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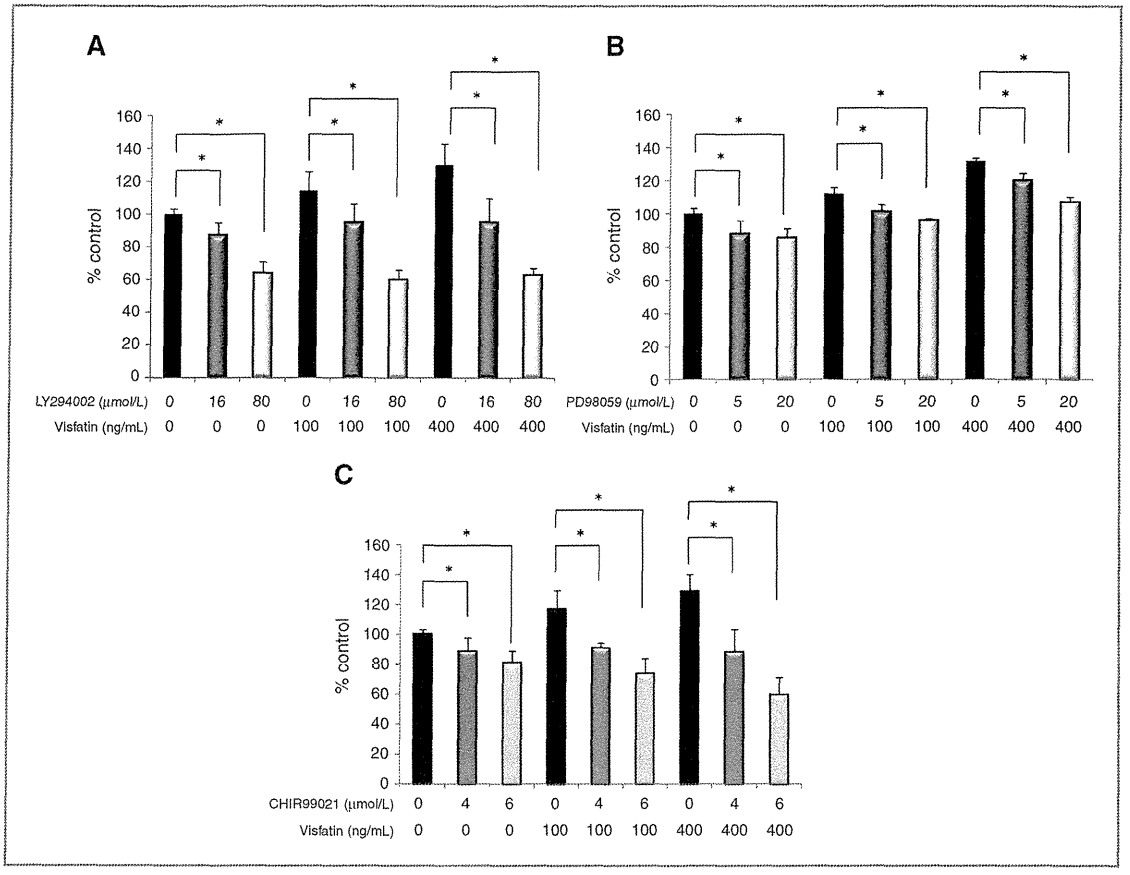

Figure 3. Effects of inhibitors of PI3K, MEK1, and GSK-3β on visfatin-induced proliferation in HepG2 cells. HepG2 cells were treated with LY294002, a PI3K inhibitor (A), PD98059, an MEK1 inhibitor (B), or CHIR99021, a GSK-3β inhibitor (C), in the absence or presence of visfatin (100 or 400 ng/mL) for 48 hours. Cell proliferation was evaluated by an XTT assay. Results were expressed as a percentage of the control value. Bars, SD of triplicate assays. *, $P < 0.05$.

were treated with similar concentrations of visfatin. BCAA also exerted no significant effect on the proliferation of Hc cells regardless of visfatin stimulation (Fig. 4C).

Effects of BCAA on visfatin-induced phosphorylation of ERK, Akt, and GSK-3β proteins in HepG2 cells

We next examined whether BCAA affected the phosphorylation of ERK, Akt, and GSK-3β proteins caused by visfatin in HepG2 cells. When the cells were stimulated by visfatin, the expression levels of phosphorylated (p)-GSK-3β protein were significantly decreased by BCAA treatment ($P < 0.05$; Fig. 5).

Effect of BCAA on cell-cycle progression, $p21^{CIP1}$ expression, and apoptosis induction in HepG2 cells in the presence and absence of visfatin

To determine whether the suppression of cell proliferation caused by BCAA (Fig. 4A and B) was associated with specific changes in cell-cycle distribution, we conducted cell-cycle analysis with DNA flow cytometry. When HepG2 cells were stimulated by visfatin for 48 hours, the percentage of cells in G_2/M phase (38%) was increased compared with that of cells not stimulated by visfatin (18%). Furthermore, regardless of visfatin stimulation, BCAA treatment increased the percentage of cells in G_0/G_1 phase; the percentage of cells in this phase was increased from 59% to 71% in the unstimulated cells and from 48% to 70% in the stimulated cells (Fig. 6A). Expression levels of $p21^{CIP1}$ protein, which suppresses tumors by promoting cell-cycle arrest (30), were also increased by BCAA treatment regardless of visfatin stimulation ($P < 0.05$; Fig. 6B). In addition, BCAA induced apoptosis in HepG2 cells because the percentage of Annexin V–positive cells was increased by the addition of BCAA in both the absence (2%–27%) and the presence (2%–10%) of visfatin stimulation (Fig. 6C).

Discussion

Obesity and related metabolic abnormalities are significant risk factors for the development of HCCs (1–5).

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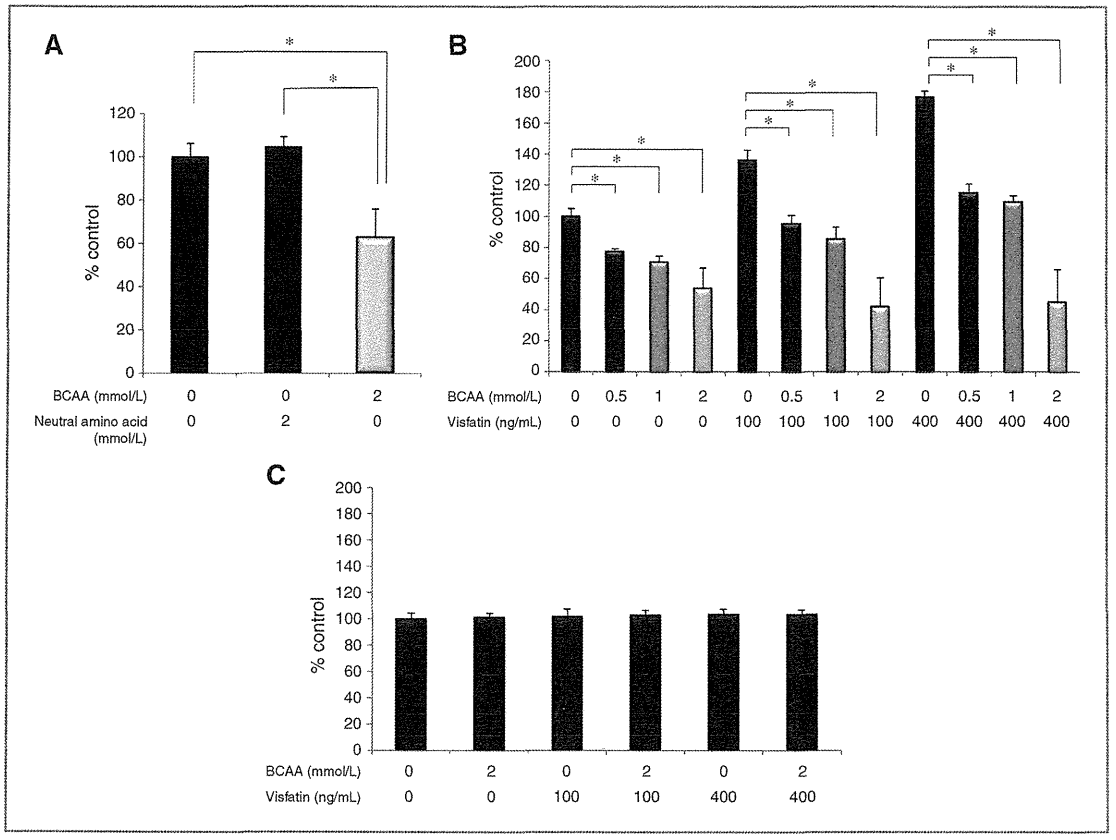

Figure 4. Effects of BCAA on visfatin-induced cell proliferation in HepG2 cells. A, HepG2 cells were treated in 2 mmol/L BCAA or 2 mmol/L neutral amino acid medium for 48 hours. Cell proliferation was evaluated by an XTT assay. HepG2 (B) and Hc (C) cells were treated with or without BCAA (0, 0.5, 1, and 2 mmol/L) in the absence or presence of visfatin (100 or 400 ng/mL) for 48 hours. Cell proliferation was evaluated by an XTT assay. Results were expressed as a percentage of the control value. Bars indicate SD values of triplicate assays. *, $P < 0.05$.

