suggested that the sensitivity and specificity are insufficient for diagnosing mild liver fibrosis.[11]

Recently, attempts have been made to combine multiple blood test data to create systems for making judgments. The serum aspartate aminotransferase to serum alanine aminotransferase (AST/ALT) ratio[12] and AST to platelet ratio index (APRI),[13] which is the ratio between AST and Plt, are simple but involve significant variations. There have also been reports on FibroTest,[14] which uses α2-macroglobulin, haptoglobin, γ-globulin, apolipoprotein AI, γ-glutamyl transpeptidase (GGT), and total bilirubin (T.Bil), but this includes items that are usually not measured and is difficult to use easily in everyday medical care.

Non-invasive Liver Fibrosis Measurement Methods Using Imaging Technique

Recently, methods have been established for non-invasively quantifying liver fibrosis using imaging technique. The method that has been implemented most quickly for clinical applications is transient elastography (TE) using a FibroScan (EchoSens, Paris, France).[15] TE is a device that refers to M-mode and A-mode images to cause low-frequency elastic waves to oscillate from a transducer on the tip of a probe, and these elastic waves cause strains in the hepatic tissue and induce shear waves, and the velocity of the shear waves is measured using ultrasonic waves. There have been several reports comparing measurement values from TE with liver biopsy tissue, and it has been shown that there is a correlation with the grade of fibrosis of the hepatic tissue. Reports about CLD related to HCV include the 2003 report by Sandrin et al.[15] and the subsequent reports by Castéra et al.,[16] Ziol et al.[17] and Ogawa et al.[18] The usefulness of this method is not limited to HCV and has also been recognised for CLD related to HCV complicated by HIV,[19] chronic hepatitis B,[18] primary biliary cirrhosis, primary sclerosing cholangitis,[20] non-alcoholic fatty liver diseases[21] and liver transplantation donors.[22] Based on these reports on TE, Shaheen et al.[23] and Talwalkar et al.[24] performed a meta-analysis and demonstrated that cirrhosis can be diagnosed with sufficient sensitivity and specificity. However, in low-grade fibrosis (i.e. from F0 to F3), the separation capacity is not as great as in cases of cirrhosis and this is an issue for future research. Performing these measurements is difficult in some patients, such as those with ascites, severe hepatic atrophy, fat deposition, obesity or narrow intercostal spaces. Foucher et al. showed body mass index (BMI)>28 to be a risk factor associated with failure of liver stiffness measurement using TE in patients with CLD.[25]

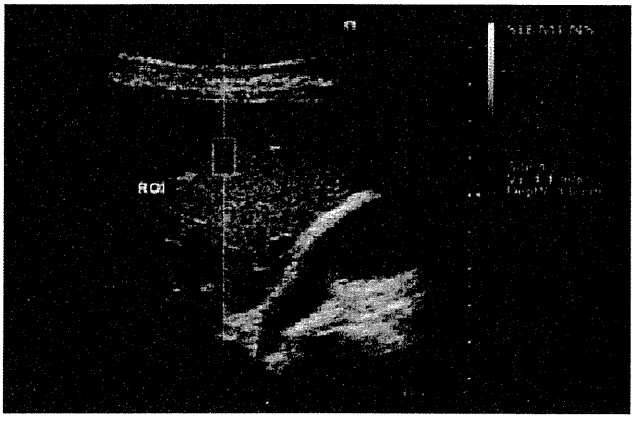
In the past several years, non-invasive liver fibrosis measurement methods mounted on ultrasonic devices have been actualised. ARFI imaging has been incorporated into a conventional ultrasonographic device (Acuson S2000; Siemens Medical Solutions, Mountain View, CA, US). ARFI imaging measures shear wave velocity (SWV) generated when tissue, having been distorted by acoustic radiation pressure from a focused ultrasonic pulse, returns to the original position. Tissue stiffness can thus be quantitatively evaluated and the results displayed on a screen. In general, the amount of change in response to a push pulse is lower in hard tissue and higher in soft tissue.[26-29] SWV is proportional to the square root of tissue elasticity.[27] Several recent reports have examined the utility of ARFI imaging for diagnosing liver fibrosis in patients with CLD.[30-32] Lupsor et al. showed that ARFI imaging is strongly associated with fibrotic stage in patients with CLD related to HCV and offers a useful modality for diagnosing liver fibrosis.[30] In addition, they reported that steatosis does not affect SWV.[30] Furthermore, Friedrich-Rust et al. reported that ARFI imaging is a highly promising method for evaluating liver fibrosis in patients with chronic hepatitis, and that diagnostic accuracy is as good as that of TE.[31]

Table 1: Non-invasive Methods for Assessment of Liver Fibrosis

Serum fibrosis markers	Hyaluronic acid[39]
	Type IV collagen[40]
	Type IV collagen 7S[41]
	Type III procollagen peptide (PIIIP)[39]
Fibrosis score	FibroTest[14]
	APRI[13]
	Forns' index[38]
	AST/ALT ratio[12]
	Hepascore[42]
	ELF[43]
	FPI[44]
	FibroSpect[45]
	Fibrometer[46]
Transient elastography	FibroScan[15-25]
Imaging technique using US	ARFI imaging[26-32]
	RTE[33]
MRI	Diffusion-weighted MRI[35]
	MR elastography[36,37]

ALT = alanine aminotransferase; APRI = aspartate to platelet ratio index; AST = aspartate aminotransferase; ELF = European Liver Fibrosis score; FPI = fibrosis probability index; US = ultrasonography; ARFI = acoustic radiation force impulse; RTE = realtime tissue elastrography; MRI = magnetic resonance imaging.

Figure 1: Acoustic Radiation Force Impulse Imaging

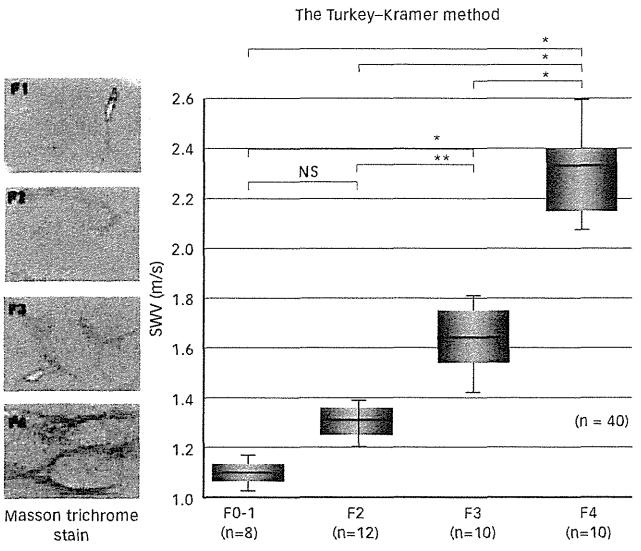


Propagation velocity of the shear wave is around 1.1 m/s and is clearly raised in a patient with chronic hepatitis C. The ultrasound image is used to quantify shear wave velocity 2 cm below the liver capsule.

Realtime tissue elastrography (RTE) is a method that has been mounted on ultrasonic devices manufactured by Hitachi Medical Corporation. RTE images the strain distribution, which represents the tissue transformation ratio when pressure is applied from the body surface. RTE performs a colouring process on this strain distribution and displays the distribution, and the degree of patchiness of the colour image is digitalised using an analysis tool to calculate the strain of the objective region. The strain decreases as liver fibrosis develops.[33]

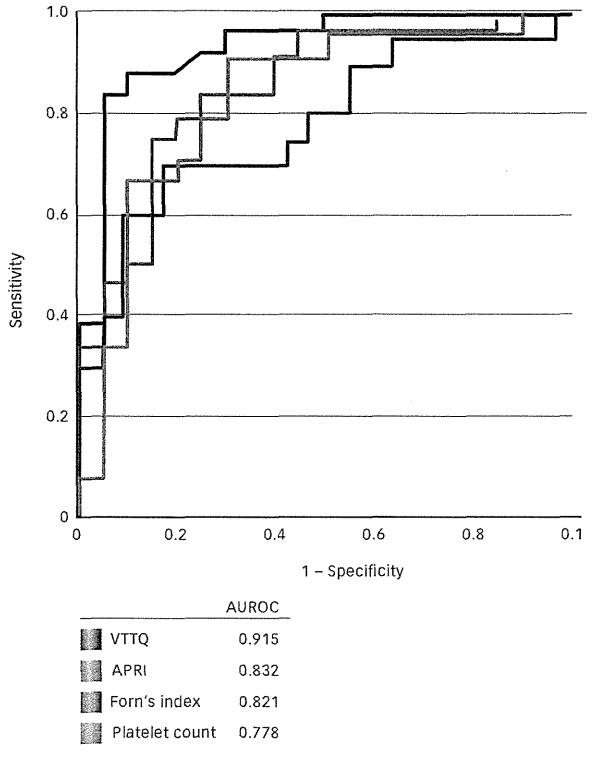
Methods for measuring liver fibrosis through contrast magnetic resonance imaging (MRI) and diffusion-weighted MRI using

Figure 2: Relationship Between Liver Stiffness Measurement and Fibrosis Stage



SWV = shear wave velocity.

