D-related kinase activity by binding to Cdk4 or Cdk6.[4–6]

The CdkI proteins, potent negative regulators for the cell cycle, are potential tumor-suppressor gene products, and their loss might play an important role in the development of human cancers.[4–11] In fact, loss of INK4 family members such as $p15^{INK4B}$, $p16^{INK4A}$, and $p18^{INK4C}$ by gene mutation, deletion, and/or methylation has been observed in a variety of human cancers.[6–11] Clinical findings have revealed that the loss of $p16^{INK4A}$ expression is associated with poor prognosis for some types of human cancers.[9–11] These studies have suggested that INK4 family members might play a role in the progression and prognosis of human cancers.

Abbreviations: HCC, hepatocellular carcinoma; Cdk, cyclin-dependent kinase; CdkI, cyclin-dependent kinase inhibitor, INK4, inhibitor of Cdk4; NL, normal liver; CH, chronic hepatitis; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HRP, horseradish peroxidase; IgG, immunoglobulin G; pRb; retinoblastoma protein; LI, labeling index; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GST, glutathione S-transferase; HBV, hepatitis B virus; TNM, tumor regional lymph nodes, distant metastasis.

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Table 1. Relationship Between $p18^{INK4C}$ Immunoreactivity and Clinicopathological Features of HCC

		$p18^{INK4C}$ Staining Status			
	Patient No. (L.I. [%])	Positive (L.I. [%])	Negative (L.I. [%])	Negative Rate (%)	P Value
Sex					
Male	37 (8.5 ± 7.5)	19 (14.6 ± 5.3)	18 (2.3 ± 2.2)	48.6	
Female	14 (7.8 ± 6.7)	8 (12.6 ± 4.4)	6 (1.3 ± 1.2)	42.9	.762
Age (y)					
<65	27 (7.5 ± 6.7)	15 (11.2 ± 3.7)	12 (2.9 ± 1.3)	44.4	
≥65	24 (6.7 ± 5.2)	12 (10.9 ± 3.7)	12 (2.5 ± 1.7)	50.0	.577
Viral infection					
HCV-positive	45 (8.7 ± 7.2)	26 (13.8 ± 5.0)	19 (1.7 ± 1.6)	42.2	
HBs Ag-positive	6 (6.2 ± 6.1)	1 (21.0 ± 0)	5 (3.2 ± 3.1)	83.3	.195
*Histological grade					
WD/MD	45 (9.5 ± 7.0)	27 (14.0 ± 5.1)	18 (2.7 ± 2.1)	41.3	
PD	6 (0 ± 0)	0 (0 ± 0)	6 (0 ± 0)	100	.024
†TNM stage					
I/II	21 (11.9 ± 7.4)	16 (14.8 ± 5.8)	5 (2.6 ± 2.1)	23.8	
III/IV	30 (5.9 ± 6.1)	11 (12.9 ± 3.8)	19 (2.2 ± 1.8)	63.3	.025

Abbreviations: WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.
*Histological grading of HCC was determined using the criteria of the International Working Party.[17]
†TNM stage was determined using the classification proposed by the Internal Union Against Cancer and the American Joint Committee on Cancer.[16]

Some studies on the relationship between $p18^{INK4C}$ and HCC, on topics such as gene methylation of $p18^{INK4C}$ in HCC,[12] involvement of $p18^{INK4C}$ in troglitazone-induced cell cycle arrest of human hepatoma cell lines,[13] and the rate of carcinogen-induced liver tumor in $p18^{INK4C}$ mutant mice,[14] have been reported. However, to our knowledge, the expression of $p18^{INK4C}$ has not yet been thoroughly examined in various liver diseases, including HCC. In this study, therefore, we focused on the relationship between $p18^{INK4C}$ expression and clinical significance in various liver diseases including HCC.

We examined the expression of $p18^{INK4C}$ immunohistochemically by using an avidin-biotin complex plus tyramide signal amplification method for signal enhancement in normal liver (NL), chronic hepatitis (CH), cirrhosis, and HCC. We also evaluated the relationships between $p18^{INK4C}$ expression and the levels of Cdk4 and Cdk6 activity in HCC. In addition, we examined whether the expression of $p18^{INK4C}$ was an important predictor of outcome in patients with HCC. In this article, we report that loss of $p18^{INK4C}$ expression is involved in hepatocarcinogenesis.

Patients and Methods

Patients. Liver biopsy specimens were obtained from 30 patients with CH (22 males and 8 females; mean age, 48.4 ± 15.5 years; range, 21-80 years). Twenty-seven patients with CH were positive for hepatititis C virus (HCV) RNA, and 3 patients with CH were positive for the hepatitis B surface antigen (HBsAg). Of these 30 patients, 8 were in F1, 7 in F2, 6 in F3, and 9 in F4 according to Desmet's classification.[15] Seven NL tissues were obtained from corresponding surgical cases of liver metastasis of colon cancer (5 males and 2 females; mean age, 58.1 ± 4.9 years; range, 52-67 years). These patients were negative for HCV RNA and HBsAg. Tissue samples of HCC were obtained from 51 patients with HCC during surgery (37 males and 14 females; mean age, 62.9 ± 7.2 years; range, 44-75 years). Forty-five patients with HCC were positive for HCV RNA, and 6 patients with HCC HBsAg. The clinical–pathological data for the patients with HCC is shown in Table 1. Of these 51 patients, 8 were in stage I, 13 in stage II, 11 in stage III, and 19 in stage IV according to the criteria of the International Union against Cancer and the American Joint Committee on Cancer.[16] Histological grade of HCC was determined according to the criteria of the International Working Party.[17] The numbers of patients with well-, moderately, and poorly differentiated HCCs were 16, 29, and 6, respectively (Table 1). Tissues were frozen immediately at −70°C. Informed consent was obtained from each patient prior to participation, and the experimental protocol was approved beforehand by the Human Subjects Committee of Kagawa Medical University.