Among obesity-related metabolic disorders, adipocytokine dysbalance is considered to play a role in liver carcinogenesis (7–9); however, the detailed relationship remains unclear. The results of the present study provide the first evidence that higher levels of serum visfatin, which are frequently found in obese individuals (11, 12), are

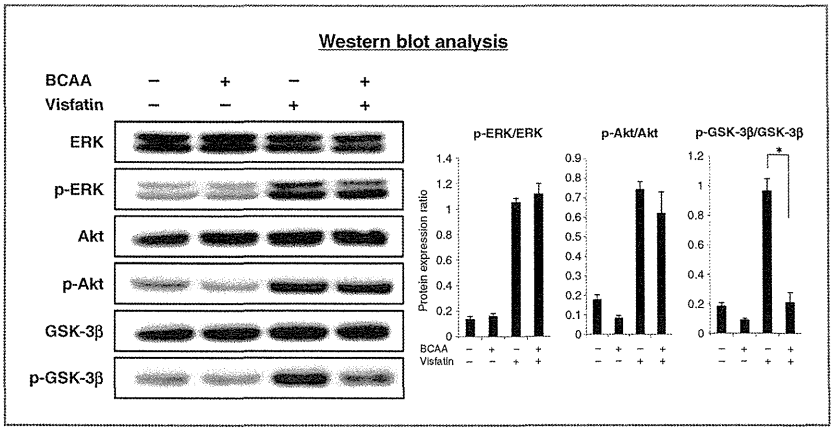

Figure 5. Effects of BCAA on visfatin-induced phosphorylation of ERK, Akt, and GSK-3β proteins in HepG2 cells. HepG2 cells were treated with or without BCAA in the absence or presence of 100 ng/mL visfatin for 30 minutes, and cell lysates were prepared. The cell lysates were then analyzed by Western blotting using corresponding antibodies (left). The intensities of the blots were quantified with densitometry. Columns and lines indicate mean ± SD (right). Repeated Western blotting produced similar results. *, $P < 0.05$. p-ERK, phosphorylated ERK; p-Akt, phosphorylated Akt; p-GSK-3β, phosphorylated GSK-3β.

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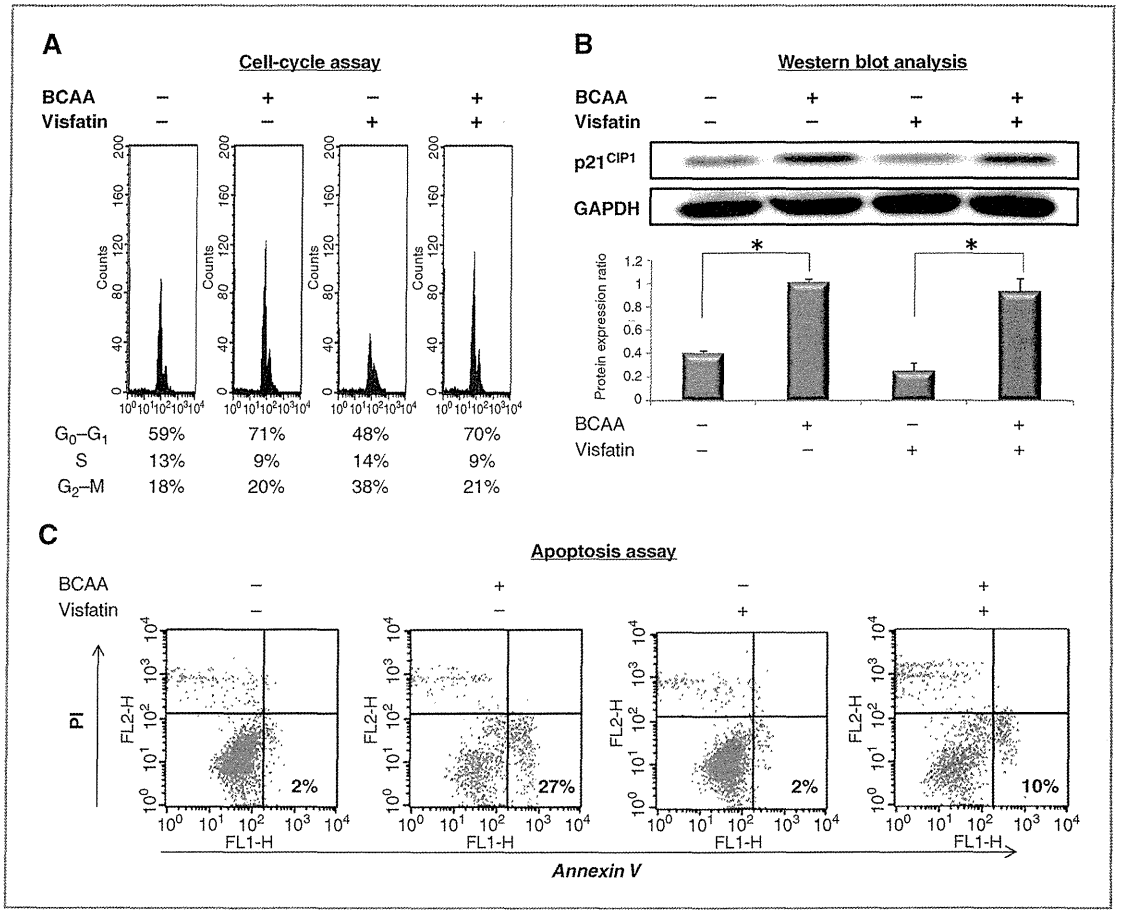

Figure 6. Effect of BCAA on the progression of cell cycle, expression of $p21^{CIP1}$, and induction of apoptosis in HepG2 cells in the presence and absence of visfatin. After treatment with and without BCAA in the presence and absence of 100 ng/mL visfatin for 48 hours, the cells were corrected and then used for cell-cycle assay (A), Western blot analysis (B), and apoptosis assay (C). A, the cells were stained with PI to analyze cell-cycle progression. B, total proteins were extracted from the cells, and the cell extracts were analyzed with a Western blot using anti-$p21^{CIP1}$ and GAPDH antibodies (top). The intensities of the blots were quantitated with densitometry. Columns and lines indicate mean and SD (bottom). *, $P < 0.05$. C, the cells were incubated with Annexin V–FITC to evaluate induction of apoptosis. Annexin V–FITC–positive and PI-negative cells were counted as apoptotic cells.

positively involved in stage progression and tumor enlargement in HCCs. On the other hand, the serum levels of other adipocytokines, including leptin, adiponectin, and resistin, are not associated with the stage progression of this malignancy (data not shown). Furthermore, visfatin stimulation strongly induced proliferation in a series of human HCC cells but not in Hc normal human hepatocytes. These findings suggest that visfatin, which might act as a growth factor in HCC cells, is one of the key adipocytokines that links obesity and the progression of HCCs. In addition, this study revealed that serum visfatin levels are significantly correlated with tumor enlargement of HCCs in patients who are not obese and do not have diabetes mellitus. A recent report has shown that visfatin is constitutively released from human HCC cells (31). This finding raises the possibility that visfatin is produced by HCC tissue itself, which might also explain the positive correlation between tumor size and serum visfatin levels observed in the present study. Therefore, our findings and the results of a previous report (31) together suggest that visfatin-dependent autocrine or paracrine loops contribute to abnormal proliferation in HCC cells.

The present study showed that visfatin induced cell proliferation in HepG2 cells by activating PI3K and MAPK signaling pathways because visfatin stimulation significantly increased phosphorylation of Akt, ERK, and GSK-3β proteins in these cells. These findings are consistent with previous reports that visfatin regulates a variety of signaling pathways, including PI3K/Akt, MAPK/ERK, and Stat3 (20, 32, 33). Visfatin stimulation also increases cell proliferation and ERK activity in prostate cancer cells (20). Moreover, recent experimental studies have shown that the activation of PI3K/Akt, MAPK/ERK, and Stat3 pathways is

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significantly associated with the development of liver tumors in obese mice, and that inhibiting the activation of these signaling pathways is critical to the prevention of obesity-related liver tumorigenesis (34, 35). These reports (34, 35), together with the present findings that specific inhibitors of PI3K, MEK1, and GSK-3β significantly suppress visfatin-induced proliferation in HCC cells, suggest that visfatin and its related signaling pathways might be effective targets for inhibiting obesity-related liver carcinogenesis.

BCAA, which was originally developed to improve protein malnutrition in patients with liver cirrhosis (23), produces improvements in metabolic abnormalities, especially insulin resistance and glucose tolerance (36, 37). BCAA supplementation also reduces the weights of white adipose tissue and improves liver steatosis in mice fed with a high-fat diet (38). In addition, long-term oral supplementation with BCAA is associated with a reduced frequency of HCCs in obese individuals (4). In rodent models, BCAA prevents obesity-related liver and colorectal carcinogenesis, and their beneficial effects are involved in the amelioration of insulin resistance and reduction of serum leptin levels (25, 26, 39). In the present study, BCAA significantly inhibited the proliferation of HCC cells stimulated by visfatin without affecting that of normal hepatocytes. This mechanism is a new one of BCAA that might explain the suppressive effects of this agent on obesity-related tumorigenesis. Therefore, the evidences in the present and previous studies (4, 25, 39) strongly support the active administration of BCAA as an HCC chemopreventive agent in patients with liver cirrhosis, especially obese patients who are at an increased risk for this malignancy. We are currently trying to gather evidence that BCAA prevents obesity-related liver carcinogenesis by targeting visfatin, in an ongoing animal study.

GSK-3β phosphorylation plays a critical role in cell survival, prevention of apoptosis, and progression of cell cycle in tumors (40). Therefore, the results of the present study suggest that BCAA might have inhibited visfatin-induced proliferation in HCC cells by, at least in part, inhibiting the phosphorylation of GSK-3β protein, which induces apoptosis and cell-cycle arrest in the G_0/G_1 phase in HepG2 cells. These findings are significant when considering the possibility of BCAA as a chemopreventive agent for HCCs because GSK-3β phosphorylation is closely associated with liver carcinogenesis (41). Phosphorylation of GSK-3β is also involved in the development of liver tumors in obese mice, and inhibition of this kinase effectively suppresses obesity-related liver tumorigenesis (35). Conversely, a recent study has shown that visfatin exerts antiapoptotic effects in HCC cells, and this might be associated with the enzymatic synthesis of NAD^+ (15). FK866, a visfatin inhibitor, effectively inhibited cell growth and induced apoptosis in human HCC cells by reducing cellular levels of NAD^+ (22). Further studies are required to clarify the effects of BCAA on the synthesis and regulation of NAD^+ and their relevance to the chemopreventive characteristics of this agent.