Figure 3: Receiver Operating Characteristic Curve for the Diagnosis of F4 by ARFI, APRI, Forns' Index and Platelet Count



APRI = aspartate to platelet ratio index; ARFI = acoustic radiation force impulse; AUROC = area under the receiver operating characteristic; VTTQ = virtual touch tissue quantification.

gadolinium and superparamagnetic iron oxide particles are also being developed. It has also been reported that apparent diffusion coefficient (ADC) values obtained using diffusion-weighted MRI decrease as hepatic fibrosis develops. Furthermore, diagnoses based on these ADC values have equivalent diagnostic capability as other non-invasive liver fibrosis measurement methods.[34,35] MR elastography, which vibrates biological tissue from outside to generate shear waves and images those shear waves, has been developed by Muthupillai et al.[36] and there have been subsequent reports of correlations with liver fibrosis.[37] Although similar non-invasive liver fibrosis measurement is possible with MRI, compared with methods using ultrasonic waves, it is more complex.

Acoustic Radiation Force Impulse Imaging

We evaluated the validity, accuracy and flexibility of the ARFI imaging method in CLD. Forty patients provided informed consent and underwent abdominal ultrasound, haematological examination and ARFI imaging between April and December 2009 at Iwate Medical University Hospital. Ten patients had liver cirrhosis and 30 had chronic hepatitis; all patients had HCV infection. Histopathological studies were conducted in all patients. Liver biopsy was performed percutaneously using a 14-G biopsy needle. Histological diagnosis of the liver was made by two highly experienced pathologists. Staging of liver fibrosis was evaluated according to the METAVIR classification.[5] ARFI imaging technology involves the mechanical excitation of tissue using short-duration acoustic pulses (push pulses) in a region of interest (ROI) chosen by the examiner, producing shear waves that spread away from the ROI, perpendicular to the acoustic push pulse, generating localised, micron-scale displacements in the tissue. ARFI imaging was carried out with a curved array at 4.5 MHz for B-mode imaging. The examination was performed in the right lobe of the liver, through the intercostal space, at the same site as TE measurement. An area where liver tissue was <5 cm thick and free of large blood vessels was chosen. The measurement depth was 2 cm below the liver capsule to standardise the examination (see *Figure 1*). SWV was measured 10 times in succession using ARFI imaging. Mean SWV excluding outliers was used to measure liver stiffness. Relationships between mean SWV measured using ARFI imaging and clinical diagnosis, sex, age, BMI and the results of liver function tests were studied. In addition, the time required to perform ARFI imaging was recorded for all patients. Biochemical tests (i.e. Plt, T.Bil, albumin [Alb], AST, ALT, GGT, total cholesterol [TC], prothrombin time [PT], HA and type IV collagen [IV-C]) were carried out using routine laboratory methods for all patients on the day of ARFI imaging. APRI and Forns' index were calculated for each patient from the results of haematological examination and age.[13,16,33] Clinical diagnostic abilities for F0-3 and F4 were compared for four modalities using receiver operating characteristic (ROC) curves: Forns' index, APRI, Plt and SWV.

The mean time required to perform ARFI imaging was 4.9 ± 2.7 minutes, and the actual operation was very easy as well. Mean SWV in all patients was 1.56 ± 0.89 m/s. No significant correlations with sex, age or BMI were observed. Mean SWV in each fibrosis stage was 1.10 ± 0.22 m/s for F0-1, 1.27 ± 0.52 m/s for F2, 1.62 ± 0.79 m/s for F3 and 2.36 ± 1.11 m/s for F4 (see *Figure 2*). SWV was significantly higher for F4 than for F0-3 ($p<0.0001$) and for F3 than for F2 ($p=0.0072$), although no significant difference was observed between groups for F2. A steady stepwise increase in elasticity correlated with staging of liver fibrosis ($r=0.9772$; $p=0.002$). SWV correlated significantly with Plt ($r=-0.6541$; $p<0.0001$), Alb ($r=-0.6541$; $p<0.0001$), T.Bil ($r=0.5712$; $p=0.0002$), AST ($r=0.7362$; $p<0.0001$), ALT ($r=0.4791$; $p=0.0017$), PT ($r=-0.6139$; $p<0.0001$), TC ($r=-0.5881$; $p=0.0002$), HA ($r=0.5551$;

(r = 0.6432; p=0.0016). The ROC curve for the diagnosis of F4 is shown in *Figure 3*. ARFI imaging showed superior diagnostic ability for F4 (area under ROC: 0.915 for ARFI, 0.832 for APRI, 0.821 for Forns' index and 0.778 for Plt). According to the results of ROC curves, the most appropriate cut-off value of SWV for diagnosing F4 was 1.59, providing 95 % sensitivity and 83 % specificity. ARFI is extremely useful for distinguishing between F4 and F0-3, and SWV might correlate significantly with degree of progression in CLD with HCV infection.

Perspectives

The clinical management and prognosis of CLD are strongly influenced by the extent of liver fibrosis because the diagnosis of liver fibrosis is very important for assessing disease severity and thus determining the optimal therapeutic strategy. Serum fibrosis markers and fibrosis scores are therefore being used to noninvasively and quantitatively evaluate liver fibrosis. Furthermore, non-invasive liver fibrosis measurement methods using techniques such as TE or ARFI imaging are useful to estimate the degree of fibrosis in CLD. ■

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Original Article

Novel scoring system as a useful model to predict the outcome of patients with acute liver failure: Application to indication criteria for liver transplantation

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Aim: In Japan, the indication for liver transplantation in patients with acute liver failure (ALF) is currently determined according to the guideline published in 1996. However, its predictive accuracy has fallen in recent patients. Thus, we attempted to establish a new guideline.

Methods: The subjects were 1096 ALF patients enrolled in a nationwide survey. All patients showed a prothrombin time <40% of the standardized value and grade II or more severe hepatic encephalopathy. A multiple logistic regression analysis and receiver operating characteristic analysis were performed in 698 patients seen between 1998 and 2003 to identify significant parameters determining the outcome of patients. The extracted parameters were graded as numerical scores. An established scoring system was validated in patients seen between 2004 and 2008.

Results: Six parameters were identified and graded as 0, 1 and/or 2; the interval between disease onset and development of hepatic encephalopathy, prothrombin time, serum total bilirubin concentration, the ratio of direct to total bilirubin concentration, peripheral platelet count and the presence of liver atrophy. When the prognosis of the patients with total score of 5 or more was judged as "death", the predictive accuracy was 0.80 with sensitivity, specificity, positive predictive value and negative predictive value greater than 0.70. The values were similarly high in patients for validation.

Conclusion: Novel scoring system for predicting the outcome of ALF patients may be useful to determine the indication of liver transplantation, since the system showed high predictive accuracy even after validation.