Chemicals and Antibodies. Chemicals were obtained from Sigma Chemical Co. (Tokyo, Japan) or Wako Pure Chemical Co. (Tokyo, Japan). All primary antibodies were purchased from Santa Cruz Biotechnology (Tokyo, Japan). Secondary antibodies were from Amersham Life Science (Tokyo, Japan). Optimal dilutions of antibodies

used for Western blot were as follows: polyclonal antibody H303 (anti-Cdk4), 1:200; polyclonal antibody C-21 (anti-Cdk6), 1:200; polyclonal antibody serine (Ser) 780 (antiphosphoserine Rb), 1:1000; monoclonal antibody TU-02 (α-tubulin), 1:1000; horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin G (IgG), 1:2000; and HRP-conjugated anti-mouse IgG, 1:2000. The phosphorylated Rb polyclonal antibody (Ser 780) reacts only with phosphorylated retinoblastoma protein (pRb) at Ser 780, which is specifically phosphorylated by cyclin D1/Cdk4, and detects a 105 kd protein corresponding to human pRb that includes amino acids 774 to 786.[18]

Immunohistochemical Examination. We prepared 2 μm-thick sections from formalin-fixed, paraffin-embedded tissue blocks. Sections were stained by an avidin-biotin-peroxidase complex method (Funakoshi Chemical, Tokyo, Japan). The detection of $p18^{INK4C}$ was performed by immunohistochemical study using polyclonal antibody M-20 (anti-$p18^{INK4C}$), as described in our previous article.[3] All sections were examined independently by two observers (T.M., S.W.), who were blinded to the clinical information for each case. For each sample, the percentage of nuclei-immunostained cells was estimated per 1,000 cancer cells. In each case, the nuclei staining evaluation for $p18^{INK4C}$ was classified as negative or positive. The decisions made by the two pathologists were fairly consistent. Furthermore, samples that resulted in disagreement on the immunostaining data between two observers (T.M., S.W.) were discussed with a third observer (A.M.), using a multiheaded microscope until agreement between at least two observers was achieved. The mean labeling index (LI) of $p18^{INK4C}$ was 8.4 $\pm$ 7.2 in 51 patients with HCC; therefore, for assessment of the expression of $p18^{INK4C}$, we categorized HCCs into 2 groups on the basis of the percentage of HCC cells positive for $p18^{INK4C}$ immunoreactivity: $p18^{INK4C}$ expression negative ($<$8.4%), and $p18^{INK4C}$ expression positive ($\geq$8.4%). Necrotic areas and edges of the tissue sections were not included in the counting in order to avoid possible immunohistochemical false-positive results.

Tissue Lysates. The tissue lysate was prepared as described in our previous reports.[1, 2] Protein concentration was measured by a dye-binding protein assay using the Bradford method.[19]

Gel Electrophoresis and Western Blot. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli,[20] and Western blot was performed as described by Towbin et al.,[21] using primary antibodies and HRP-conjugated secondary antibodies. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham) on X-ray film.

Kinase Assay of Cdk4 and Cdk6. The kinase activities of Cdk4 and Cdk6 were examined by autoradiography, as described in our previous reports.[1, 2]

Analysis of $p18^{INK4C}$-Bound Cdk4 and Cdk6 in HCC. Immunoprecipitation using $p18^{INK4C}$ was performed as described in our previous report.[1, 2] Immunoprecipitates were then applied to 12.5% SDS-PAGE, and $p18^{INK4C}$-bound Cdk4 and Cdk6 were detected by Western blot analysis using antibody for Cdk4 and Cdk 6, respectively.

Densitometric Analysis. Density of the phosphorylated band of Rb fusion protein obtained by autoradiography was quantitated by densitometric scanning.

Statistical Analysis. We performed statistical analysis of the relationship between $p18^{INK4C}$ expression and clinical–pathological parameters by means of the Fisher t test, as appropriate. The survival curve was plotted using the Kaplan-Meier method, and differences were analyzed statistically by log-rank test. The relationship between $p18^{INK4C}$ expression and the activity of Cdk4 or Cdk6 was also determined by Scheffe test. In addition, to identify independent predictors in patients with HCC, multivariate analysis using the Cox proportional hazards model was performed.

Results

$p18^{INK4C}$ Expression in NL, CH, and Liver Cirrhosis. Representative immunostaining of $p18^{INK4C}$ in NL (Fig. 1A), CH (Figs. 1B and C), and liver cirrhosis (Fig. 1D) are shown. The stages of fibrosis in Figs. 1B, C, and D were F2, F3, and F4, respectively. The $p18^{INK4C}$ in NL and CH (Figs. 1B and C) was localized in hepatocellular nuclei and/or nonparenchymal cells such as infiltrating lymphocytes (Fig. 1B, arrowhead), fibroblasts (Fig. 1C, arrowhead), and endothelial cells. The expression of $p18^{INK4C}$ was detected in all patients (n = 30) with CH or cirrhosis, regardless of fibrosis staging. As shown in Fig. 1D, the expression of $p18^{INK4C}$ in liver cirrhosis was detected not only in the hepatocellular cytoplasm, but also in the hepatocellular nucleus in all 9 cases. The expression of $p18^{INK4C}$ was detected in the hepatocellular nucleus in all NLs examined in this study.