In summary, our data explained, for the first time, the molecular mechanisms responsible for HCC cell proliferation induced by visfatin, establishing a direct association between obesity and HCC progression. Because the evaluation of obesity-related metabolic disorders such as insulin resistance and hyperleptinemia are useful for predicting the risk of recurrence in HCCs (8, 42), we presume that, along with these metabolic abnormalities, measurement of serum visfatin levels might also have the potential to become a valuable biomarker for HCC development and progression. The results of the present study also indicate that targeting visfatin and related signaling pathways might be a promising strategy for the prevention or treatment of HCCs in obese patients with chronic liver disease. BCAA is potentially effective and critical candidate for this purpose because it can inhibit visfatin-mediated cell proliferation and activation of intracellular signaling pathways.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Original Article

Increased levels of serum leptin are a risk factor for the recurrence of stage I/II hepatocellular carcinoma after curative treatment

Naoki Watanabe, Koji Takai, Kenji Imai, Masahito Shimizu, Takafumi Naiki, Masahito Nagaki and Hisataka Moriwaki*

Department of Medicine, Gifu University Graduate School of Medicine, 1-1 Yanagido, Gifu 501-1194, Japan

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Obesity and related adipocytokine disbalance increase the risk of hepatocellular carcinoma. To determine the impact of increased levels of leptin, an obesity-related adipocytokine, on the recurrence of hepatocellular carcinoma, we conducted a prospective case-series analysis. Eighty-five consecutive primary hepatocellular carcinoma patients at our hospital from January 2006 to December 2008 were analyzed. Serum leptin level significantly correlated with Body Mass Index, total body fat, and the amount of subcutaneous fat. They included 33 with stage I/II, who underwent curative treatment. The factors contributing to recurrence of hepatocellular carcinoma, including leptin, were subjected to univariate and multivariate analyses using the Cox proportional hazards model. Body Mass Index ($p = 0.0062$), total body fat ($p = 0.0404$), albumin ($p = 0.0210$), α-fetoprotein ($p = 0.0365$), and leptin ($p = 0.0003$) were significantly associated with the recurrence of hepatocellular carcinoma in univariate analysis. Multivariate analysis suggested that leptin (hazard ratio 1.25, 95% CI 1.07–1.49, $p = 0.0035$) was a sole independent predictor. Kaplan-Meier analysis showed that recurrence-free survival was lower in patients with greater serum leptin concentrations (>5 ng/mL, $p = 0.0221$). These results suggest that the serum leptin level is a useful biomarker for predicting the early recurrence of hepatocellular carcinoma.

Key Words: **hepatocellular carcinoma, carcinogenesis, leptin, obesity, insulin resistance**

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide and is estimated to cause approximately 500,000 deaths annually.[1] HCC frequently develops and in many cases recurs in cirrhotic livers due to persistent hepatitis B virus (HBV) and hepatitis C virus (HCV) infection; this is strongly associated with poor prognosis for this particular malignancy.[2] Therefore, careful surveillance of high-risk groups for HCC is important to improve prognosis. Hence, there is a critical need to identify useful risk factors for the development of HCC. Infection with HBV and HCV, alcohol consumption, aflatoxin exposure, and immune-related hepatitis are accepted as significant risk factors for the development of primary HCC.[3] Male gender, the presence of cirrhosis, high α-fetoprotein (AFP), large tumor foci, multiplicity of tumors, pathologically high-grade atypia of tumor cells, and the presence of portal venous invasion of tumors also raise the risk for HCC recurrence.[4–8]

In addition to these factors, recent studies demonstrate that obesity[9] and related metabolic abnormalities—especially diabetes mellitus (DM) and insulin resistance[10,11]—are important risk factors for the development of HCC. For instance, insulin resistance significantly raises the risk of the recurrence of stage I HCC after curative treatment.[10] Several pathophysiological mechanisms linking obesity and HCC development have been proposed and include the emergence of insulin resistance and a state of chronic inflammation.[12,13] Adipocytokine disbalance might also be involved in obesity-related liver carcinogenesis.[14] Among the adipocytokines, it is well known that the serum levels of leptin, which regulate the homeostasis of glucose and lipid metabolism,[15] are elevated in obese individuals.[16] In addition, both *in vitro* and *in vivo* studies indicate that leptin might play a role in the development of several types of human malignancies, including HCC.[17–21] These findings suggest that the dysregulation of serum leptin levels may be a critical link between obesity and liver carcinogenesis. However, whether leptin is a significant biomarker for predicting the development and/or recurrence of HCC has not been explored.

In this study, we measured the serum leptin concentration in patients with HCC and examined whether it is correlated with obesity and insulin resistance. In addition, we designed a prospective case-series analysis to examine the recurrence-free survival in consecutive patients with stage I/II HCC, who received curative treatment by surgical resection or radiofrequency ablation (RFA), stratified by serum leptin concentrations.

Materials and Methods

Patients. From January 2006 to December 2008, 85 primary HCC patients underwent initial treatment at our hospital. We measured visceral and subcutaneous fat volume using computed tomography (CT) scans at the umbilical level according to a previously reported technique (fatAnalyses and EV Insite R, PSP Corporation, Tokyo, Japan).[22] Tumor stage was defined according to the staging system of the Liver Cancer Study Group of Japan (LCSGJ).[23] HCC nodules were detected by imaging modalities including abdominal ultrasonography (US), dynamic CT, dynamic magnetic resonance imaging (MRI), and abdominal arteriography. The diagnosis of HCC was made from a typical hypervascular tumor stain on angiography and a typical dynamic-study finding of enhanced staining in the early phase and attenuation in the delayed phase.

Treatment, follow-up, and determination of recurrence. Fifteen patients were treated with surgical resection, 41 with RFA, 19 with transarterial chemoembolization (TACE), and 10 with transarterial infusion (TAI). Among them, we selected 33 curative cases that met the following criteria: tumor stage classified as I or II; and surgical resection or RFA conducted for the initial HCC treatment. In all 33 cases, therapeutic effects were judged to be

*To whom correspondence should be addressed.
E-mail: hmori@gifu-u.ac.jp

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Table 1. Baseline demographic and clinical characteristics

Variable	Total patients (n = 85)
Sex (male/female)	54/31
Age (years)	73 [36–87]
BMI (kg/m^2)	23.2 [17.5–30.7]
Total body fat (cm^2)	188.81 [12.93–501.8]
Amount of visceral fat (cm^2)	76.43 [3.82–359.83]
Amount of subcutaneous fat (cm^2)	105.66 [9.11–265.26]
Etiology (B/C/B + C/other)*	8/55/2/20
Child-Pugh classification (A/B/C)	60/23/2
Ascites on CT imaging (present/absent)	7/78
ALB (g/dL)	3.5 [2.2–4.5]
PLT ($\times 10^4/\mu$L)	11.7 [3.0–76.4]
FPG (mg/dL)	97 [67–271]
FIRI (μU/mL)	8.115 [1.21–90.2]
HOMA-IR	2.245 [0.27–28.28]
HbA_{1c} (%)	5.3 [3.7–10.3]
Leptin (ng/mL)	5.0 [1.4–26.6]
Stage (I/II/III/IVA/IVB)	19/26/27/10/3
Initial treatment for HCC (resection/RFA/TACE/TAI)	15/41/19/10
AFP (ng/mL)	48 [0–222000]
PIVKA-II (mAU/mL)	170 [7–474000]
Follow-up period (days)	484 [14–1429]

Values are median [range]. *B means positive for hepatitis B surface antigen and C means positive for hepatitis C virus antibody. AFP, α-fetoprotein; B, hepatitis B virus; BMI, body mass index; C, hepatitis C virus; FPG, fasting plasma glucose; FIRI, fasting immunoreactive insulin; HbA_{1c}, hemoglobin A_{1c}; HCC, hepatocellular carcinoma; HOMA-IR, homeostasis model assessment of insulin resistance; PIVKA-II, protein induced by vitamin K absence or antagonists-II; RFA, radiofrequency ablation; TACE, transarterial chemoembolization; TAI, transarterial infusion.

curative using dynamic CT or MRI exhibiting a complete disappearance of the imaging characteristics of HCC described above.

Patients were thereafter followed up on a monthly outpatient basis using serum tumor markers every month, such as AFP and proteins induced by vitamin K absence or antagonists-II (PIVKA-II), and by abdominal US, dynamic CT scanning, or dynamic MRI every 3 months. Recurrent HCC was diagnosed, using the imaging modalities described earlier, by the appearance of other lesions differed from the primary lesions. The follow-up period was defined as the interval from the date of initial treatment until the date of diagnosis of recurrence or until April 2009 if HCC did not recur.

Statistical analysis. The Pearson product-moment correlation coefficient was used for measuring the linear correlation between 2 continuous variables. Recurrence-free survival was estimated using the Kaplan-Meier method, and differences between curves were examined with a log-rank test. Baseline characteristics were compared using Student's t test for continuous variables or the χ^2 test for categorical variables. There were 17 possible predictors for the recurrence of HCC after the initial curative treatment: sex, age, body mass index (BMI), total body fat, amounts of both visceral and subcutaneous fat, the presence of HCV-antibodies (HCV-Ab), Child-Pugh classification, serum albumin concentration, platelet count, homeostasis model assessment of insulin resistance (HOMA-IR = fasting plasma glucose (mg/dL) × fasting immunoreactive insulin (μU/mL)/405), hemoglobin A_{1c} (HbA_{1c}), serum tumor markers (AFP and PIVKA-II), initial treatment for HCC, tumor stage, and serum leptin concentration. Parameters that were significant as determined by univariate analysis were then subjected to multivariate analyses using the Cox proportional hazards model. Statistical significance was defined as $p<0.05$.

Results

Baseline characteristics and laboratory data of patients. The baseline characteristics and laboratory data of 85 patients (54 men and 31 women, median age 73 years) are shown in Table 1. The median follow-up period was 484 days (range, 14–1,429 days). Median BMI was 23.2 kg/m^2, which was classified in the normal range according to the WHO classification of obesity (http://www.who.int/bmi). Median free plasma glucose (FPG), free immunoreactive insulin (FIRI), HOMA-IR, and HbA_{1c} were 97 mg/dL, 8.115 μU/mL, 2.245, and 5.3%, respectively. The median serum leptin concentration was 5.0 ng/mL (range 1.4–26.6).

Association of the serum leptin concentration with obesity and insulin resistance. Four obesity-related factors were tested for possible association with the serum leptin concentration: BMI, total body fat, and the amounts of visceral and subcutaneous fat (Fig. 1). For BMI analysis, we excluded 7 patients with CT-detected ascites. The Pearson product-moment correlation coefficient and p values of BMI and the total body fat with serum leptin concentration were $r = 0.4559$ and $p<0.0001$, and $r = 0.3560$ and $p = 0.0008$, respectively; indicating that these 2 factors were significantly correlated with the serum leptin concentration. The amount of subcutaneous fat ($r = 0.5174$ and $p<0.0001$) was also strongly correlated with the serum leptin level, whereas the amount of visceral fat ($r = 0.0987$ and $p = 0.3776$) was not. In addition, no significant correlations were noted between the serum leptin concentration and insulin resistance-related factors, including FPG ($r = -0.0816$ and $p = 0.4579$), FIRI ($r = 0.1049$ and $p = 0.3378$), HOMA-IR ($r = 0.0506$ and $p = 0.6385$), and HbA_{1c} ($r = 0.0194$ and $p = 0.7820$).