Key words: acute liver failure, fulminant hepatitis, guideline, indication criteria, liver transplantation, outcome prediction

INTRODUCTION

ACUTE LIVER FAILURE is a disease entity characteristic with extensive destruction of liver parenchyma by hepatitis virus infection and other causes, and is typically represented by fulminant hepatitis. Although the outcome may differ depending on the etiology of acute liver failure, survival rates of patients receiving conventional medical care are generally low in cases with impaired liver regeneration. Hepatitis patients are diagnosed as fulminant hepatitis in Japan if grade II or deeper hepatic encephalopathy develops within 8 weeks of the onset of hepatitis symptoms due to severe abnormality of the liver function with prothrombin time lower than 40% of the standardized value. Fulminant hepatitis is further classified into two subtypes according to clinical

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course; acute type with hepatic encephalopathy developing within 10 days of disease onset and subacute type with hepatic encephalopathy at 11 days or later.[1,2] In addition, late onset hepatic failure (LOHF) is defined as a related disease of fulminant hepatitis, in which hepatic encephalopathy develops between 8 and 24 weeks after the onset of hepatitis symptoms.[2] Fulminant hepatitis in Japan is defined as acute liver failure with histological appearance of hepatic inflammation, such as lymphocyte infiltration in the liver. Thus, the etiology of fulminant hepatitis comprises viral hepatitis including persistent hepatitis B virus (HBV) carriers, autoimmune hepatitis, drug-induced/allergic hepatitis, and hepatitis with indeterminate etiologies, but excludes drug-induced/toxic liver damage, acute fatty liver in pregnancy, postoperative liver damage and ischemic liver damage. However, in Europe and the United States, the latter causes are included in the disease entity of acute liver failure, formerly called fulminant hepatic failure. Thus, for example, acetaminophen-induced liver damage is included in acute liver failure in Europe and the United States, while it is excluded from the disease entity of fulminant hepatitis in Japan.[3] Considering such differences of the definition and diagnostic criteria between fulminant hepatitis in Japan and acute liver failure in the United States and Europe, the guidelines in the latter countries to determine the indication of liver transplantation for acute liver failure are not directly applicable to fulminant hepatitis in Japan.

It is accepted worldwide that liver transplantation is the most effective therapeutic modality for patients with acute liver failure. In Japan, indication criteria for liver transplantation in patients with fulminant hepatitis were defined in 1996, as a two-step outcome prediction scoring system, by the Acute Liver Failure Study Group of Japan (Table 1).[4,5] According to this guideline, the outcome of fulminant hepatitis patients is predicted at the onset of grade II or more severe hepatic encephalopathy based on five parameters: age of patient, the interval between occurrence of hepatitis symptoms and development of hepatic encephalopathy, prothrombin time, serum total bilirubin concentration and the ratio of the serum direct to total bilirubin concentration. Then, in patients undergoing intensive medical care including artificial liver support, their prognosis is reassessed 5 days later, according to the extent of improvement of hepatic encephalopathy and prothrombin time. This guideline was prepared based on the clinical findings in fulminant hepatitis patients seen between 1988 and 1992, and was considered to be useful, since the predictive accuracy was found to be 83% in a prospective analysis conducted in patients seen between 1993 and 1995.[4] However, the predictive accuracy of the guideline fell in patients with fulminant hepatitis seen between 1998 and 2003; 68% and 78% in the acute and subacute types, respectively, and the values did not improve following reassessment at 5 days later.[6]

Table 1 Guideline to determine the indications for liver transplantation in patients with fulminant hepatitis by the Acute Liver Failure Study Group of Japan in 1996

Patients may be registered as recipients of liver transplantation when at least two of the five criteria are satisfied at the time of onset of Grade II or more severe hepatic encephalopathy.

1 Age ≥45 years.
2 Interval from the appearance of the initial symptoms to the development of hepatic encephalopathy ≥11 days.
3 Prothrombin time <10% of the standardized value.
4 Serum bilirubin concentration ≥18.0 mg/dL.
5 Ratio of the direct to total bilirubin concentration <0.67.

If liver transplantation cannot be performed within 5 days and intensive medical therapy, including artificial liver support, is undertaken, the prognosis of the patients is evaluated again. If both of the following two criteria are positive at 5 days after the onset of hepatic encephalopathy, the patients are re-predicted as "alive" and excluded from the candidate list for liver transplantation.

1 The hepatic encephalopathy shows improvement to Grade I or less or attenuation by two more grades.
2 Prothrombin time improves to over 50% of the standardized value.

English version of this guideline was published in Mochida *et al.*[5]

To improve the predictive accuracy of indication criteria for liver transplantation in patients with fulminant hepatitis, the Study Group of Intractable Hepatobiliary Diseases supported by the Ministry of Health, Labor, and Welfare of Japan organized a task force in 2006. The task force first analyzed the database obtained from patients with fulminant hepatitis and LOHF seen between 1998 and 2003 in Japan to establish the novel scoring system to predict the outcome of the patients. Then, the established system was evaluated in the patients seen between 2004 and 2008. In the present paper, we report on the usefulness of this novel scoring system. We state here that the new system is intended for use in a general cohort of acute liver failure, but is actually organized on the database of registered patients with fulminant hepatitis and LOHF. Thus, validation of

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the system for acute liver failure due to other etiologies as described earlier awaits future study.

METHODS

Patients

THE STUDY SUBJECTS are 1096 patients with acute liver failure who were enrolled in the nationwide survey by the Intractable Hepato-Biliary Disease Study Group of Japan between 1999 and 2008 (formerly the Intractable Liver Diseases Study Group of Japan before 2003). All of the patients showed grade II or more severe hepatic encephalopathy and prothrombin time of less than 40% of the standardized value and were admitted to 610 hospitals of Japan specializing in hepatology between 1998 and 2008. The patients consisted of three disease types; 505 and 449 patients, respectively, with acute and subacute types of fulminant hepatitis and 88 patients with LOHF. They were divided into two cohorts; 698 patients (316, 318 and 64 patients, respectively, with acute and subacute types of fulminant hepatitis and LOHF) seen between 1998 and 2003 (the estimation cohort) and 394 patients (189, 191 and 24 patients, respectively, of each disease type) seen between 2004 and 2008 (the validation cohort). From both cohorts, the patients with incomplete records and those treated with liver transplantation were excluded. Thus, the estimation cohort included 421 patients (201 and 178 patients, respectively, with acute and subacute types of fulminant hepatitis and 41 patients with LOHF) and the validation cohort recruited 231 patients (125, 95 and 11 patients, respectively, in each disease type).

Etiologies of hepatitis in the estimation and validation cohorts are given in Table 2. Demographic and clinical features of patients in each cohort are shown in Tables 3 and 4, respectively. These features did not differ between the two cohorts, except that the ages of the patients were greater in the validation cohort than in the estimation cohort. The survival rates of patients were equivalent between two cohorts; 37.4% in the estimation cohort and 37.7% in the validation cohort.

Table 2 Etiologies of fulminant hepatitis and late onset hepatic failure (LOHF) in the estimation cohort and validation cohort

	Estimation cohort 1998–2003	Validation cohort 2004–2008
HAV	33	15
HBV (acute onset)	104	51
HBV (career)	65	33
HBV (unclassified)	9	16
HCV	8	3
Other Virus	3	5
AIH	26	23
Drug	35	33
Undetermined	132	49
No record	6	3
Total	421	231

Data are expressed as the number of patients.
AIH, autoimmune hepatitis; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus.

Identification of prognostic factors responsible for the outcome of patients with fulminant hepatitis and LOHF in the estimation cohort

First, univariate logistic analysis was performed in patients of the estimation cohort to identify possible prognostic factors among demographic and clinical features at the onset of grade II or more severe hepatic encephalopathy as follows. (i) *Demographic features*; sex and age of patients, the types of disease (acute and subacute types of fulminant hepatitis and LOHF), the interval (days) between the onset of hepatitis symptoms and the development of hepatic encephalopathy. (ii) *Symptoms*; fever of 37.5°C or more, convulsion, tachycardia, disappearance of liver dullness on physical examination, flapping tremor, hepatic odor and edema. (iii) *Laboratory parameters*; prothrombin time (%), hepaplastin test (%), antithrombin III activity (%), serum concentrations of albumin (g/dL) and total and direct bilirubin (mg/dL), the ratio of direct to total bilirubin concentration, serum levels of aspartate aminotransferase (AST: IU/L) and alanine aminotransferase (AST: IU/L), serum α-fetoprotein concentration (ng/mL), blood ammonia concentration (μg/dL), plasma concentration of hepatocyte growth factor (HGF: ng/mL), peripheral platelet and white blood cell counts (/mm^3). (iv) *Imaging*; liver atrophy diagnosed by ultrasound sonography and/or computed tomography/magnetic resonance imaging (CT/MRI).