$p18^{INK4C}$ in Malignant Liver Tissues. As shown in Table 1, $p18^{INK4C}$-negative HCCs were observed in 24 of 51 HCCs (47%; LI, 2.3 $\pm$ 2.2%) examined, and $p18^{INK4C}$-positive HCCs were detected in the remaining 27 cases (53%; LI, 14.0 $\pm$ 5.1%). In well-differentiated HCCs, 10 of 15 tumor samples were positively stained in the nucleus of cancer cells for $p18^{INK4C}$ (67%; LI, 15.2 $\pm$

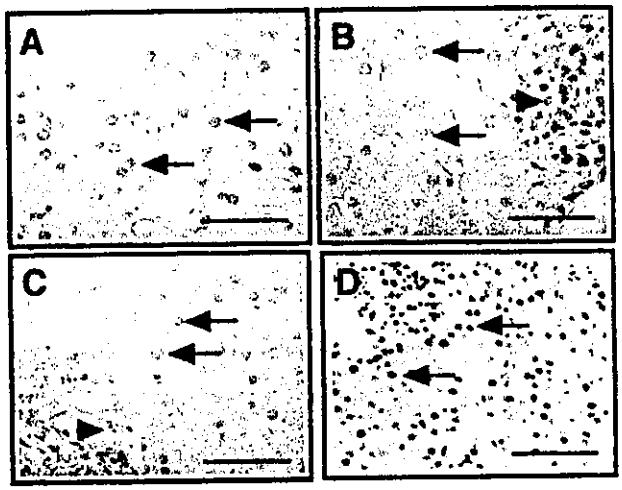

Fig. 1. Immunohistochemistry of $p18^{INK4C}$ in liver tissues of (A) normal liver, (B and C) chronic hepatitis, and (D) liver cirrhosis. Fibrosis stages in panels B, C, and D were grades F2, F3, and F4, respectively, according to Desmet's classification. Staining for $p18^{INK4C}$ in normal liver and chronic hepatitis was seen (A-C) in the hepatocellular nucleus (**arrows**)), (B) in lymphocytes (**arrowhead**), and (C) in fibroblasts (**arrowhead**). Expression of $p18^{INK4C}$ in liver cirrhosis was positive not only for $p18^{INK4C}$ staining in the hepatocellular cytoplasm but also for $p18^{INK4C}$ staining in (D) the hepatocellular nucleus (**arrows**). **Scale bars**, 50 μm. (Original magnification [A-D], ×200.)

6.7%; Figs. 2A-C), and the remaining cases were negative for staining (33%; LI, 3.8 ± 1.6%). In moderately differentiated HCCs, $p18^{INK4C}$-positive HCCs were detected in 17 of 30 cases (57%; LI, 13.4 ± 3.8%) and not in the remaining cases (43%; LI, 2.7 ± 2.2%; Figs. 2D-F). In poorly differentiated HCCs, $p18^{INK4C}$-positive HCCs were not detected in any of the cases examined in this study (Figs. 2G-I: LI, 0.0 ± 0.0 %).

Correlations between $p18^{INK4C}$ expression and clinical–pathological factors as determined by univariate analysis are summarized in Table 1. The ratio of $p18^{INK4C}$-positive HCCs in poorly differentiated tissues (LI, 0.0 ± 0.0%) was significantly lower than that in well-differentiated and moderately differentiated tissues (LI, 14.0 ± 5.1%; Table 1, $P = .024$). In addition, the ratio of $p18^{INK4C}$-negative HCCs in tumor stages III and IV (63.3%) was significantly higher than that in tumor stages I and II (23.8 %; $P = .025$). However, no significant relationship was seen between $p18^{INK4C}$ expression and gender, age, or viral markers.

Prognostic Significance of $p18^{INK4C}$. Survival analysis of HCC was performed by the Kaplan-Meier method. The tumors were divided into $p18^{INK4C}$-positive and $p18^{INK4C}$-negative groups. Patients with $p18^{INK4C}$-negative HCC had a significantly worse prognosis than those with $p18^{INK4C}$-positive HCC ($P = .0007$; Fig. 3). Multivariate analysis using the Cox proportional hazards model was performed. Among age, sex, histological grade, tumor stage, and hepatitis viral infection, only tumor stage and $p18^{INK4C}$ expression were shown to be independent prognostic factors for overall survival of patients with HCC (Table 2).

Activities and Amounts of Cdk4 and Cdk6 in HCC. $p18^{INK4C}$ has been shown to inhibit cyclin D1- related