Possible risk factors for the recurrence of HCC. In all 33 curative cases of stage I/II HCC, 12 patients experienced recur-

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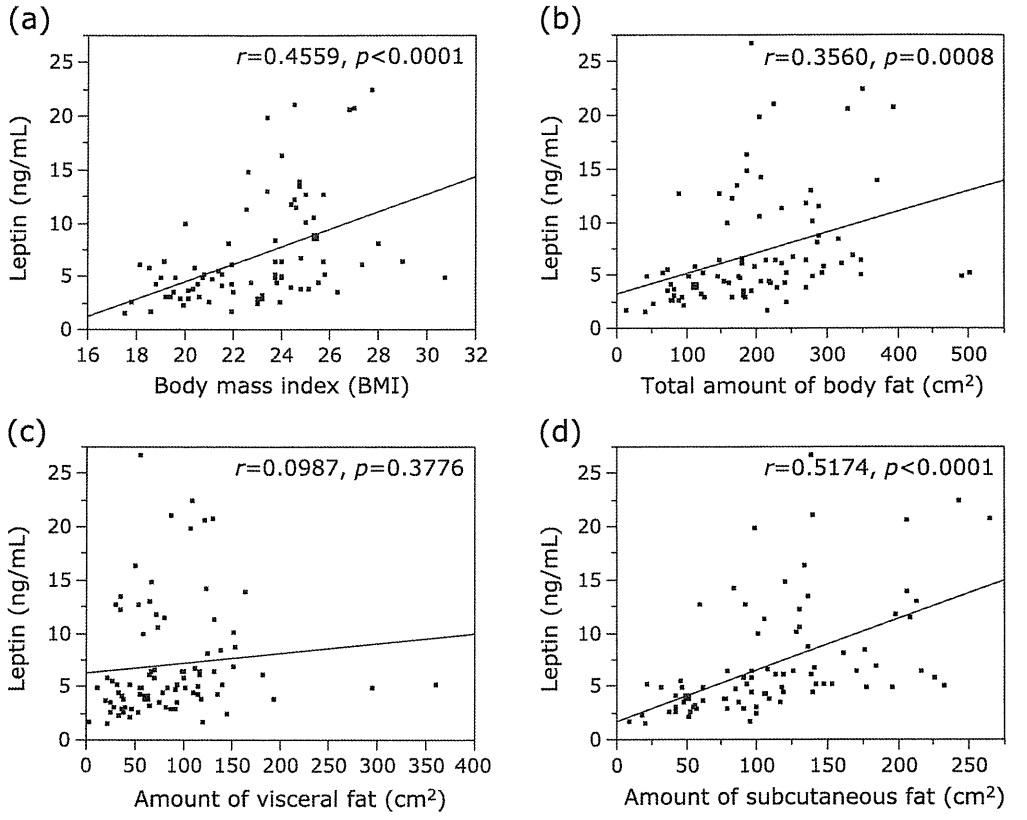

Fig. 1. Correlation between the serum levels of leptin and (a) BMI, (b) total body fat, (c) amount of visceral fat, and (d) amount of subcutaneous fat in patients with HCC (n = 85). For BMI analysis, we excluded 7 patients with CT-detected ascites.

rence in the liver, but none exhibited distant metastasis. The 1-year recurrence-free survival in the 33 patients was 79% (Fig. 2a). Fig. 2b shows Kaplan-Meier curves for recurrence-free survival divided into 2 subgroups on the basis of median serum leptin concentration (≤5 or >5 ng/mL), which results in a significant difference (p = 0.0221).

The Cox proportional hazards model was used to analyze risk factors for the recurrence of stage I/II HCC after curative treatments using the 17 variables listed in Table 2. BMI (hazard ratio 1.30, 95% CI 1.08–1.56, p = 0.0062), total body fat (hazard ratio 1.00, 95% CI 1.00–1.01, p = 0.0404), serum albumin concentration (hazard ratio 0.26, 95% CI 0.08–0.81, p = 0.0210), AFP (hazard ratio 0.99, 95% CI 0.99–0.99, p = 0.0365), and serum leptin concentration (hazard ratio 1.29, 95% CI 1.12–1.50, p = 0.0003) were identified as significant risk factors by univariate analysis. Multivariate analysis only identified serum leptin concentration (hazard ratio 1.25, 95% CI 1.07–1.49, p = 0.0035) as significant independent risk factor for the recurrence of HCC (Table 3).

Table 4 shows the baseline characteristics and laboratory data of patients divided on the basis of the serum leptin concentration (≤5 and >5 ng/mL). No significant differences were noted between the 2 subgroups, except the amount of subcutaneous fat (p = 0.0461).

Discussion

Leptin regulates body weight by signaling information to the brain regarding the availability of energy stored as fat; this negative feedback loop is disrupted in most obese individuals and results in a state known as leptin resistance.[16,24] Consistent with the results of previous studies,[16,24] the serum leptin concentration was significantly correlated with BMI and total body fat in the present study (Fig. 1 a and b). These parameters were also significant risk factors for the recurrence of HCC as determined by univariate analysis (Table 2); however, the serum leptin concentration was the most significant biomarker (p = 0.0003).

In addition, we clearly showed for the first time that patients with greater serum leptin concentrations were susceptible to HCC recurrence (Fig. 2b); thus, increased serum leptin levels are a significant independent risk factor for the recurrence of this malignancy (Table 3). This finding indicates that increased serum leptin concentration, which might link obesity with liver carcinogenesis, is a preferable and useful biomarker for screening high-risk groups for the recurrence of HCC. We previously reported that a state of insulin resistance associated with obesity is an independent risk factor for the recurrence of HCC after curative treatment.[10] Furthermore, no significant correlations between serum leptin levels and insulin resistance-related factors were noted in the present study, suggesting these two conditions might be independent from each other in HCC patients. Therefore, a combination evaluation for both the serum leptin level and insulin resistance would be more effective for screening high-risk groups for HCC, and requires future confirmation.

Several studies report that leptin is a risk factor for carcinogenesis at various organ sites, including the liver.[17–21] Leptin can stimulate cellular proliferation in various types of cancer cells such as HCC cells.[19–21,25] In addition, when focusing on the liver,

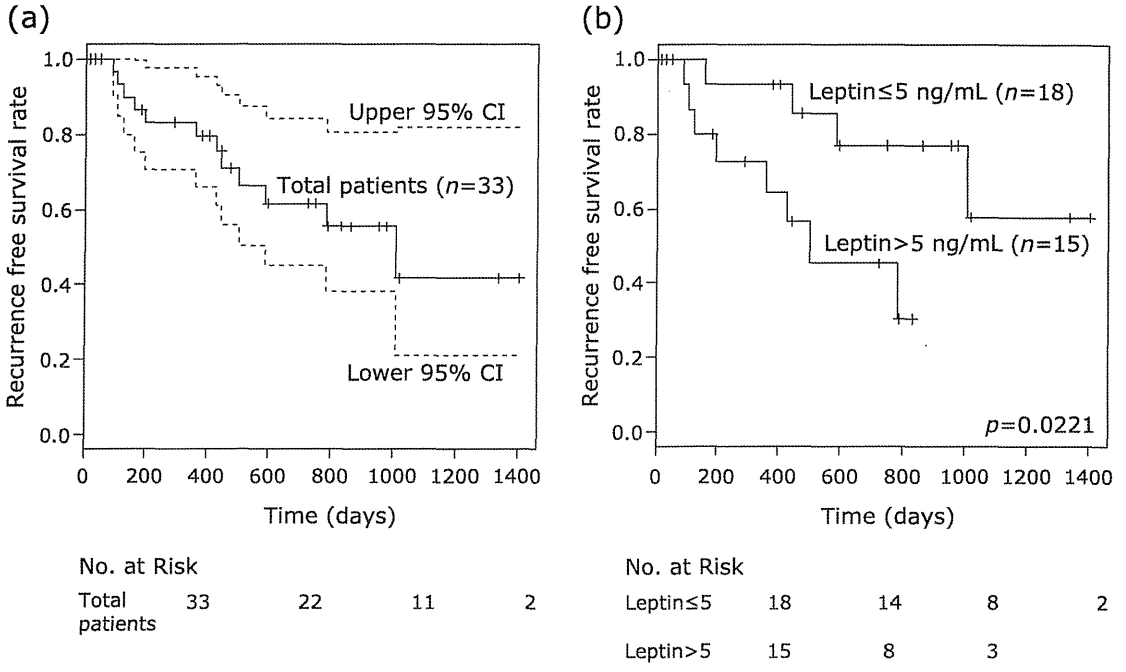

Fig. 2. Kaplan-Meier curves for recurrence-free survival in (a) total patients and in (b) subgroups divided on the basis of the serum leptin concentration (≤5 or >5 ng/mL).