Extracted factors were subjected to multivariate logistic analyses through a stepwise elimination manner. Then a receiver operating characteristic (ROC) curve was constructed for each significant variable.

Scoring of prognostic factors and predicted mortality of patients with fulminant hepatitis and LOHF in the estimation and validation cohorts

The grading of variables was determined as numerical scores based on the inflection points of each ROC curve.

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Table 3 Demographic and clinical features of patients with fulminant hepatitis and late onset hepatic failure (LOHF) seen between 1998 and 2003 (estimation cohort)

	Total ($n = 421$)	Dead patients ($n = 260$)	Surviving patients ($n = 161$)
Sex (Male : Female)	218:202:(1)†	148:111:(1)†	70:91
Age	48.6 ± 16.3‡	53.2 ± 14.8**	41.3 ± 16.0
HBV Carrier	15.2% (64/421)	18.8%** (49/260)	9.3% (15/161)
Disease Type (FHA : FHS : LOHF)	201:178:41	86:138:36**	115:40:5
HGF (ng/mL)	6.0 ± 11.4	7.6 ± 13.9*	3.9 ± 6.0
TB (mg/dL)	14.0 ± 9.1	16.6 ± 9.6**	9.7 ± 6.2
D/T ratio	0.63 ± 0.13	0.62 ± 0.14**	0.66 ± 0.12
PT (%)	22.7 ± 12.6	22.5 ± 13.8*	23.5 ± 11.3
AT (%)	37.3 ± 20.3	35.9 ± 20.3*	41.5 ± 19.9
NH3 (μg/dL)	138.7 ± 82.8	151.6 ± 87.8**	118.1 ± 69.6
PLT (10^4/μL)	12.7 ± 7.7	12.1 ± 8.1*	13.6 ± 6.9
Liver Atrophy (present : absent)	265:156	210:50**	55:106
O-C (days)	21.2 ± 26.7	26.4 ± 29.3**	12.8 ± 19.2

*$P < 0.05$, **$P < 0.01$ versus alive.
†A value in parenthesis means the number of patients with no record regarding the sex.
‡Values are expressed as mean ± standard deviation (SD).
AT, antithrombin III; D/T ratio, ratio of direct to total bilirubin concentrations; FHA, acute type of fulminant hepatitis; FHS, subacute type of fulminant hepatitis; HBV, hepatitis B virus; HGF, hepatocyte growth factor; O-C, intervals between hepatitis onset and hepatic encephalopathy development; PLT, platelet; PT, prothrombin time; TB, total bilirubin.

The total scores were calculated in each patient belonging to the estimation cohort, and the mortality rates were evaluated depending on total scores. Then, ROC analysis was performed again to identify the cut-off value of the total score that can discriminate sharply between survived and dead patients. Finally, sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and predictive accuracy of the established scoring system were calculated when the predicted outcome of patients with the total score greater than the cut-off value was judged as "death".

Predictive accuracies of the established system were confirmed similarly in the validation cohort.

Statistical analysis

All statistical analyses were performed with JMP v7.0 for Macintosh (SAS Institute Inc., Cary, NC, USA). Univariate analyses were performed with analysis of variance (ANOVA) and χ^2 test. Multivariate analyses were performed by multiple logistic regression analysis with stepwise selection.

RESULTS

Prognostic factors responsible for the outcome of patients with fulminant hepatitis and LOHF

UNIVARIATE LOGISTIC ANALYSIS revealed 18 variables including demographic features and clinical characteristics at the appearance of grade II or more severe hepatic encephalopathy may affect the mortality of the patients; age of patients, the interval between disease onset and the development of hepatic encephalopathy, presence of tachycardia and edema, disappearance of liver dullness on physical examination and presence of liver atrophy on imaging examination, serum concentrations of albumin and total bilirubin, the ratio of direct to total bilirubin concentration, serum levels of AST and ALT, blood ammonia level, plasma HGF concentration, prothrombin time, hepaplastin test, antithrombin III activity and peripheral platelet count. These factors were subjected to multivariate logistic analysis with stepwise elimination manner, and 10 variables were identified as significant as shown in Table 5. At this step,

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Table 4 Demographic and clinical features of patients with fulminant hepatitis and late onset hepatic failure (LOHF) seen between 2004 and 2008 (validation cohort)

	Total ($n = 231$)	Dead patients ($n = 144$)	Surviving patients ($n = 87$)
Sex (Male : Female)	120:111	74:70	46:41
Age	54.7 ± 15.0†	59.8 ± 12.2**	46.3 ± 15.6
HBV Carrier	14.2% (33/231)	19.4% (28/144)	5.7% (5/87)
Disease Type (FHA : FHS : LOHF)	125:95:11	61:74:9**	64:21:2
HGF (ng/mL)	6.0 ± 9.2	7.1 ± 10.4	3.2 ± 3.6
TB (mg/dL)	13.7 ± 8.9	16.6 ± 9.3**	8.8 ± 5.3
D/T ratio	0.64 ± 0.14	0.64 ± 0.13	0.63 ± 0.16
PT (%)	24.5 ± 12.7	22.0 ± 12.4**	28.4 ± 12.4
AT (%)	38.2 ± 20.4	34.4 ± 21.1*	44.3 ± 17.7
NH3 (μg/dL)	162.0 ± 141.8	191.1 ± 168.8**	116.4 ± 60.7
PLT (10^4/μL)	12.6 ± 7.2	11.5 ± 6.9**	14.3 ± 7.2
Liver Atrophy (Present/absent)	146:85	114:30**	32:50
O-C (days)	15.9 ± 17.0	19.0 ± 18.5**	10.6 ± 12.2

*$P < 0.05$, **$P < 0.01$ versus alive.
†Values are expressed as mean ± standard deviation (SD).
AT, antithrombin III; D/T ratio, ratio of direct to total bilirubin concentrations; FHA, acute type of fulminant hepatitis; FHS, subacute type of fulminant hepatitis; HBV, hepatitis B virus; HGF, hepatocyte growth factor; O-C, intervals between hepatitis onset and hepatic encephalopathy development; PLT, platelet; PT, prothrombin time; TB, total bilirubin.

"age of patients" was excluded from the list of candidate variables in order to facilitate the system to be available in pediatric patients. Then ROC curve was constructed for each variable, and six variables with the greatest area under the curve (AUC) were identified; the interval between disease onset and the development of hepatic encephalopathy, prothrombin time, serum concentration of total bilirubin, the ratio of direct to total bilirubin concentration, peripheral platelet count, and liver atrophy.

Table 5 Prognostic factors to affect the outcome of patients with fulminant hepatitis and late onset hepatic failure (LOHF): multivariate logistic analysis in those seen between 1998 and 2003 (estimation cohort)

	Odds ratio†	(95% confidence interval)	*P*-value
Liver atrophy	9.777		<0.0001
TB	1.0993	(1.043–1.168)	0.0009
D/T ratio	0.000446	(0.0001146–0.081412)	0.0062
NH3	1.007	(1.002–1.014)	0.0098
Age	1.0654	(1.010–1.136)	0.0113
PT%	0.9773	(0.959–0.995)	0.0115
HGF	1.1837	(1.049–1.374)	0.0139
O-C	1.0687	(1.014–1.141)	0.0270
ALB	0.0409	(0.129–0.906)	0.0312
PLT	0.9648	(0.931–0.999)	0.0489

†Odds ratio of dead patients to survived patients in relation to the presence or absence of liver atrophy and a unit increase of each continuous parameter.
ALB, albumin; D/T ratio, ratio of direct to total bilirubin concentration; HGF, hepatocyte growth factor; O-C, the interval between hepatitis onset and the development of hepatic encephalopathy; PLT, platelet; PT, prothrombin time; TB, total bilirubin.

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Table 6 Scores for Predictive Variables Affecting the Mortality of Patients with Fulminant Hepatitis and late onset hepatic failure (LOHF)

Score	0	1	2
O-C (days)	≤5	6–10	11≤
PT (%)	20<	5< ≤20	≤5
TB (mg/dL)	<10	10≤ <15	15≤
D/T ratio	0.7≤	0.5≤ <0.7	<0.5
PLT (104/μL)	10<	5< ≤10	≤5
Liver atrophy	Absent	Present	

D/T ratio, ratio of direct to total bilirubin concentrations; O-C, the interval between hepatitis onset and the development of hepatic encephalopathy; PLT, platelet; PT, prothrombin time; TB, total bilirubin.