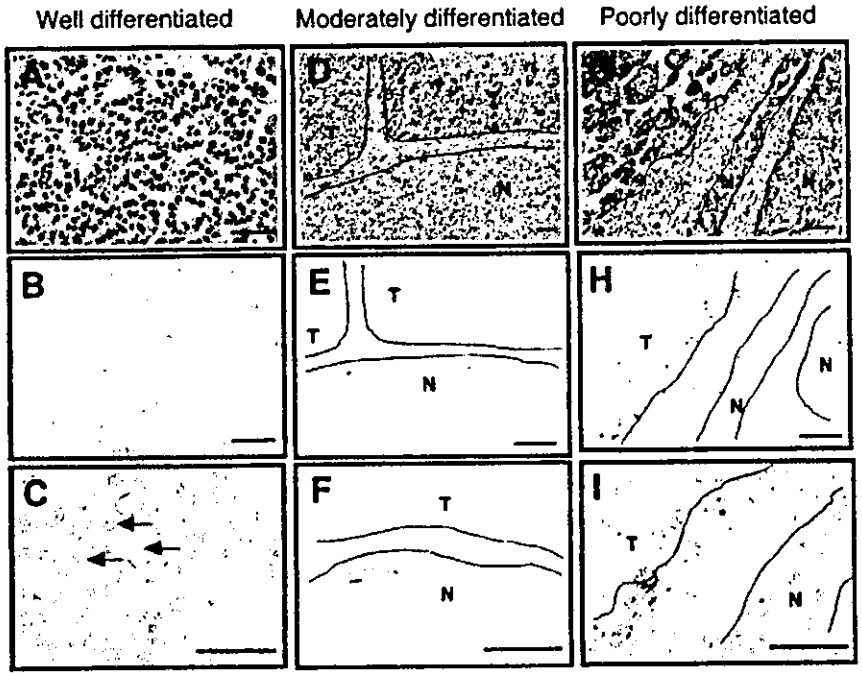

Fig. 2. Immunohistochemistry of $p18^{INK4C}$ in (B and C) well-, (E and F) moderately, and (H and I) poorly differentiated HCCs. A, D and G show hematoxylin-eosin staining of the section adjacent to B, E, and H, respectively. C, F, and I represent higher magnifications of the sections of B, E, and H, respectively. T and N indicate HCC and nontumorous tissues with cirrhosis, respectively. The expression of $p18^{INK4C}$ 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. **Scale bars**, 50 μm.(Original magnification: A, B, E, and H, ×100; D and G, ×50; and C, F, and I, ×200.)

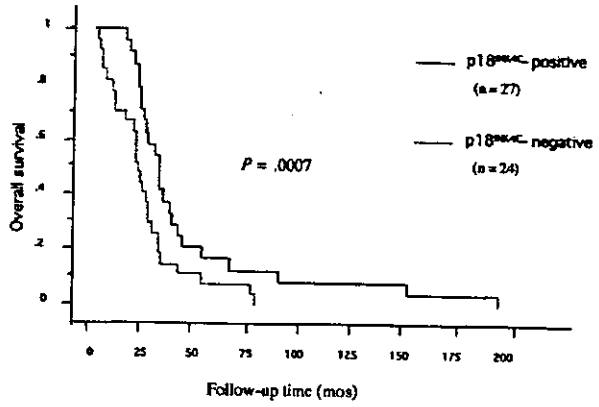

Fig. 3. Kaplan-Meier curves of overall survival according to $p18^{INK4C}$ expression (positive staining vs. negative staining) in 51 patients with HCC. Time to death was significantly shorter in patients with $p18^{INK4C}$-negative HCCs than in those with $p18^{INK4C}$-positive HCCs ($P < .0007$).

kinase activity specifically by binding Cdk4 or Cdk6. Therefore, to examine the role of $p18^{INK4C}$ in the control of cell proliferation via inhibition of Cdk4 or Cdk6 activities in the G1 to S phase, we studied the activity of Cdk4 and Cdk6 in 38 HCC cases, including 12 well-differentiated, 20 moderately differentiated, and 6 poorly differentiated tumors. Clinical–pathological data for the 38 patients with HCC are shown in Table 3. $p18^{INK4C}$-negative HCCs were 21 (55 %) out of 38, with the remaining 17 (45 %) $p18^{INK4C}$-positive. Glutathione S-transferase (GST)-Rb fusion protein was used as a substrate to measure the Cdk4 and Cdk6 activity. As shown in Fig. 4A, staining of the GST-Rb fusion protein after separation by SDS-PAGE showed a single band with a molecular size of 46 kd. The activities of Cdk4 and Cdk6 were measured by an *in vitro* kinase assay using the GST-Rb fusion protein as a substrate (Figs. 4B and C). A single band of phosphorylated GST-Rb fusion protein resulting from the level of Cdk4 and Cdk6 activity was detected in all HCCs studied. Additionally, phosphorylated GST-Rb fusion protein was not observed in the immunoprecipitate product when nonimmune rabbit IgG was used as a control (data not shown). Extremely enhanced Cdk4 activity was detected in a subset of $p18^{INK4C}$-negative HCCs (Fig 4B, patients 6 and 7). In moderately differentiated HCCs, the number of $p18^{INK4C}$-negative HCCs and $p18^{INK4C}$-positive HCCs was 10 and 10, respectively. In moderately differentiated HCCs, Cdk4 activity of $p18^{INK4C}$-negative and $p18^{INK4C}$-positive HCCs were 9.8 ± 6.4 and 4.1 ± 1.4 times higher, respectively, than those of control NLs (Fig. 4D). Cdk4 activity in $p18^{INK4C}$-negative moderately differentiated HCCs was significantly higher than that in $p18^{INK4C}$-positive HCCs ($^*P < .02$). Conversely, Cdk6 activity of moderately differentiated HCCs did not change regardless of expression of $p18^{INK4C}$ (Fig. 4 D). In well-differentiated HCCs, the number of $p18^{INK4C}$-negative HCCs and positive HCCs was 7 and 5, respectively. Although Cdk 6 activity remained at a similar level regardless of $p18^{INK4C}$ expression in well-differentiated HCCs, Cdk4 activity in $p18^{INK4C}$-negative HCCs was significantly higher than that in $p18^{INK4C}$-positive HCCs ($^*P < .05$; Fig. 4E). The kinase activity levels of Cdk4 and Cdk6 in poorly differentiated HCCs were 16.6 ± 3.8 and 2.8 ± 1.3, respectively (data not shown). We then ana-

Table 2. Multivariate Cox Model Analysis of Overall Survival of Patients With HCC

	Hazards Ratio	Overall Survival 95% CI	P Value
Age (<65/≥65)	0.870	0.407–1.860	.719
Sex (male/female)	0.821	0.354–1.902	.645
Histology (PD/WD, MD)	1.380	0.443–4.306	.579
Tumor stage (III–IV/I–II)	2.595	1.077–6.257	.034
Infection (HCV/HBV)	0.772	0.300–1.982	.590
$p18^{INK4C}$ (negative/positive)	2.725	1.155–6.427	.022

Abbreviations: PD, poorly differentiated; WD, well-differentiated; MD, moderately differentiated.