Table 2. Univariate analyses of possible risk factors for recurrence-free survival of HCC using the Cox proportional hazards model

Variable	95% CI			
	HR*	lower	upper	*p* value
Sex (male vs female)	0.9	0.28	3.09	0.8726
Age (years)	0.96	0.89	1.03	0.277
BMI (kg/m^2)	1.3	1.08	1.56	0.0062
Total body fat (cm^2)	1	1	1.01	0.0404
Amount of visceral fat (cm^2)	1	0.99	1.01	0.0909
Amount of subcutaneous fat (cm^2)	1	0.99	1.01	0.0601
The presence of HCV-Ab (yes vs no)	0.42	0.12	1.98	0.2501
Child-Pugh classification (B + C vs A)	1.33	0.35	4.3	0.6482
ALB (g/dL)	0.26	0.08	0.81	0.021
PLT (×10^4/μL)	0.87	0.75	1.01	0.0714
HOMA-IR	1.03	0.94	1.1	0.4
HbA_{1c} (%)	0.87	0.37	1.6	0.7108
AFP (ng/mL)	0.99	0.99	0.99	0.0365
PIVKA-II (mAU/mL)	0.99	0.99	1	0.7448
Initial treatment for HCC (RFA vs resection)	1.61	0.42	10.5	0.5128
Stage (II vs I)	1.08	0.32	3.78	0.89
Leptin (ng/mL)	1.29	1.12	1.5	0.0003

*HR represents the values with a unit increase in continuous variables. AFP, α-fetoprotein; BMI, body mass index; CI, confidence interval; HbA_{1c}, hemoglobin A_{1c}; HCC, hepatocellular carcinoma; HCV-Ab, hepatitis C virus antibody; HOMA-IR, homeostasis model assessment of insulin resistance; HR, hazard ratio; PIVKA-II, protein induced by vitamin K absence or antagonists-II; RFA, radiofrequency ablation.

leptin is a potent profibrogenic cytokine and thus plays a key role in the progression of cirrhosis,[26] which is a precancerous condition of HCC. Indeed, increased serum leptin concentration has been documented in cirrhotic patients.[27,28] Moreover, increased leptin expression is associated with increased intratumor microvascular density. Consequently, it is hypothesized that leptin plays a stimulatory role in the development of HCC via neovascularization.[29] In addition to using leptin as a biomarker for the risk of HCC recurrence, the present findings suggest that targeting leptin might be an effective strategy for the prevention and treatment of

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Table 3. Multivariate analyses of possible risk factors for recurrence-free survival of HCC using the Cox proportional hazards model

Variable	95% CI			
	HR*	lower	upper	*p* value
BMI (kg/m^2)	1.2	0.83	1.81	0.3278
Total body fat (cm^2)	1	0.99	1.01	0.8003
ALB (g/dL)	0.54	0.12	2.28	0.4018
AFP (ng/mL)	0.99	0.99	1	0.1416
Leptin (ng/mL)	1.25	1.07	1.49	0.0035

*HR represents the values with a unit increase in continuous variables. AFP, α-fetoprotein; BMI, body mass index; CI, confidence interval; HR, hazard ratio.

Table 4. Baseline demographic and clinical characteristics of patients classified on the basis of the serum leptin concentration

Variable	Leptin ≤ 5 ng/mL (*n* = 18)	Leptin > 5 ng/mL (*n* = 15)	*p* value
Sex (male/female)	13/5	6/9	0.0604
Age (years)	72.5 [59–87]	70 [50–85]	0.2565
BMI (kg/m^2)	21.5 [17.8–30.7]	24.5 [18.5–27.7]	0.1111
Total body fat (cm^2)	167.5 [73.9–490.9]	207.3 [112.2–350.8]	0.2591
Amount of visceral fat (cm^2)	69.4 [19.9–294.4]	98.9 [21.9–181.6]	0.9479
Amount of subcutaneous fat (cm^2)	90.2 [42.0–232.3]	134.3 [79.6–242.5]	0.0461
Etiology (C/others)	14/4	11/4	0.767
Child-Pugh classification (A/B/C)	15/3/0	10/5/0	0.2657
ALB (g/dL)	3.6 [2.6–4.2]	3.3 [2.4–4.4]	0.2708
PLT (×10^4/μL)	12.45 [7.7–26.1]	9.5 [3.0–20.6]	0.0895
FPG (mg/dL)	97.5 [83–271]	105 [75–154]	0.7424
FIRI (μU/mL)	6.05 [2.57–65.2]	14.3 [7.3–27.4]	0.3657
HOMA-IR	1.51 [0.53–24.8]	3.41 [1.45–9.40]	0.641
HbA_{1c} (%)	5.3 [4.5–10.3]	5.2 [3.7–6.8]	0.3351
Stage (I/II)	7/11	9/6	0.2253
Initial treatment for HCC (resection/RFA)	6/12	2/13	0.1726
AFP (ng/mL)	8 [0–20500]	28 [1–2530]	0.1687
PIVKA-II (mAU/mL)	22.7 [8–201000]	26 [7–29800]	0.4385

Values are median [range]. AFP, α-fetoprotein; C, hepatitis C virus; FPG, fasting plasma glucose; FIRI, fasting immunoreactive insulin; HbA_{1c}, hemoglobin A_{1c}; HCC, hepatocellular carcinoma; HOMA-IR, homeostasis model assessment of insulin resistance; PIVKA-II, protein induced by vitamin K absence or antagonists-II; RFA, radiofrequency ablation.

HCC. Ribatti *et al.* state that anti-leptin antibodies reduce the angiogenic response in HCC biopsy specimens.[29] Decreases in serum leptin are also associated with the prevention of obesity-related liver tumorigenesis in obese and diabetic mice models.[14]

In conclusion, we report that patients with high serum leptin concentrations are susceptible to HCC recurrence in stage I/II cases curatively treated by surgical resection or RFA. Increased serum leptin concentration may be a useful biomarker for predicting the recurrence of HCC in high-risk patients.

Acknowledgments

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Abbreviations

AFP	α-fetoprotein
BMI	body mass index
CT	computed tomography
DM	diabetes mellitus
FPG	fasting plasma glucose
FIRI	fasting immunoreactive insulin
HbA_{1c}	hemoglobin A_{1c}
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HOMA-IR	homeostasis model assessment of insulin resistance
LCSGJ	Liver Cancer Study Group of Japan
MRI	magnetic resonance imaging
PIVKA-II	protein induced by vitamin K absence or antagonists-II
RFA	radiofrequency ablation
TACE	transarterial chemoembolization
TAI	transarterial infusion
US	ultrasonography

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CLINICAL STUDIES

Up-regulated aldo-keto reductase family 1 member B10 in chronic hepatitis C: association with serum alpha-fetoprotein and hepatocellular carcinoma

Shunsuke Sato[1], Takuya Genda[1], Katsuharu Hirano[1], Hironori Tsuzura[1], Yutaka Narita[1], Yoshio Kanemitsu[1], Tetsu Kikuchi[1], Katsuyori Iijima[1], Ryo Wada[2] and Takafumi Ichida[1]

1 Department of Gastroenterology and Hepatology, Juntendo University Shizuoka Hospital, Shizuoka, Japan
2 Department of Pathology, Juntendo University Shizuoka Hospital, Shizuoka, Japan

Keywords
AKR1B10 – alpha-fetoprotein – carcinogenesis – chronic hepatitis C – liver – microarray – risk factor

Correspondence
Takuya Genda, MD, PhD, Department of Gastroenterology and Hepatology, Juntendo University Shizuoka Hospital, 1129 Nagaoka Izunokuni-shi Shizuoka 410-2295 Japan
Tel: +81-559-48-3111
Fax: +81-559-48-5088
e-mail: genda@rice.ocn.ne.jp

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Abstract

Background: Elevated serum alpha-fetoprotein (AFP) is not only a diagnostic marker for hepatocellular carcinoma (HCC), but is also a risk factor for HCC in chronic hepatitis C patients who do not have HCC. *Aim:* The aim was to analyse the hepatic gene expression signature in chronic hepatitis C patients with elevated AFP, who were at high risk for HCC. *Methods:* Liver tissue samples from 48 chronic hepatitis C patients were stratified by their serum AFP levels and analysed for gene expression profiles. The association between aldo-keto reductase family 1 member B10 (AKR1B10) expression and serum AFP was confirmed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemical analyses. A matched case-control study was performed to evaluate the risk of AKR1B10 expression for HCC development. *Results:* Distinct hepatic gene expression patterns were demonstrated in patients with elevated AFP ($\geq$10 ng/mL) and normal AFP (<10 ng/mL). Of the 627 differently expressed genes, the most abundantly expressed gene in patients with elevated AFP was AKR1B10 (fold change, 26.2; $P < 0.001$), which was originally isolated as an overexpressed gene in human HCC. The qRT-PCR and immunohistochemical studies confirmed a proportional correlation between AKR1B10 expression and serum AFP. A matched case-control study identified that AKR1B10 up-regulation (>6%) was an independent risk factor for HCC development (hazard ratio, 21.4; $P = 0.001$). *Conclusion:* AKR1B10 was up-regulated in association with serum AFP, and was an independent risk factor for HCC in chronic hepatitis C patients, suggesting its possible involvement at a very early stage of hepatocarcinogenesis.

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer-related death worldwide (1). Approximately 70–90% of patients with HCC have an established background of chronic liver disease and cirrhosis (1). Persistent infection with the hepatitis C virus (HCV) is one of the major causes of chronic liver disease leading to the development of HCC. Persistent HCV infection is responsible for 27–75% of the HCC cases in Europe and the United States and >80% of the HCC cases in Japan (2, 3). The annual incidence of HCC development is 2–8% in cirrhotic patients with chronic HCV infection (4, 5). Persons with anti-HCV positivity were shown to have a 20-fold increased risk of developing HCC in comparison with those who were negative for anti-HCV (6). Thus, the carcinogenic role of persistent HCV infection appears to be significant. However, the molecular mechanism of HCV-related hepatocarcinogenesis is not completely understood, particularly in its early stages.

Alpha-fetoprotein (AFP) is the most thoroughly characterized carcinofetal gene product and its usefulness in the surveillance and diagnosis of HCC is well established. On the other hand, AFP elevation is recognized not only in patients with HCC but also in patients with chronic viral hepatitis or cirrhosis, who have no evidence of HCC. AFP elevation was observed in over 15% of patients with chronic hepatitis C in the absence of HCC (7). In addition, several studies have indicated that AFP elevation is a significant

predictor of HCC development (8–10). Recent reports reveal that the estimated HCC risk in patients with elevated AFP is over three-fold higher than that in patients with normal AFP (11, 12). These observations suggest that the molecular alterations associated with the very early stages of hepatocarcinogenesis have occurred in the livers of patients with chronic hepatitis C with AFP elevation. In this study, we attempted to identify a specific gene expression signature by performing microarray analysis on the livers of patients with chronic hepatitis C and AFP elevation, which is considered high risk for development of HCC.