Scoring system to predict the possible outcome of patients with fulminant hepatitis and LOHF

Variables extracted through ROC curve analysis were graded as shown in Table 6, according to the inflection points of each curve. The interval between hepatitis onset and the development of hepatic encephalopathy, prothrombin time, serum concentration of total bilirubin, the ratio of direct to total bilirubin concentration and peripheral platelet count were classified into three grades (0, 1 and 2), and liver atrophy into two grades (0 and 1).

As shown in Table 7, the mortality rates rose in relation to total scores calculated in patients seen between 1998 and 2003 (estimation cohort). When the predictive outcome of patients showing total scores of 5 or more was judged as "death", PPV and NPV of the system were 0.84 and 0.70, respectively (Table 8), suggesting that total scores of 5 is sufficient enough as a cut-off value that can discriminate between dead and survived patients. The scoring system with such cut-off value showed sensitivity and specificity of 0.80 and 0.76, respectively, and resulted in predictive accuracy of 0.78 in patients in the estimation cohort. Predictive accuracies did not differ depending on the disease types; 0.75 in patients with acute type of fulminant hepatitis and 0.87 in those with subacute type of fulminant hepatitis.

Table 7 The outcome of patients with fulminant hepatitis and late onset hepatic failure (LOHF) seen between 1998 and 2003 (estimation cohort) depending on total scores calculated through established scoring system

Total scores	Mortality (%)	FHA/FHS/LOHF
9≤	9/10 (90.0%)	2/4/4
8	26/27 (96.3%)	2/20/5
7	42/46 (91.3%)	10/30/6
6	71/83 (85.5%)	20/52/11
5	59/80 (73.8%)	26/42/12
4	31/55 (56.3%)	32/22/1
3	12/50 (24.0%)	44/4/2
2	8/40 (20.0%)	35/5/0
1	2/25 (8.0%)	25/0/0
0	0/5 (0.0%)	5/0/0

FHA, acute type of fulminant hepatitis; FHS, subacute type of fulminant hepatitis.

Table 8 Accuracies of established scoring system in patients with fulminant hepatitis and late onset hepatic failure (LOHF) seen between 1998 and 2003 (estimation cohort) when predictive outcome of patients showing total scores of 5 or more are diagnosed as "death"

	Total scores		
	≥5	<5	Total
Number of patients			
Dead patients	207	53	260
Surviving patients	39	122	161
Total	246	175	421
Mortality	84.1%	30.3%	61.8%
The accuracies			
Positive predictive value (PPV)	207/246		0.84
Negative predictive value (NPV)	122/175		0.70
Sensitivity	207/260		0.80
Specificity	122/161		0.76
Predictive accuracy (PA)	(207+122)/421		0.78

The accuracies of the established scoring system were validated in patients with fulminant hepatitis and LOHF seen between 2004 and 2008 (validation cohort). As shown in Table 9, the mortality rate of patients in each total score was almost equivalent to that obtained in analysis with patients in the estimation cohort. Thus, predictive accuracy through analysis in the validation cohort was 0.75 with sensitivity, specificity, PPV and NPV of 0.75, 0.80, 0.86 and 0.65, respectively (Table 10).

DISCUSSION

LIVER TRANSPLANTATION IS regarded worldwide as the most effective therapeutic procedure for patients with end-stage liver diseases including acute liver

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Table 9 The outcome of patients with fulminant hepatitis and late onset hepatic failure (LOHF) seen between 2004 and 2008 (validation cohort) depending on total scores calculated through established scoring system

Total scores	Mortality (%)	FHA/FHS/LOHF
9≤	4/4 (100.0%)	0/3/1
8	9/9 (100.0%)	1/6/2
7	26/30 (86.7%)	9/20/1
6	35/39 (89.7%)	9/29/1
5	33/42 (78.6%)	18/19/5
4	21/39 (53.8%)	28/10/1
3	10/30 (33.3%)	22/8/0
2	4/26 (15.4%)	26/0/0
1	2/8 (25.0%)	8/0/0
0	0/4 (0.0%)	4/0/0

FHA, acute type of fulminant hepatitis; FHS, subacute type of fulminant hepatitis.

failure. Japanese Society for the Study of Liver Transplantation revealed that survival rate at 1 year after liver transplantation was 72.7% in patients with acute liver failure,[7] while conventional medical care yielded insufficient prognosis in such patients; survival rates were 54.0% and 24.0%, respectively, in patients with fulminant hepatitis of acute and subacute types and 15.0% in LOHF patients according to the nationwide survey by the Study Group of Intractable Hepatobiliary Diseases.[8] In general, in Japan, patients with acute liver failure visit clinics or hospitals at the onset of hepatitis symptoms and derangement of liver function was diagnosed by physicians specialized in general medicine. Next, the patients were transferred to hospitals with specialists in the fields of hepatology and emergency medicine around the periods of the development of hepatic encephalopathy. Conventional medical care including artificial liver support with plasma exchange and hemodiafiltration was performed, and then the patients were introduced to transplant surgeons regarding the indication of liver transplantation. Thus, the simple criteria to predict the outcome of patients with fulminant hepatitis and LOHF with sufficient accuracies are required to facilitate communication among general physicians, hepatologists and transplant surgeons.

Table 10 Accuracies of established scoring system in patients with fulminant hepatitis and late onset hepatic failure (LOHF) seen between 2004 and 2008 (validation cohort) when predictive outcome of patients showing total scores of 5 or more are diagnosed as "death"

	Total scores		
	≥5	<5	Total
Number of patients			
Dead patients	107	17	124
Surviving patients	37	70	107
Total	144	87	231
Mortality	74.3%	19.5%	53.7%
The accuracies			
Positive predictive value (PPV)	107/144		0.75
Negative predictive value (NPV)	70/87		0.80
Sensitivity	107/124		0.86
Specificity	70/107		0.65
Predictive accuracy (PA)	(107+70)/231		0.77

In Europe and the United States, the indication of liver transplantation in patients with acute liver failure has been determined according to the guideline proposed by King's Collage Hospital[9] and Beaujon Hospital.[10] In addition, a scoring system of model for end-stage liver disease (MELD), initially designed for patients with chronic liver failure, has recently been applied also to those with acute liver failure.[11] However, these guidelines are not directly applicable to patients with fulminant hepatitis and LOHF in Japan, since social environment as well as demographic and clinical features of the patients differ among Japan, Europe and the United States; for example liver transplantation with brain death-related donor is hardly available and artificial liver support is routinely performed in Japan. Thus, novel guidelines should be established for Japanese patients with fulminant hepatitis and LOHF instead of the previous guideline proposed by the Acute Liver Failure Study Group in Japan at 1996,[4] which shows decline of predictive accuracy when applied to recent patients.[5]

In the present paper, a novel scoring system to predict the outcome of patients with fulminant hepatitis and LOHF was established based on demographic and clinical features of patients seen between 1998 and 2008. The predictive mortality rates were estimated through six variables at the occurrence of grade 2 or more severe hepatic encephalopathy; the interval between the onset of hepatitis symptoms and the development of hepatic encephalopathy, prothrombin time, serum concentration of total bilirubin, the ratio of direct to total bilirubin concentration, peripheral platelet count and presence of liver atrophy on imaging. When total scores were calculated through six variables in patients belonging to the estimation cohort, the mortality rate was 84.1% in those with scores of 5 or more, while it was 30.3% in those with scores of 4 or less. Thus, the cut-off value of total scores to discriminate possible dead

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patients from surviving patients was set between 4 and 5. Consequently, excellent accuracies were obtained through analysis in patients belonging to the estimation cohort; predictive accuracy was 0.78 with either of PPV, NPV, sensitivity and specificity greater than 0.7. Such high predictive accuracy was also found through analysis in patients belonging to the validation cohort. It is noteworthy that peripheral platelet count and the presence of liver atrophy were added to the list of predictive variables in the present scoring system. Also, cut-off values to grade other variables, such as the interval between the onset of hepatitis symptoms and the development of hepatic encephalopathy, differ between the present system (Table 6) and previous guidelines (Table 1). These modifications may contribute to improve predictive accuracy of the novel scoring system when applied to recent patients.