Table 3. Characteristics of HCC Patients Examined for Cdk4 and Cdk6 Activity

Sex	
Male	25
Female	13
Age (y)	
Mean ± SD	63.8 ± 6.5
Range	44–75
Viral infection	
HCV-positive	33
HBsAg-positive	5
*Histological background	
F3	6
F4	32
†Histological differentiation	
WD	12
MD	20
PD	6
$p18^{INK4C}$ expression	
Positive	17
Negative	21
‡TNM stage	
I	13
II	10
III	8
IV	7

Abbreviations: WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated; TNM, tumor regional lymph nodes, distant metastasis.

*The fibrosis stage of the tissue surrounding HCC was assessed according to Desmet's classification.

†Histological grading of HCC was determined using criteria of the International Working Party.[17]

‡TNM stage was determined using the classification proposed by the International Union Against Cancer and the American Joint Committee on Cancer.[16]

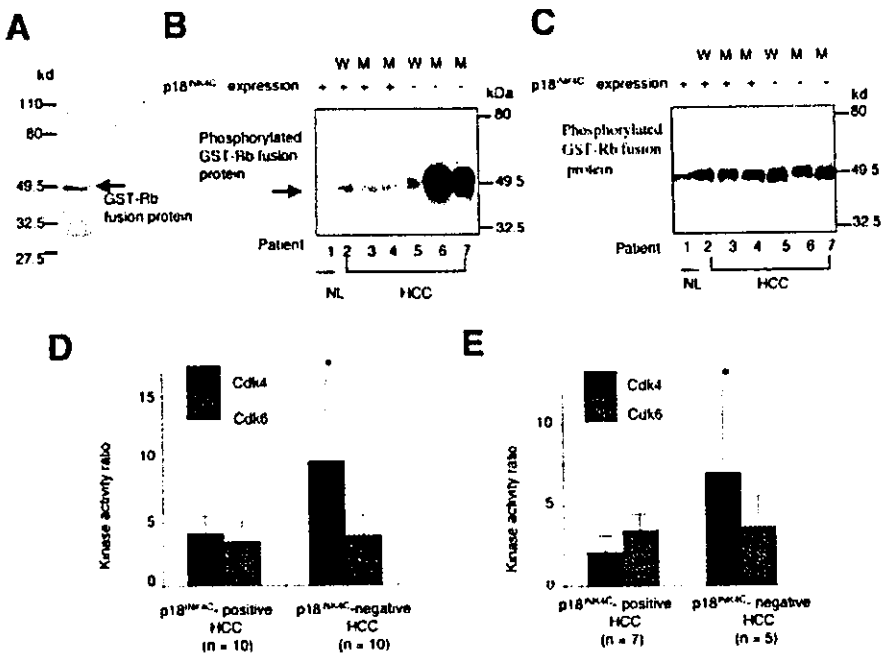

Fig. 4. Activity of Cdk4 and Cdk6 in HCC. (A) SDS-PAGE profile of a glutathione-S transferase (GST)-Rb fusion protein. Staining showed a single band of 46 kd (**arrow**). (B) Cdk4 and (C) Cdk6 kinase assays in NL and HCC. Lysates containing 500 μg of total cellular protein were prepared as described in Materials and Methods. The protein was precipitated with excess Cdk4 or Cdk6 antibodies, incubated for 10 minutes at 30°C with [γ-^{32}P] ATP and GST-Rb fusion protein, then resolved on 12.5% SDS-polyacrylamide gel. (B) The **arrow** indicates the band corresponding to the phosphorylation of GST-Rb fusion protein. (B and C) The immunostaining pattern of $p18^{INK4C}$ in NL and HCC is indicated as positive (+) or negative (−). W, well-differentiated HCC; M, moderately differentiated HCC. Note that extremely enhanced Cdk4 activity was seen in a subset of $p18^{INK4C}$-negative HCCs, but Cdk6 activity level was similar regardless of $p18^{INK4C}$ expression. The relative levels of Cdk4 and Cdk6 activity in (D) moderately differentiated and (E) well-differentiated HCCs in $p18^{INK4C}$-positive and -negative HCCs. Cdk4 activity of $p18^{INK4C}$-negative HCCs was significantly higher than that of $p18^{INK4C}$-positive HCCs when measured by the phosphorylation of GST-Rb fusion protein, whereas Cdk6 activity was not significantly different between $p18^{INK4C}$-positive and $p18^{INK4C}$-negative HCCs, regardless of differentiation. *$P < .05$ compared with HCC $p18^{INK4C}$-positive HCCs.

lyzed whether one of the pRb phosphorylation sites, Ser 780, was phosphorylated. The phosphorylated pRb band was detected in $p18^{INK4C}$-negative HCCs (Fig. 5A). A band corresponding to the pRb phosphorylated at Ser 780 was detected in 18 (86%) of 21 $p18^{INK4C}$-negative HCCs, and in 2 (12%) of 17 $p18^{INK4C}$-positive HCCs. The expression levels of Cdk4 and Cdk6 were measured by Western blot. In HCC, Cdk4 (Fig. 5B) and Cdk6 (Fig. 5C) immunoreactive bands were detected at molecular weights of 34 kd and 38 kd, respectively. As an internal control, the amount of α-tubulin was almost the same in each lane (Fig. 5D). Protein levels of Cdk4 and Cdk6 were almost the same in $p18^{INK4C}$-negative and $p18^{INK4C}$-positive HCCs in not only moderately differentiated HCCs (Fig. 5E) but also in well-differentiated HCCs (Fig. 5F).