Materials and methods

Patients and sample preparation

Liver tissues were obtained from patients with chronic hepatitis C via percutaneous liver biopsy at Juntendo University Shizuoka Hospital (Shizuoka, Japan). Chronic hepatitis C was diagnosed on the basis of anti-HCV status and detectable serum HCV RNA. All the patients were negative for hepatitis B surface antigen and showed no evidence of HCC on ultrasonography or computed tomography before biopsy. Histological grading and staging were performed according to the Metavir classification system (13). Blood chemistry values for the following factors were determined: complete blood count, alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γGTP) and AFP. In this study, we defined elevated AFP as a value of $\geq$10 ng/mL, according to previous reports (10, 12). Normal liver tissues without unusual histological features were obtained from nontumoral parts of livers complicated with colorectal metastasis and were used as control liver tissues.

This study was approved by the Ethical Committee of Juntendo University Shizuoka Hospital in accordance with the Helsinki Declaration, and written informed consent was obtained from all patients.

RNA preparation and microarray hybridization

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and evaluated by the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Only high-quality RNA with RNA integrity numbers greater than 7.0 was used for experiments. The Agilent Whole Human Genome Microarray (design ID, 014850), which contains 44,000 60-mer oligonucleotide probes representing 41,000 genes and transcripts, was used to generate gene expression profiles. Total RNA (250 ng) was labelled and hybridized according to the One-Color Microarray-Based Gene Expression Analysis protocol ver.5.7. Hybridization signals were detected using the DNA microarray scanner G2505B (Agilent Technologies).

Microarray data analysis

The intensity values of each scanned feature were quantified using Agilent feature extraction software (v10.7.1.1, Agilent Technologies). We only used features that were flagged as no errors (present flags) and excluded features that were not positive, not significant, not uniform, not above background, saturated and population outliers (marginal and absent flags). Normalization was performed using GeneSpring GX 10.0.2 software (Agilent Technologies) (per chip, normalization to 75 percentile shift; per gene, normalization to median of all samples). Data filtration resulted in 30,150 probes as a valid expressed gene set in which at least 24 of the 48 samples had present flags for further analysis. Raw microarray data were deposited in Gene Expression Omnibus (GSE32221) and are available to the public.

The altered transcripts were quantified using the comparative method. Statistical analysis between groups was performed by the unpaired unequal variance Welch's *t*-test, and multiple testing corrections were performed by determining the false discovery rate (FDR) using GeneSpring software. Altered gene expression was considered significant if the transcript had an FDR corrected to $P \leq 0.05$ and $\geq$ 2-fold change in signal intensity. Principal component analysis (PCA), hierarchical clustering analysis (HCA) and gene ontology (GO) analysis were performed using GeneSpring software.

Quantitative real-time RT-PCR for RNA quantification (qRT-PCR)

TaqMan real-time RT-PCR was performed to quantify the relative expression levels of aldo-keto reductase family 1, member B10 (AKR1B10) (assay ID, Hs01546975_gH) and the beta-actin housekeeping gene (assay ID, Hs99999903_m1) (Applied Biosystems, Foster City, CA, USA). cDNA was synthesized from 1 μg total RNA by using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo dT primers, according to the manufacturer's instructions. Specific mRNA was quantified with a LightCycler 480 (Roche, Mannheim, Germany) by using 2 × Premix Ex Taq (TaKaRa BIO, Shiga, Japan). All PCR reactions were performed in triplicate. The relative expression of AKR1B10 was calculated using the comparative cycle threshold (delta C_T) method as described previously (14).

Immunohistochemistry

AKR1B10 immunohistochemical analysis was performed as described previously with some modifications (14). In brief, deparaffinized and rehydrated sections were processed by heat-induced antigen retrieval in 0.1 M citrate buffer at pH 6.0. After blocking the endogenous peroxidase activity, the sections were incubated with a mouse monoclonal antibody against AKR1B10

(Ab 57547; Abcam, Cambridge, UK) with a 1:100 dilution at room temperature, followed by incubation with biotinylated secondary antibody (Ventana iVIEW DAB Universal Kit; Ventana Medical Systems Inc., Tucson, AZ, USA). Staining was visualized using 3'-3-diaminobenzidine tetrahydrochloride and hematoxylin counterstain. Negative controls were performed by replacing the primary antibody with mouse immunoglobin (Sigma–Aldrich Biochemicals, St. Louis, MO, USA). AKR1B10 immunostaining was based on positive cytoplasmic staining and was quantitatively assessed as the average percentage of AKR1B10 positive areas in two independent fields at 100 × magnification by using Lumina Vision 2.4 Bio-imaging software (Mitani Corporation, Tokyo, Japan).

Matched case-control study

From January 2005 to April 2010, 278 consecutive patients with chronic hepatitis C underwent liver biopsy followed by periodic HCC surveillance by using ultrasonography or computed tomography at least every 4 months. All the patients had a minimum follow-up duration of 12 months after liver biopsy. HCC was diagnosed by histological examination and/or triphasic computerized tomography, in which hyperattenuation in the arterial phase with washout in the late phase is pathognomonic for HCC (15). For each patient who developed HCC, two control patients who matched the HCC patient in terms of gender, age (within 5 years) and histological fibrosis stage were randomly selected from the patients who did not develop HCC during the follow-up period.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test for comparison of continuous variables between groups and the corrected Chi-squared method for comparison of qualitative data. Univariate and multivariate Cox proportional hazard models were used to assess factors that were significantly associated with HCC development. The hazard ratio and 95% confidence interval (CI) were also calculated. All statistical analyses were performed using IBM SPSS 13.0 (IBM SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Gene expression profiling by microarray analysis

The baseline characteristics of the 48 patients who were enrolled in the microarray analysis are summarized in Table 1. Fifteen (31%) of the 48 patients showed elevation of serum AFP ($\geq$ 10.0 ng/mL). There were no significant differences between patients with elevated AFP and those with normal AFP in terms of age, gender, body mass index or histological grade of necroinflammation. Patients with elevated AFP showed higher serum ALT, lower platelet count and further progression of liver fibrosis compared to those with normal AFP.

Among the 30,150 valid genes, 627 were identified as differentially expressed genes with a minimal fold change of 2.0. Using these 627 genes, PCA and HCA were used to successfully distinguish samples according to their AFP status. Patients with elevated AFP were divided from the cluster of those with normal AFP in scatter spot graphics of PCA (Fig. 1A). HCA resulted in the formation of two main clusters, one comprising 27 of the 33 (81.8%) patients with normal AFP and the other comprising 14 of the 15 (93.3%) patients with elevated AFP (Fig. 1B). Classification of these 627 genes according to GO function demonstrated that patients with elevated AFP showed up-regulation of genes asso-

Table 1. Baseline characteristics of patients enrolled in the microarray analysis

Variables	All patients	Patients with normal AFP (<10 ng/mL)	Patients with elevated AFP ($\geq$ 10 ng/mL)	*P* value†
Number	*N* = 48	*N* = 33	*N* = 15	
Age (years)*	59.5 (32–78)	57.0 (32–78)	65.0 (44–78)	0.142
Gender (male/female)	28/20	21/12	7/8	0.349
Body mass index (kg/cm^2)*	22.7 (17.5–30.4)	22.8 (18.6–30.4)	22.5 (17.5–29.0)	0.755
ALT (IU/L)*	61.5 (10–290)	39.0 (10–270)	162.0 (54–290)	<0.001
γGTP (IU/L)*	38.5 (9–540)	28.0 (9–119)	16 (28–540)	<0.001
Platelet count (10^4/μL)*	18.8 (5.2–35.4)	20.5 (8.3–35.4)	14.8 (5.2–22.4)	<0.001
AFP (ng/μL)*	5.0 (2–120)	4.0 (2–9)	19.0 (10–120)	<0.001
Staging (F0-F2/F3-F4)	41/7	33/0	8/7	<0.001
Grading (A0-A1/A2-A3)	12/26	10/23	2/13	0.292

ALT, alanine aminotransferase; AFP, alpha-fetoprotein; γGTP, γ-glutamyl transpeptidase.

*Data are expressed as median (range).

†The *P* value was determined using the Mann–Whitney *U*-test, the Chi-square test, and Fisher's exact probability test.

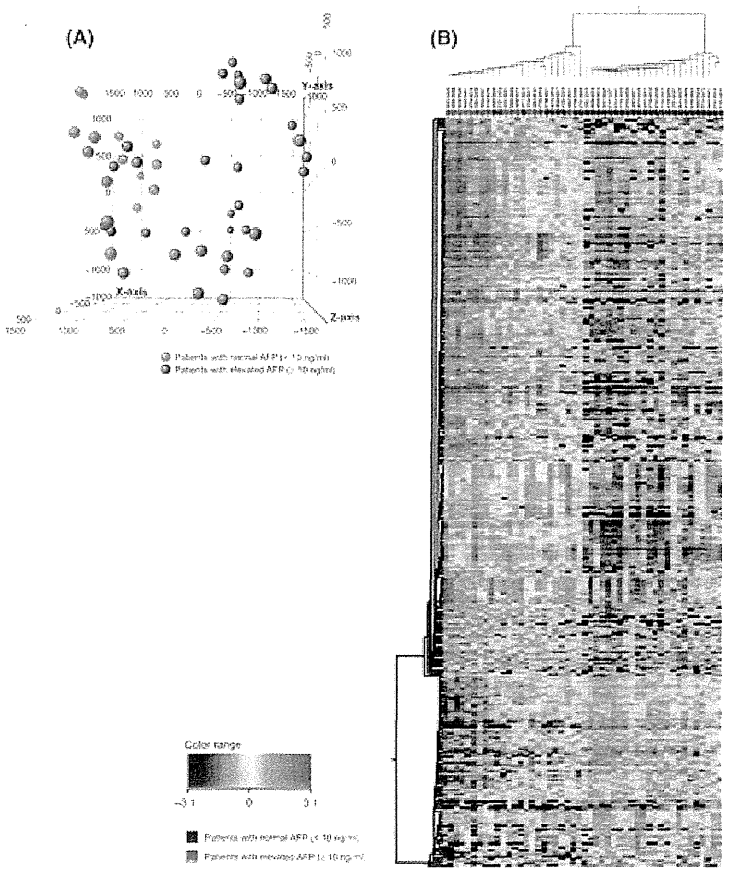

Fig. 1. Analysis of gene expression data. (A) Principal component analysis for the 627 differentially expressed genes between the patients with normal alpha-fetoprotein (AFP) (*N* = 33, *blue dots*) and elevated AFP (*N* = 15, *red dots*). (B) Hierarchical clustering for the 627 differentially expressed genes between the patients with normal AFP (*N* = 33, *blue squares*) and elevated AFP (*N* = 15, *red squares*). Red and blue cells indicate the ratio of each expression level above and below the median respectively.

ciated with the GO terms 'immune response,' 'DNA replication,' 'biological adhesion' and 'cell adhesion' (Table S1).