In the present study, the age of patients was excluded from the list of predictive variables to facilitate the use of the system in pediatric patients. In our database, a patient showing total score of 6 died, while three cases with total scores of 4 or less survived, when the system was applied for patients aged less than 15 years old. Furthermore, the most recent report by Fujisawa showed 100% specificity and PPV by the scoring system in 40 pediatric patients.[12] Thus, the system seems to be useful even in such patients. Also, plasma HGF concentration was deleted from the list of predictive variables, because it is difficult to obtain the results within a day in most of the hospitals in Japan. In contrast, the presence of liver atrophy was included in the predictive variable list, but the quantitative criteria for liver atrophy were not specified in the present scoring system. The estimated liver volume is measured on CT examination, and the ratio of the value to the standardized liver volume was reported to correlate with mortality in patients with acute liver failure in Japan.[13] These problems, regarding age of patients, significance of plasma HGF concentration and diagnostic criteria to determine liver atrophy should be further investigated.

In conclusion, a novel scoring system for predicting outcome of patients with fulminant hepatitis and LOHF was established. This system may be useful to determine the indication of liver transplantation in patients with acute liver failure, since the system showed high predictive accuracies even after the validation.

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Research Article

Cancer Prevention Research

Acyclic Retinoid Inhibits Diethylnitrosamine-Induced Liver Tumorigenesis in Obese and Diabetic C57BLKS/J- +Lepr^{db}/+Lepr^{db} Mice

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Abstract

Obesity and the related metabolic abnormalities are associated with increased risk of hepatocellular carcinoma (HCC). Malfunctioning of retinoid X receptor (RXR) α due to phosphorylation by Ras/MAPK also plays a critical role in liver carcinogenesis. In the present study, we examined the effects of acyclic retinoid (ACR), which targets RXRα, on the development of diethylnitrosamine (DEN)-induced liver tumorigenesis in C57BLKS/J- +Lepr^{db}/+Lepr^{db} (*db/db*) obese mice. Male *db/db* mice were given tap water containing 40 ppm DEN for 2 weeks, after which they were fed a diet containing 0.03% or 0.06% of ACR throughout the experiment. In mice treated with either dose of ACR for 34 weeks, the development of liver cell adenomas was significantly inhibited as compared with basal diet-fed mice. ACR markedly inhibited the activation of Ras and phosphorylation of the ERK (extracellular signal-regulated kinase) and RXRα proteins in the livers of experimental mice. It also increased the expression of *RARβ* and *p21^{CIP1}* mRNA while decreasing the expression of *cyclin D1*, *c-Fos*, and *c-Jun* mRNA in the liver, thereby restoring RXRα function. Administration of ACR improved liver steatosis and activated the AMPK protein. The serum levels of insulin decreased by ACR treatment, whereas the quantitative insulin sensitivity check index (QUICKI) values increased, indicating improved insulin sensitivity. The serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice were decreased by ACR treatment, suggesting attenuation of the chronic inflammation induced by excessive fatty deposits. ACR may be, therefore, useful in the chemoprevention of obesity-related HCC. *Cancer Prev Res; 4(1); 128–36. ©2010 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is a serious health-care problem worldwide. The risk factors associated with the development of HCC include chronic hepatitis B and/or hepatitis C infection, particularly with subsequent cirrhosis. Recent evidence also indicates that obesity and the related metabolic abnormalities, especially diabetes mellitus, increase the risk of HCC (1–3). In a rodent model, the occurrence of diethylnitrosamine (DEN)-induced liver tumorigenesis was found to be significantly higher in obese and diabetic C57BLKS/J- +Lepr^{db}/+Lepr^{db} (*db/db*) mice than in genetic control mice (4). Diabetes mellitus has been shown to increase the risk of primary HCC in patients with viral hepatitis (5). Insulin resistance is also significantly associated with the recurrence of stage I HCC after curative treatment (6). Nonalcoholic fatty liver disease (NAFLD) is a hepatic manifestation of the insulin resistance syndrome, and in a subset of NAFLD patients, the condition progresses to nonalcoholic steatohepatitis, which involves severe inflammation and therefore poses the threat of HCC (7, 8). Coexistent obesity or steatosis exacerbates liver injury and fibrosis and thus is involved in liver tumorigenesis (9). Therefore, patients with obesity and insulin resistance comprise a high-risk group for HCC, and their treatment must target the prevention of this malignancy.

Acyclic retinoid (ACR, the same substance as NIK-333), a synthetic retinoid, apparently exerts chemopreventive effects on the development of HCC (10). It inhibits experimental liver carcinogenesis and suppresses the

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Note: Supplementary data for this article are available at Cancer Prevention Research Online (http://cancerprevres.aacrjournals.org/).

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growth of HCC-derived cells by inducing apoptosis and causing cell-cycle arrest in G_0–G_1 (11–15). These effects of ACR are associated with its agonistic activity for distinct nuclear retinoid receptors—retinoid X receptors (RXR) and retinoic acid receptors (RAR), both of which have 3 subtypes (α, β, and γ; 16)—and subsequent expression of the ACR target genes *RAR*β and $p21^{CIP1}$ (12–15). A clinical trial revealed that oral administration of ACR significantly reduced the incidence of postthertherapeutic HCC recurrence and improved the survival rates of patients (17, 18). A phase II/III trial of ACR confirmed its effectiveness in preventing second primary HCC in hepatitis C virus–positive patients in a large-scale (n = 401) randomized, placebo-controlled trial; hazard ratio for recurrence-free survival with ACR 600 mg/d versus placebo was 0.27 (95% CI, 0.07–0.96) after 2 years randomization (19).

Among the retinoid receptors, RXRα is considered as one of the most important receptors with respect to the regulation of fundamental cell activities because it forms a heterodimer with other nuclear receptors and thereby acts as the master regulator of nuclear receptors (20). Recent studies indicate that phosphorylation of RXRα abolishes its ability to form a heterodimer with RARβ, and the accumulation of phosphorylated RXRα (p-RXRα, i.e., nonfunctional RXRα), which is caused by activation of the Ras/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling pathway, plays a critical role in the development of HCC (10, 21, 22). On the other hand, the effects of ACR in suppressing growth and inducing apoptosis in HCC cells depend on the inactivation of Ras-ERK signaling system and subsequent RXRα dephosphorylation (15, 23, 24). In the present study, we examined the effects of ACR on obesity-related liver tumorigenesis by focusing on the inhibition of RXRα phosphorylation. We also examined whether ACR treatment improves the insulin resistance, liver steatosis, and inflammatory condition caused by obesity with DEN-treated *db/db* mice, a useful preclinical model, to evaluate the mechanisms underlying the inhibition of obesity-related liver tumorigenesis by chemopreventive drugs (4).

Materials and Methods

Animals and chemicals

Four-week-old male *db/db* mice were obtained from Japan SLC, Inc. All mice received humane care and were housed at Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. DEN was purchased from Sigma Chemical Co. ACR was supplied by Kowa Pharmaceutical Co.

Experimental procedure

The experimental protocol, which was approved by the Institutional Committee of Animal Experiments of Gifu University, was as described previously (4). At 5 weeks of age, 40 *db/db* mice were randomly divided into 5 groups. All the mice in groups 1 (n = 10), 2 (n = 10), and 3 (n = 10) were given tap water containing 40 ppm of DEN for the first 2 weeks, which is sufficient to develop liver neoplasms in *db/db* mice (4). After DEN treatment, the mice in groups 2 and 3 were fed the basal diet CRF-1 (Oriental Yeast Co.) containing 0.03% ACR (group 2) or 0.06% ACR (group 3), respectively, with free access to the feed till the end of experiment. Group 4 (n = 5) was fed the CRF-1 diet containing 0.06% ACR. The mice in groups 1 and 5 (n = 5) were fed the CRF-1 diet throughout the experiment. The rationale for the doses (0.03% and 0.06%) selection of ACR was based on previous studies, in which similar doses of ACR inhibited experimental liver carcinogenesis induced by chemical agents (25, 26). At 41 weeks of age (after 34 weeks of ACR treatment), all the mice were sacrificed by CO_2 asphyxiation to check for the development of HCC, liver cell adenoma, and foci of cellular alteration (FCA).