Detection of $p18^{INK4C}$-Bound Cdk4 and Cdk6 in HCC. Immunoprecipitates with an anti-$p18^{INK4C}$ antibody obtained from 17 HCCs were immunoblotted with an anti-Cdk4 or anti-Cdk6 antibody. Clinical–pathological data for these 17 patients with HCC are shown in Table 4. Patients 2, 3, and 4 in Fig. 5G correspond to F.O., N.S., and Y.K., respectively. As shown in Fig. 5G and Table 4, $p18^{INK4C}$-bound Cdk4 was detected in 16 patients (94%), including HCCs from patients 2 (F.O.), 3 (N.S.), and 4 (Y. K.), and was not detected in one case (R.K.). $p18^{INK4C}$-bound Cdk6 was detected in 2 cases (12%), including HCCs from patient 2 (F.O.), but not in any other cases (Fig. 5G, Table 4). $p18^{INK4C}$-bound Cdk4 was also detected in 2 cases (F.O. and F.E.) with interaction between Cdk6 and $p18^{INK4C}$ (Table 4).

Discussion

CdkIs regulate the progression of the cell cycle by modulating the activity of Cdks.[9] Inactivation of CdkIs has been associated with neoplastic transformation in a large number of human epithelial tissues.[5,6] Moreover, recent studies have extensively demonstrated that the inactivation of $p16^{INK4A}$ in the members of the INK4 family leads to the development and aggression of a number of human malignancies including HCC.[7–11] These previous studies

Table 4. Characteristics of HCC Patients Examined for Detection of $p18^{INK4C}$-Bound Cdk4 and Cdk6

Patient	Sex	Age	Virus	*Histological Background	†Histological Grade	‡TNM Stage	$p18^{INK4C}$-Bound Cdk4	$p18^{INK4C}$-Bound Cdk6
F.O.	M	70	C	F4	WD	I	D	D
S.K.	M	54	C	F4	WD	I	D	ND
M.T.	M	62	C	F4	WD	II	D	ND
T.K.	F	66	C	F4	WD	I	D	ND
Y.S.	M	56	B	F3	WD	I	D	ND
K.H.	F	65	C	F4	WD	II	D	ND
T.K.	M	58	C	F3	WD	I	D	ND
N.S.	M	74	C	F4	MD	II	D	ND
Y.K.	M	61	C	F4	MD	I	D	ND
R.K.	M	52	B	F3	MD	I	ND	ND
T.K.	F	71	C	F4	MD	II	D	ND
Y.K.	M	60	C	F4	MD	III	D	ND
F.E.	M	66	C	F4	MD	I	D	D
S.N.	F	64	C	F4	MD	II	D	ND
T.M.	M	58	B	F3	MD	II	D	ND
Y.M.	M	74	C	F4	MD	II	D	ND
K.N.	M	67	C	F4	MD	III	D	ND

Abbreviations: B, hepatitis B; C, hepatitis C; WD, well-differentiated; MD, moderately differentiated; D, detection; ND, no detection.
*The fibrosis stage of the tissue surrounding HCC was assessed according to Desmet's classification.
†Histological grading of HCC was determined using the criteria of the International Working Party.[17]
‡TNM stage was determined using the classification proposed by the International Union Against Cancer and the American Joint Committee on Cancer.

has also been observed in testicular cancer and oligodendroglia.[22,23] These previous reports are consistent with the results of reduced $p18^{INK4C}$ expression in HCC found in this study. Roncalli et al.[12] reported that $p18^{INK4C}$ was not methylated in any of the HCCs examined. Therefore, there is little possibility that the cause of loss of $p18^{INK4C}$ expression is related to the promoter methylation of $p18^{INK4C}$. However, because there have been no other papers examining methylation of the $p18^{INK4C}$ gene in HCC, more studies are needed to draw any conclusions. In addition, loss of $p18^{INK4C}$ protein at the level of translation, or proteosome-mediated degradation, might be responsible for the down-regulation of $p18^{INK4C}$ in HCC.

All tissue samples used in this study were either HCV- or hepatitis B virus (HBV)-positive. In this study, the expression of $p18^{INK4C}$ in HCV- or HBV-induced CH was detected in all cases, whereas expression was not detected in a subset of HCV- or HBV-induced HCCs. These data suggest that the changes in $p18^{INK4C}$ expression in the process of HCC from CH are not affected by hepatitis viral infection but that its changes are affected by the malignant process.

Identification of the grade of tumor malignancy would facilitate treatment selection for patients and provide important information for predicting their prognosis. In HCC, clinical–pathological prognostic factors, such as tumor size, the number of tumor nodules, capsule formation, capsule invasion, and vascular invasion, have been studied.[34] Cell cycle-related molecules, such as proliferating nuclear antigen, p53, $p21^{CIP1}$, $p27^{KIP2}$, and p73, were shown to be prognostic biomarkers in various types of human cancer including HCC.[35–39] To date, the relationship between $p18^{INK4C}$ and prognosis in cancers has been reported in only one study on oligodendroglioma.[23] However, there have been no reports on the relationship between $p18^{INK4C}$ expression and prognosis in human HCC. In the present study, survival analysis by the Kaplan-Meier method revealed that $p18^{INK4C}$ expression was associated with the overall survival of patients with HCC. Of particular importance is the finding that the loss of $p18^{INK4C}$ expression was significantly associated with short survival of patients with HCC. According to the multivariate analysis, $p18^{INK4C}$ and tumor stage were independent prognostic factors for overall survival. These data suggest that loss of $p18^{INK4C}$ in HCC might serve as an indicator of poor prognosis.