Up-regulation of AKR1B10 in patients with elevated AFP

The top 20 differentially expressed genes are shown in Table 2. Among them, AKR1B10 was the most abundantly up-regulated gene in patients with elevated AFP (26.1-fold change, $P < 0.001$). The microarray data were validated by qRT-PCR. The expression of AKR1B10 mRNA was significantly higher in patients with elevated AFP than in patients with normal AFP: median 22.3 arbitrary units vs. 0.58 arbitrary units respectively ($P < 0.001$) (Fig. 2A). In addition, regression analysis showed a significant correlation between AKR1B10 mRNA and serum AFP (Fig. 2B).

To further analyse AKR1B10 expression, immunohistochemical analysis was performed using a monoclonal antibody against AKR1B10. In the normal control liver tissues, immunoreactivity was mainly observed in bile duct cells, whereas reactivity was rarely observed in the cytoplasm of these hepatocytes (Fig. 3A). In contrast, hepatocytes in livers with chronic hepatitis C showed prominent AKR1B10 immunoreactivity in the cytoplasm, or in the cytoplasm and nucleus (Fig. 3B). The AKR1B10-positive hepatocytes were mostly localized in the periportal zone. Quantitative image analysis revealed that the median percentage of the AKR1B10 positive area was 0.16, 0.16 and 12.57% in control subjects, patients with normal AFP and patients with elevated AFP respectively. The AKR1B10 positive areas were significantly greater in patients with elevated AFP than in control subjects and patients with normal AFP ($P < 0.001$) (Fig. 3C). Although several patients with normal AFP exhibited elevated AKR1B10 immunoreactivity, these differences were not statistically significant between control subjects and patients with normal AFP. Similar to the qRT-PCR results, regression analysis showed a significant correlation between AKR1B10 immunoreactivity and serum AFP (Fig. 3D).

Table 2. Differentially expressed genes in patients with elevated alpha-fetoprotein by fold change ranking (Top20)

Symbol	Title	Up or down	*P* value	Fold change
AKR1B10	Aldo-keto reductase family 1, member B10	Up	9.19E-07	26.2
KRT23	Keratin 23	Up	4.17E-07	22.6
GPC3	Glypican 3	Up	3.54E-09	12.3
FAM3B	Family with sequence similarity 3, member B	Up	1.67E-06	10.5
HKDC1	Hexokinase domain containing 1	Up	3.89E-07	8.8
EpCAM	Epithelial cell adhesion molecule (TACSTD1)	Up	3.07E-06	7.6
DHRS2	Dehydrogenase/reductase (SDR family) member 2	Down	2.39E-04	7.0
OSTbeta	Organic solute transporter beta	Up	3.14E-06	7.0
NRXN3	Neurexin 3	Up	1.40E-04	6.7
CHI3L1	Chitinase 3-like 1	Up	2.92E-06	6.5
TMEM125	Transmembrane protein 125	Up	9.21E-06	6.3
KCNN2	Potassium intermediate/small conductance calcium-activated channel, subfamily N	Down	5.68E-04	6.2
PDZK1IP1	PDZK1 interacting protein 1	Up	1.39E-05	6.1
RAB25	RAB25, member RAS oncogene family	Up	1.73E-05	6.0
DIO3OS	Deiodinase, iodothyronine, type 3 opposite strand	Down	1.87E-06	5.6
LYPD1	LY6/PLAUR domain containing 1	Up	3.97E-05	5.5
STMN2	Stathmin-like 2	Up	5.53E-04	5.1
LOXL4	Lysyl oxidase-like 4	Up	3.91E-06	5.1
KLHL29	Kelch-like protein 29	Up	3.68E-08	5.0
TMC4	Transmembrane channel-like 4	Up	3.64E-06	5.0

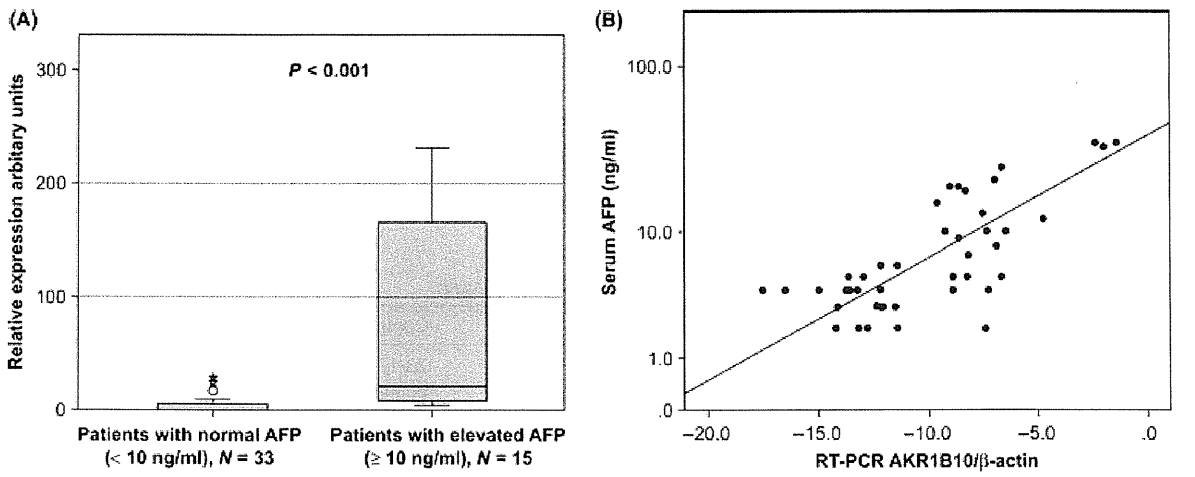

Fig. 2. Quantitative real-time RT-PCR analysis. (A) Comparison of AKR1B10 mRNA expression between the patients with normal alpha-fetoprotein (AFP) and the patients with elevated AFP (Mann–Whitney *U*-test, $P < 0.001$). (B) Regression of AKR1B10 mRNA and serum AFP ($N = 48$, $R^2 = 0.326$, $P < 0.001$).

AKR1B10 expression and risk of HCC

A matched case-control study was performed to evaluate the risk of AKR1B10 expression for HCC development. During the follow-up period after liver biopsy, 20 of 278 chronic hepatitis C patients developed HCC. A comparison of patient characteristics between HCC and control cases is shown in Table 3. According to the case-match design, age, gender and fibrosis stage were similar in both groups, but serum ALT and AFP were significantly higher in HCC cases than in control cases. Immunohistochemical analysis demonstrated that AKR1B10 expression was significantly higher in HCC cases than in control cases (17.7% vs. 1.2%, $P = 0.001$). Table 4 shows the Cox proportional hazard ratios for HCC development estimated with univariate and multivariate models. Univariate analysis identified four factors that were significantly associated with HCC development: ALT ($\geq$90 IU/L, $P = 0.037$), platelet count ($\leq 10 \times 10^4/\mu L$, $P = 0.005$), AFP ($\geq$13 ng/mL, $P = 0.012$) and AKR1B10 expression ($\geq$6%, $P = 0.006$). Multivariate analysis identified two independent

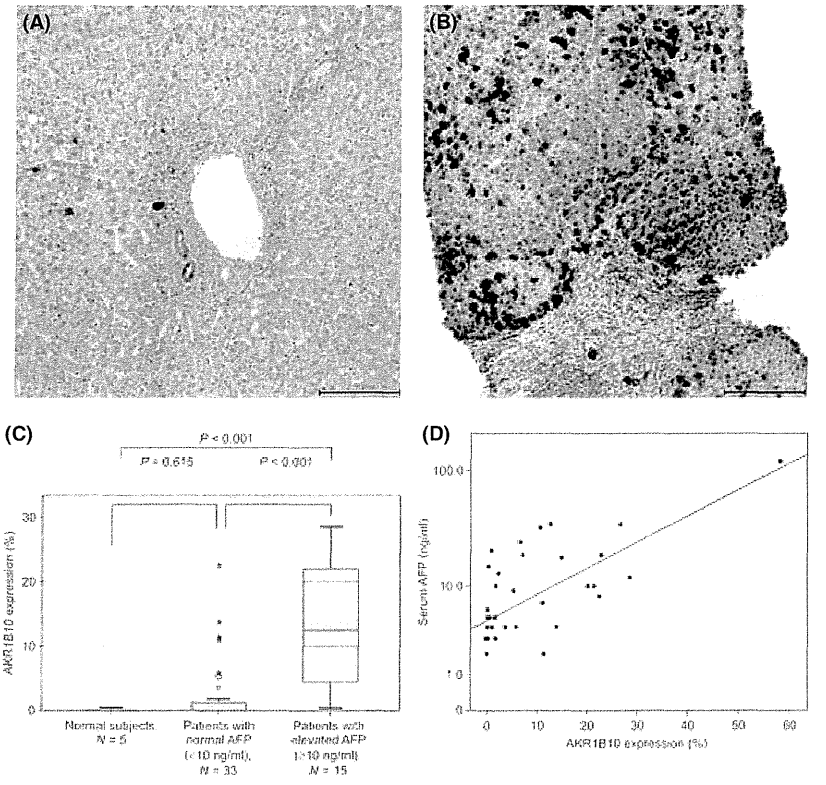

Fig. 3. AKR1B10 immnohistochemical analysis. (A) Representative immunohistochemical staining of normal control liver tissue. Bile duct epithelium served as the positive control. (B) Representative immunohistochemical staining of liver tissue with chronic hepatitis C (magnification × 100). (C) Quantification of AKR1B10 immunoreactivity (Mann–Whitney *U*-test). (D) Regression of AKR1B10 immunoreactivity and serum alpha-fetoprotein in patients with chronic hepatitis C ($N = 48$, $R^2 = 0.613$, $P < 0.001$).