Histopathologic analysis

At sacrifice, the livers were immediately removed and macroscopically inspected for the presence of neoplasms. Maximum sagittal sections of each lobe (6 lobes) were used for histopathologic examination. For all experimental groups, 4-μm thick sections of formalin-fixed, paraffin-embedded livers were stained routinely with hematoxylin and eosin (H&E) for histopathologic examination. The presence of HCC, liver cell adenoma, and FCA was judged according to previously described criteria (27). The multiplicity of FCA was assessed on a per unit area (cm^2) basis.

Ras activation assay

Ras activity was determined using a Ras activation assay kit (Upstate Biotechnology) according to the manufacturer's instructions. Ras was precipitated in equivalent amounts of liver extract (50 μg) from DEN-treated mice (groups 1–3) by using Raf-1/Ras-binding domain-immobilized agarose, which was then subjected to Western blot analysis using anti-Ras antibody (24). The intensity of the blots was quantified using NIH imaging software Version 1.62.

Protein extraction and Western blot analysis

Total protein was extracted from the nontumor site of livers of DEN-treated mice, and equivalent amounts of proteins (30 μg per lane) were examined by Western blot analysis (4). Previously described primary antibodies for RXRα (ΔN-197 and D-20), ERK, phosphorylated ERK (p-ERK), Stat3, p-Stat3, AMP-activated kinase (AMPK), p-AMPK, and GAPDH were used (15, 22, 28, 29). The ΔN-197 antibody is considered a specific antibody for the p-RXRα protein (22, 23). The GAPDH antibody served as a loading control.

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RNA extraction and quantitative real-time reverse transcription PCR

Total RNA was isolated from the nontumor site livers of DEN-treated mice by using the RNAqueous-4PCR kit (Ambion Applied Biosystems). cDNA was amplified from 0.2 μg of total RNA by using the SuperScript III First-Strand Synthesis System (Invitrogen), and quantitative real-time reverse transcription PCR (RT-PCR) analysis was carried out as described previously (4). The specific primers used for amplification of the *TNF-α*, *IL-6*, *IL-1β*, and *β-actin* genes were as described previously (30). The primers for the amplification of *RARβ*, *p21^{CIP1}*, *cyclin D1*, *c-Jun*, and *c-Fos* genes are listed in Supplementary Table S1.

Clinical chemistry

Before sacrifice, the mice were fasted for 6 hours, and at sacrifice, blood samples were collected for assaying the serum concentrations of insulin, glucose, and TNF-α, which was as described previously (4, 29). The serum TNF-α (Shibayagi) levels were determined using an enzyme immunoassay according to the manufacturer's protocol. Insulin resistance was estimated by determining the quantitative insulin sensitivity check index (QUICKI) as follows: QUICKI = $1/[\log(I_0) + \log(G_0)]$, where I_0 is the fasting insulin level and G_0 is the fasting glucose level, which correlates with the glucose clamp method (31).

Hepatic lipid analysis

Approximately 200 mg of frozen liver was homogenized, and lipids were extracted using Folch's method (32). The levels of triglyceride in the liver were measured using the triglyceride E-test kit (Wako Pure Chemical Co.) according to the manufacturer's protocol. To visualize the intrahepatic lipids, Sudan III staining was conducted using the standard procedure with frozen sections.

Statistical analysis

The results are presented as the mean ± SD and were analyzed using the GraphPad Instat software program Version 3.05 (GraphPad Software) for Macintosh. Differences among the groups were analyzed by either 1-way ANOVA or, as required, by 2-way ANOVA. When the ANOVA showed a statistically significant effect ($P < 0.05$), each experimental group was compared with the control group by using the Tukey–Kramer multiple comparisons test. The differences were considered significant when the 2-sided P value was less than 0.05.

Results

General observations

As shown in Table 1, no significant differences were observed in the body, kidney, and fat weights among the groups at the end of the study. A significant decrease in the liver weight was observed in the ACR-treated groups as compared with the basal diet-fed group ($P < 0.05$ or $P < 0.01$), irrespective of DEN treatment. Histopathologic examination showed the absence of ACR toxicity in the liver, kidney, and spleen (data not shown).

Effects of ACR on DEN-induced liver tumorigenesis in *db/db* mice

Table 2 summarizes the incidence and multiplicity of liver neoplasms (adenoma and HCC) and FCA in the mice from all groups. FCA developed in the livers of mice from all groups, irrespective of DEN treatment. On the other hand, liver cell adenomas developed only in the DEN-treated *db/db* mice. HCCs also developed in all DEN-treated groups; however, the incidence (10% in each group) wan not high. These findings might be associated with experimental protocol because the duration of the experiments (41 weeks) was sufficient to develop adenoma but not HCC. In mice treated with either dose (0.03% and 0.06%) of ACR, the incidence ($P < 0.01$ in each comparison) and multiplicity of adenoma ($P < 0.05$ or $P < 0.01$) were significantly inhibited compared to ACR-untreated mice. The number of FCA was also significantly decreased by ACR treatment, irrespective of DEN treatment ($P < 0.001$ or $P < 0.05$).

Effects of ACR on Ras activity and phosphorylation of RXRα, ERK, and Stat3 proteins in the livers of DEN-treated *db/db* mice

ACR prevents the growth of HCC cells by inactivating Ras-ERK and dephosphorylating RXRα, thereby restoring RXRα function (10, 15, 23, 24). Stat3 is also an ACR target for the inhibition of cancer cell growth (28). Therefore, the effects of ACR on the inhibition of Ras activity and phosphorylation of the RXRα, ERK, and Stat3 proteins were examined in this study by using an obesity-related liver tumorigenesis model. As shown in Figure 1A, the activity of Raf-1–bound Ras in the liver was significantly inhibited by treatment with either dose of ACR ($P < 0.01$). The expression levels of the p-ERK and p-RXRα proteins were also decreased by ACR treatment (Fig. 1B), indicating that ACR inhibits the development of obesity-related liver neoplasms, at least in part, by dephosphorylating RXRα and thereby restoring its function. At both doses, ACR also decreased the expression levels of the p-Stat3 protein in the livers of DEN-treated *db/db* mice (Fig. 1B).

Effects of ACR on the expression levels of RARβ, p21^{CIP1}, cyclin D1, c-Fos, and c-Jun mRNA in the livers of DEN-treated *db/db* mice

ACR inhibits the growth of HCC cells by increasing the cellular levels of RARβ and p21^{CIP1} but decreasing the levels of cyclin D1, and these effects might be associated with the restoration of RXRα function (12–15). It also suppresses the growth of cancer cells by inhibiting the activity of AP-1, which comprises the Jun and Fos oncoprotein families (28). Therefore, the effect of ACR on the mRNA levels of these molecules was examined next. As shown in Figure 1C, quantitative real-time RT-PCR analysis indicated that ACR treatment

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Table 1. Body, liver, kidney, and fat weights of the experimental mice

Group no.	Treatment	No. of mice	Weight, g			
			Body	Liver	Kidney	Fat[a]
1	DEN alone	10	71.2 ± 8.8[b]	4.5 ± 0.8	0.9 ± 1.0	7.5 ± 2.2
2	DEN + 0.03% ACR	10	65.7 ± 7.2	3.3 ± 1.1[c]	0.5 ± 0.1	6.0 ± 1.5
3	DEN + 0.06% ACR	10	66.0 ± 7.4	3.0 ± 0.7[d]	0.5 ± 0.1	5.7 ± 1.3
4	0.06% ACR alone	5	66.0 ± 7.4	3.0 ± 0.7[e]	0.5 ± 0.1	5.7 ± 1.3
5	Basal diet	5	67.9 ± 7.8	4.8 ± 1.0	0.6 ± 0.1	6.2 ± 1.4

[a]White adipose tissue of the periorchis and retroperitoneum.
[b]Mean ± SD.
[c]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[d]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.01$).
[e]Significantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.05$).

significantly increased the expression levels of *RARβ* and $p21^{CIP1}$ mRNA, especially *RARβ* mRNA, in the livers of DEN-exposed *db/db* mice ($P < 0.01$). On the other hand, the expression levels of *cyclin D1*, *c-Fos*, and *c-Jun* mRNA were significantly decreased by ACR treatment ($P < 0.01$).