In the univariate analysis, $p18^{INK4C}$ expression correlated with the differentiation status and the tumor stage of HCCs, whereas only tumor stage and $p18^{INK4C}$ expression level, and not the differentiation status, were independent prognostic markers in the multivariate analysis. In recent reports, Hu et al.[40,41] showed that after multivariate analysis, the differentiation of HCC is not an independent prognostic marker for overall survival, although it was a prognostic marker for overall survival in the univariate analysis; this is consistent with the results obtained in the present study. Collectively, the differentiation of HCC is a prognostic marker dependent on other factors, such as TNM stage and $p18^{INK4C}$ expression.

The $p18^{INK4C}$ protein has been shown to interact with, and subsequently inactivate Cdk4. Half of the HCCs in

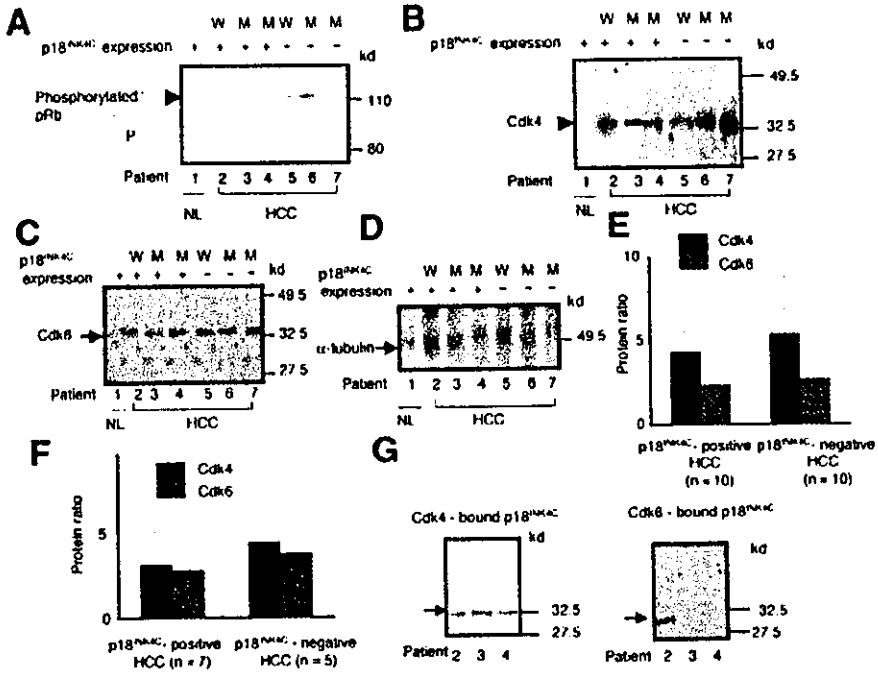

Fig. 5. Amounts of Cdk4 and Cdk6 in HCC. (A) Representative Western blot of retinoblastoma protein (pRb) using an antibody against phosphorylated pRb (Ser 780) in normal liver (NL) and HCC. The phosphorylation of pRb was detected in HCCs negative for $p18^{INK4C}$ expression, but not in those positive for $p18^{INK4C}$ expression. Western blot of (B) Cdk4, (C) Cdk6, and (D) α-tubulin in NL and HCC. The relative amount of Cdk4 and Cdk6 in (E) moderately differentiated HCC and (F) well-differentiated HCC in $p18^{INK4C}$-positive and - negative HCCs. Note that the amounts of Cdk4 and Cdk6 in $p18^{INK4C}$-positive and $p18^{INK4C}$-negative HCCs did not significantly differ. (G) Amounts of Cdk4-bound $p18^{INK4C}$ and Cdk6-bound $p18^{INK4C}$ in $p18^{INK4C}$- positive HCCs. Note that the complex of Cdk6 and $p18^{INK4C}$ was not seen in the liver tissues of patients 3 and 4 with $p18^{INK4C}$-positive HCC but was detected in the liver tissue of patient 2 (**arrow**). Cdk4-bound $p18^{INK4C}$ was detected in these same patients (2, 3, and 4; **arrow**). W, well-differentiated HCC; M, moderately differentiated HCC.

suggested that the other INK4 family might also play a role in the progression and prognosis of human cancers. Recently, inactivation of $p18^{INK4C}$ has been reported in various human cancers.[22–25] To date, very little data is available on the relationship between $p18^{INK4C}$ and HCC,[12] though previous reports have investigated gene methylation of $p18^{INK4C}$ in HCC, involvement of $p18^{INK4C}$ in troglitazone-induced cell cycle arrest of hepatoma cell lines,[13] and the rate of carcinogen-induced liver tumor in $p18^{INK4C}$ mutant mice.[14] The expression of $p18^{INK4C}$ protein in HCC is not yet known. In the present study, therefore, we evaluated the expression of $p18^{INK4C}$ in various liver diseases including HCC. To our knowledge, this study is the first to assess the involvement of $p18^{INK4C}$ protein in hepatocarcinogenesis.

The major finding in this study was that the loss of $p18^{INK4C}$ does not occur in most NL, CH, and cirrhosis, but does occur in a subset of HCCs, especially in poorly differentiated HCCs, suggesting that loss of $p18^{INK4C}$ is involved in hepatocarcinogenesis. The levels of Cdk4 activity in $p18^{INK4C}$-negative HCCs were significantly higher than those in $p18^{INK4C}$- positive HCCs, underscoring the functional importance of $p18^{INK4C}$ as an inhibitor of Cdk4 in HCC. It has been shown that the loss of $p18^{INK4C}$ expression in HCC is a poor prognostic marker.