Table 3. Baseline characteristics of patients enrolled in the matched case-control study

Variables	*HCC cases (N = 20)*	*Control cases (N = 40)*	*P value†*
Gender (male/female)	14/6	28/12	Matched
Age (years)*	65 (44–79)	66 (44–80)	Matched
Staging (F1/F2/F3/F4)	3/2/11/4	6/4/22/8	Matched
Body mass index (kg/m^2)*	23.0 (17.5–31.3)	23.1 (17.3–28.6)	0.878
ALT (IU/L)*	97 (32–489)	53 (17–699)	0.017
γGTP (IU/L)*	79 (24–161)	45 (13–375)	0.063
Platelet count (×10^4/μL)	9.9 (5.1–17.3)	15.4 (9.1–24.4)	<0.001
AFP (ng/mL)*	20 (3–142)	6 (1–576)	0.007
Interferon therapy (Yes/No)	16/4	37/3	0.208
Viral clearance (Yes/No)	6/14	21/19	0.099
AKR1B10 expression (%)*	17.7 (0–66.6)	1.2 (0–41.0)	0.001
Follow-up duration (days)*	920 (164–2079)	1534 (406–2118)	0.004

ALT, alanine aminotransferase; AFP, alpha-fetoprotein; γGTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma.
*Data are shown as median (range).
†The *P* value was determined using the Mann–Whitney *U*-test, the chi-square test and Fisher's exact probability test.

factors that were significantly associated with HCC development: expression of AKR1B10 (hazard ratio, 21.45; $P = 0.023$) and platelet count (hazard ratio, 17.46; $P = 0.029$). Kaplan-Meier plot analysis and the log-rank test showed a significant difference in cumulative incidence of HCC development between cases with high ($\geq 6\%$) or low (<6%) expression of AKR1B10 (Fig. 4).

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Table 4. Univariate and multivariate analysis for predictors of hepatocellular carcinoma development

Variable	Category	Univariate analysis		Multivariate analysis	
		Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value*
Body mass index (kg/m^2)	0: <25.2	1	0.332		
	1: ≥25.2	1.75 (0.57–5.42)			
ALT (IU/L)	0: <90	1	0.037		
	1: ≥90	3.53 (1.08–11.56)			
γGTP (IU/L)	0: <65	1	0.061		
	1: ≥65	3.20 (0.95–10.81)			
Platelet count (×10^4/μL)	0: ≥10.0	1	0.005	1	0.029
	1: <10.0	19.08 (2.44–149.10)		17.46 (1.34–226.81)	
AFP (ng/mL)	0: <13	1	0.012		
	1: ≥13	5.26 (1.43–19.31)			
AKR1B10 expression (%)	0: <6	1	0.006	1	0.023
	1: ≥6	17.79 (2.29–138.33)		21.45 (1.54–310.86)	
Interferon therapy	0: Yes	1	0.149		
	1: No	5.26 (0.55–50.02)			
Viral clearance	0: Yes	1	0.120	1	0.057
	1: No	2.41 (0.80–7.32)		10.34 (0.93–114.40)	

ALT, alanine aminotransferase; AFP, alpha-fetoprotein; γGTP, γ-glutamyl transpeptidase.
*The *P* value was determined using the Cox proportional hazard model.

Discussion

Although AFP is widely used in the surveillance and diagnosis of HCC, the AFP level is sometimes elevated in chronic liver disease patients who have no evidence of HCC. Measurement of AFP is clinically important despite its lack of specificity because elevated serum AFP in benign liver disease is a significant predictor of HCC (7–12). Therefore, livers with elevated AFP are at a higher carcinogenic risk than those with normal AFP. In this study, to precisely investigate the molecular alteration in the very early stages of hepatocarcinogenesis, we used a cDNA microarray-based strategy to compare the gene expression profiles of chronic hepatitis C patients who were stratified according to their AFP levels. The PCA and HCA of our microarray data demonstrated a clear difference in the intrahepatic molecular signatures between patients with elevated AFP and those with normal AFP. GO analysis revealed that genes up-regulated in patients with elevated AFP were enriched in the GO terms 'immune response' (e.g., *IFI6*, *TREM2*, *ISG15* and *CXCL10*) and 'DNA replication' (e.g., *CDC6* and *CDC45L*). Serum AFP is thought to be increased both by hepatocyte injury and up-regulation of its turnover, because it is correlated with serum ALT and histological necroinflammation in patients with chronic hepatitis C (7). The hepatic gene signatures in patients with elevated AFP observed in our GO analysis substantiate this theory.

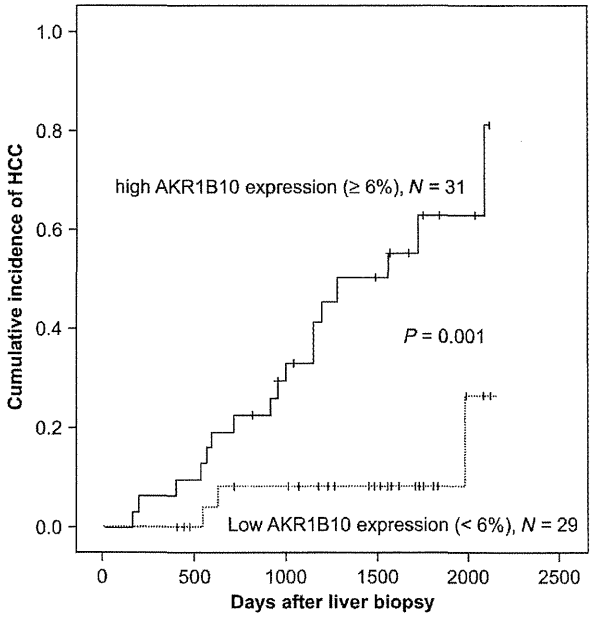

Fig. 4. Cumulative incidence of hepatocellular carcinoma development according to AKR1B10 expression (log-rank test, $P = 0.001$).

Among the 627 differentially expressed genes, AKR1B10 was the most highly up-regulated gene in patients with elevated AFP. AKR1B10 is a member of the AKR superfamily and was originally isolated as a gene whose expression was increased in human HCC. Previous studies reported faint AKR1B10 expression in the normal liver and frequent over-expression in human HCC (14, 16, 17). However, it remained unclear whether AKR1B10 expression was altered in patients with chronic liver disease, particularly in that associated with chronic hepatitis C. In this study, AKR1B10 expression was significantly up-regulated in patients with chronic hepatitis C and elevated AFP compared to normal liver control subjects or patients with chronic

hepatitis C and normal AFP. More importantly, regression analysis revealed a significant correlation between AKR1B10 expression and serum AFP. To our knowledge, this is the first report to show up-regulation of intrahepatic AKR1B10 expression in association with serum AFP in patients with chronic hepatitis C. The mechanism by which intrahepatic AKR1B10 was up-regulated in chronic hepatitis C remains largely unknown. Although previous studies reported that AKR1B10 expression was regulated by the transcription factors AP-1 and Nrf-2 (18, 19), changes of AP-1 and Nrf-2 gene expression were not observed in our microarray analysis (data not shown). Further studies are warranted to better understand the mechanism of AKR1B10 regulation in chronic hepatitis C.

It is not clear why AKR1B10 expression is correlated with AFP. Aldo-keto reductase enzymes are NAD(P)H-dependent oxidoreductases that catalyse the reduction of carbonyl compounds, and various physiological substrates have been proposed for many AKR enzymes. Recently, AKR1B10 was shown to have a high catalytic efficiency for the reduction of all-*trans*-, 9-*cis*- and 13-*cis*-retinals to their corresponding retinols *in vitro* and *in vivo* (20, 21). Conversion of retinals to retinols via AKR1B10 can deprive retinoic acid receptors of their ligands, and can presumably inhibit the retinoic acid signalling pathway (22). Retinoic acid is thought to be essential for the maintenance of normal epithelial differentiation. Retinoic acid depletion causes cell proliferation and loss of differentiation, thereby inducing preneoplastic phenotypes in normal epithelium (23–25). On the other hand, retinoic acid exposure inhibits proliferation of normal and transformed cells *in vitro* (26, 27), and dietary retinoic acid reduced the development of premalignant and malignant lesions in a chemically induced mouse carcinogenesis model (28). In human HCC, oral administration of acyclic retinoids is reported to prevent HCC (29). Collectively, these data indicate that up-regulation of AKR1B10 is linked to the depletion of retinoic acid levels, subsequent loss of differentiation and induction of the carcinofetal phenotype in hepatocytes, resulting in elevated serum AFP. Interestingly, our microarray analysis identified that dehydrogenase/reductase member 2 (DHRS2) was the most down-regulated gene in patients with elevated AFP. DHRS2 was previously known as a nuclear protein Hep27 and functions in inhibition of cell proliferation through p53 stabilization (30). Therefore, DHRS2 down-regulation is likely to result in hepatocyte proliferation. Taken together, not only AKR1B10 up-regulation but also alteration of other molecules, such as DHRS2, might be involved in the mechanisms of serum AFP elevation.

In the matched case-control study, AKR1B10 expression and platelet count were identified as independent predictors of HCC development. In particular, a $\geq 6\%$ up-regulation of AKR1B10 was associated with a ≥ 21-fold relative risk. Many studies have shown AKR1B10 up-regulation in several types of cancers, including recent reports of HCC (14, 31, 32), as well as in precancerous conditions, such as squamous metaplasia and Barrett's oesophagus (33, 34). Furthermore, several reports have shown that down-regulation of AKR1B10 by using small interfering RNA inhibited cancer cell proliferation both *in vitro* and *in vivo* (31, 35). Thus, the involvement of AKR1B10 in carcinogenesis is intriguing. Collectively, our data and these studies indicate that AKR1B10 is not only a useful predictive marker of HCC but also might play an important role in hepatocarcinogenesis, particularly in the very early stages. Consistently, previous studies reported that AKR1B10 up-regulation was mainly observed in early-stage HCC with well differentiation, and rarely observed in advanced stage HCC with poor differentiation (32, 36), indicating AKR1B10 up-regulation is an early event in the process of hepatocarcinogenesis.

In conclusion, this study demonstrated that intrahepatic AKR1B10 expression was up-regulated in association with AFP and significantly reflected the risk of HCC in patients with chronic hepatitis C. AKR1B10 is not only a clinically useful predictive marker for HCC development but may also hold the key to elucidating the mechanism of the very early stages of hepatocarcinogenesis. Our findings might reveal a new insight into the molecular mechanism of hepatocarcinogenesis and provide a novel therapeutic target for the prevention of HCC.

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