Evidence suggests that $p18^{INK4C}$ functions as a tumor suppressor. Some studies using gene knockout mice indicated that the loss of $p18^{INK4C}$ displays a variety of aberrant phenotypes including lymphoproliferative disorders, organomegaly, and pituitary gland hyperplasia. Double knockout mice for $p18^{INK4C}$ and other members of the CdkI family display more varied and pronounced phenotypes,[26–28] indicating that $p18^{INK4C}$ is important for the proliferative control of various cell lineages. On the relationship between hepatocarcinogenesis and p 18^{INK4C}, Bai et al.[14] reported that $p18^{INK4C}$-null and -heterozygous mice with chemical carcinogen results in tumor development at an accelerated rate, suggesting that loss and decrease of $p18^{INK4C}$ leads to malignant transformation. In addition, the $p18^{INK4C}$ gene has been mapped to chromosome 1q32, where chromosomal translocation or loss has been found in various human cancers including HCC.[29–33] Such events might explain the loss in $p18^{INK4C}$ protein expression found in a subset of HCC in this study. A marked decrease in $p18^{INK4C}$ protein level

this study lacked detectable levels of $p18^{INK4C}$ protein when assayed by immunohistochemistry. Regarding Cdk4, we found previously that increase in its activity was particularly important in the development of HCC in humans and in the Long-Evans Cinnamon rat, an animal HCC model.[1,2] In addition, in our previous report[2] we found that the kinase activity of Cdk4 was markedly increased in poorly differentiated HCC compared to that in well- and moderately differentiated HCC, suggesting that Cdk4 activation may be closely related to the histopathological grade of HCC. Therefore, to determine whether reduced $p18^{INK4C}$ in HCC is related to increased cell proliferative activity, we studied the activities of the target proteins of $p18^{INK4C}$, Cdk4, and Cdk6 in HCCs with the same degree of differentiation (*i.e.*, in order to not reflect the differentiation in HCC) in $p18^{INK4C}$-positive and $p18^{INK4C}$-negative HCCs. Although Cdk6 activity in $p18^{INK4C}$-positive and $p18^{INK4C}$-negative HCCs was not significantly different between well- and moderately differentiated HCCs, Cdk4 activity was significantly higher in well- and moderately differentiated $p18^{INK4C}$-negative HCCs than in $p18^{INK4C}$-positive HCCs. On the other hand, $p18^{INK4C}$-positive HCCs were not detected in any cases of poorly differentiated HCCs used in this study. Thus, we could not study the relationship between $p18^{INK4C}$ expression and the activities of Cdk4 and Cdk6 in poorly differentiated HCC. The kinase activity levels of Cdk4 and Cdk6 in poorly differentiated HCCs were 16.5 ± 3.8 and 2.8 ± 1.3, respectively (data not shown). Based on these data, Cdk4 activity level was also markedly increased in poorly differentiated HCCs of $p18^{INK4C}$-negative HCCs. These data suggest that the up-regulation of Cdk4 activity in $p18^{INK4C}$-negative HCCs might not be related to the increase of its protein but rather to the reduction of $p18^{INK4C}$ relative to that in $p18^{INK4C}$-positive HCCs.

Why does the reduced $p18^{INK4C}$ lead to the up-regulation of Cdk4 activity but not of Cdk6? We hypothesized that $p18^{INK4C}$ might bind to Cdk4 rather than to Cdk6 in HCC tissues. To study the formation of these complexes ($p18^{INK4C}$/Cdk4 and $p18^{INK4C}$/Cdk6), we immunoprecipitated $p18^{INK4C}$ from HCC lysates with $p18^{INK4C}$ expression and performed Western blot analysis of Cdk4 and Cdk6 (Fig. 5G). Based on these data, $p18^{INK4C}$ was shown to substantially bind to Cdk4 but not to Cdk6 in HCC. Therefore, it was suggested that, in HCC, reduced $p18^{INK4C}$ contributed only to the up-regulation of Cdk4. The $p18^{INK4C}$ protein has been shown to interact with, and subsequently inactivate, Cdk4.[5,6] Conversely, there have been no reports that activation of Cdk4 does not directly decrease $p18^{INK4C}$ expression. Therefore, it is difficult to guess whether increased Cdk4 activity in a subset of HCC does decrease $p18^{INK4C}$ expression.

To date, $p18^{INK4C}$ has been reported to play an important role in the regulation of cell differentiation.[22,23] To investigate the possible involvement of this protein in the differentiation of HCCs, we studied the relationship between the level of $p18^{INK4C}$ expression and the histological grade of HCC. Expression of $p18^{INK4C}$ was reduced in poorly differentiated HCCs compared with levels in well- and moderately differentiated HCCs, supporting the finding in previous reports that $p18^{INK4C}$ accumulates at high levels in terminally differentiated cells.[22,42,43] However, our studies have not yet clarified whether the loss of $p18^{INK4C}$ in HCC promotes or inhibits differentiation. These data suggest that decreased expression of $p18^{INK4C}$ may play a role in the regulation of tumor differentiation as well as in the malignant transformation leading to HCC. In the present study, $p18^{INK4C}$ was expressed in the nuclei of hepatocytes in NL and CH, and $p18^{INK4C}$ was also detected in the cytoplasm in liver cirrhosis. $p18^{INK4C}$ has been shown to localize both in the nucleus and in the cytoplasm of cells.[23,44] However, the significance of the cytoplasmic expression of $p18^{INK4C}$ in hepatocytes of livers with cirrhosis remains unclear.

In conclusion, loss of $p18^{INK4C}$ expression may be important in the process of malignant transformation and appears to be closely related to histological differentiation of HCC. In addition, loss of $p18^{INK4C}$ was shown to be associated with up-regulation of Cdk4 activity. Furthermore, expression of $p18^{INK4C}$ may be an effective predictor of clinical behavior in HCC, and, therefore, a new prognostic marker for HCC.

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