Effects of ACR on hepatic steatosis and the activation of AMPK in the livers of DEN-treated *db/db* mice

Hepatic steatosis is considered a promoter of the development of HCC (8, 9). Therefore, whether ACR treatment enhances the accumulation of lipids in the liver of experimental mice was examined. Examination of Sudan III–stained sections revealed that ACR treatment significantly improved macrovesicular steatosis in the livers of DEN-treated *db/db* mice (Fig. 2A, top panels). The triglyceride levels in the liver were also significantly decreased in mice treated with ACR at either dose ($P < 0.05$) in comparison with those fed the basal diet (Fig. 2A, bottom graph). Moreover, ACR markedly phosphorylated (activated) the AMPK protein, which is a critical serine/threonine kinase that monitors cellular energy status (33), in the livers of the experimental mice (Fig. 2B).

Effects of ACR on insulin resistance in DEN-treated *db/db* mice

Insulin resistance plays a critical role in the development of HCC (1–6). Therefore, the effects of ACR on the levels of serum insulin and QUICKI values, which indicate the degree of insulin sensitivity, were examined in DEN-treated *db/db* mice. As shown in Figure 2C, the serum insulin level was decreased ($P < 0.05$) whereas the QUICKI value was increased in mice treated with 0.06% ACR ($P < 0.05$)

Table 2. Incidence and multiplicity of hepatic neoplasms and FCA in the experimental mice

Group no.	Treatment	No. of mice	Incidence		Multiplicity[a]		FCA
			Adenoma	HCC	Adenoma	HCC	(No./cm^2)
1	DEN alone	10	7/10 (70%)	1/10 (10%)	1.3 ± 1.2[b]	0.1 ± 0.3	15.1 ± 3.5[c]
2	DEN + 0.03% ACR	10	1/10 (10%)[e]	1/10 (10%)	0.2 ± 0.6[e]	0.1 ± 0.3	6.6 ± 2.5[f]
3	DEN + 0.06% ACR	10	1/10 (10%)[e]	1/10 (10%)	0.1 ± 0.3[g]	0.1 ± 0.3	2.8 ± 1.8[f]
4	0.06% ACR alone	5	0/5 (0%)	0/5 (0%)	0	0	3.0 ± 2.8[h]
5	Basal diet	5	0/5 (0%)	0/5 (0%)	0	0	8.0 ± 1.2

[a]Number of neoplasms per mouse.
[b]Mean ± SD.
[c]Significantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.001$).
[d]Significantly different from group 1 by Fisher's exact probability test ($P < 0.01$).
[e]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.05$).
[f]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.001$).
[g]Significantly different from group 1 by Tukey–Kramer multiple comparison test ($P < 0.01$).
[h]Significantly different from group 5 by Tukey–Kramer multiple comparison test ($P < 0.05$).

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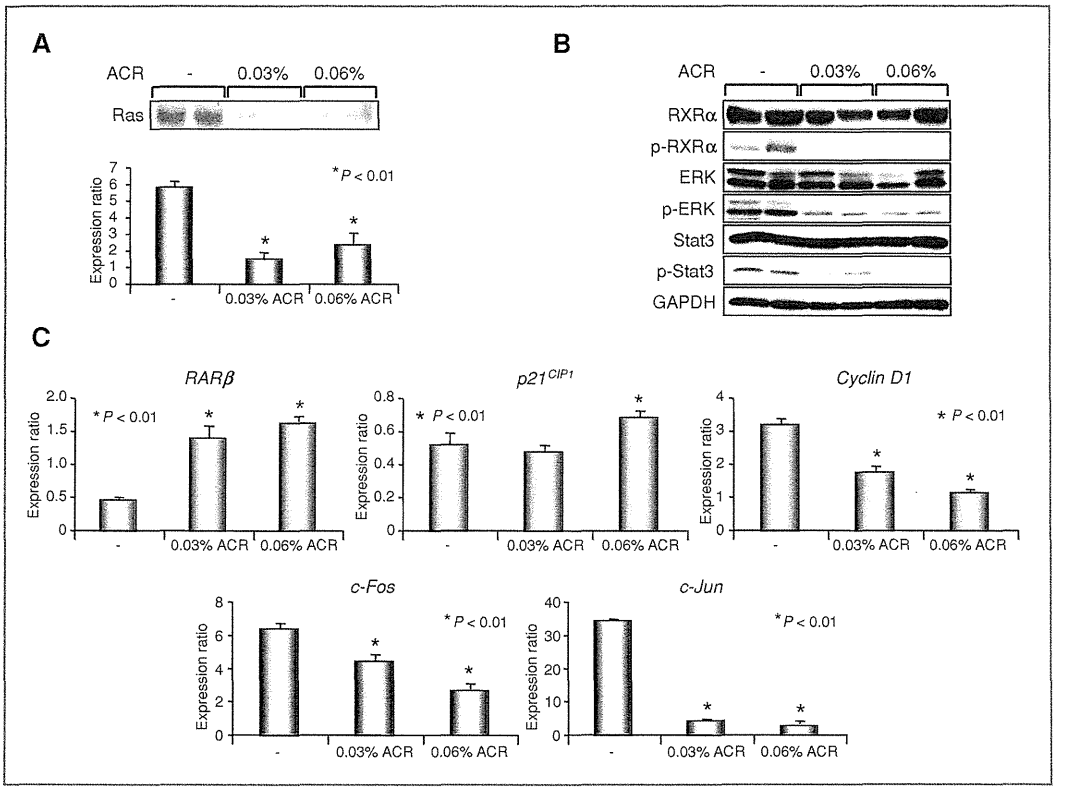

Figure 1. Effects of ACR on Ras activity; phosphorylation of RXRα, ERK, and Stat3 proteins; and the expression of target genes in the livers of DEN-treated *db/db* mice. The total proteins and mRNAs were extracted from the livers of DEN-treated mice. A, the Ras activities were determined using a Ras activation assay kit (top). The relative intensity of the blots was quantified by densitometry and is displayed in the bottom graph. B, the expression levels of the RXRα, p-RXRα, ERK, p-ERK, Stat3, and p-Stat3 proteins were examined by Western blot analysis, using the respective antibodies. Equal protein loading was verified by the detection of GAPDH. Two lanes represent protein samples from two different mice from each group. Repeat Western blots yielded similar results. C, the expression levels of *RARβ*, *p21^{CIP1}*, *cyclin D1*, *c-Fos*, and *c-Jun* mRNA were examined by quantitative real-time RT-PCR using specific primers. β-Actin was used as a control. Each experiment was performed in triplicate, and the average value was calculated. Values are the mean ± SD. *, $P < 0.01$ vs. ACR-untreated group.

compared with those in the basal diet–fed group. These findings suggest that ACR improves insulin resistance in obese and diabetic *db/db* mice.

Effects of ACR on the serum levels of TNF-α and hepatic expression of TNF-α, IL-6, and IL-1β mRNA in DEN-treated *db/db* mice

Because a state of chronic inflammation induced by excessive production of storage lipids and insulin resistance is associated with obesity-related liver carcinogenesis (34), the effects of ACR on the levels of the proinflammatory cytokines TNF-α, IL-6, and IL-1β in DEN-treated *db/db* mice were examined. As shown in Figure 3A, the serum levels of TNF-α were decreased after ACR treatment ($P < 0.01$). Furthermore, the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice were also significantly decreased by ACR treatment ($P < 0.01$). The decrease was most apparent in the levels of *IL-6* mRNA: the inhibition rates were about 85% at both doses of ACR (Fig. 3B).

Discussion

In the present health care scenario, the effects of obesity, including the promotion of cancer, are critical issues that need to be resolved and HCC is one of the representative malignancies influenced by excessive body weight and related metabolic abnormalities (1–3, 5, 6). A recent clinical trial revealed that supplementation of food with branched-chain amino acids (BCAA), which improves insulin resistance (35), reduced the risk of HCC in obese patients with chronic viral liver disease (3). BCAA supplementation also suppresses liver tumorigenesis in obese and diabetic *db/db* mice by improving insulin resistance and attenuating liver steatosis and fibrosis (4). The results of the present study clearly indicated that ACR also effectively

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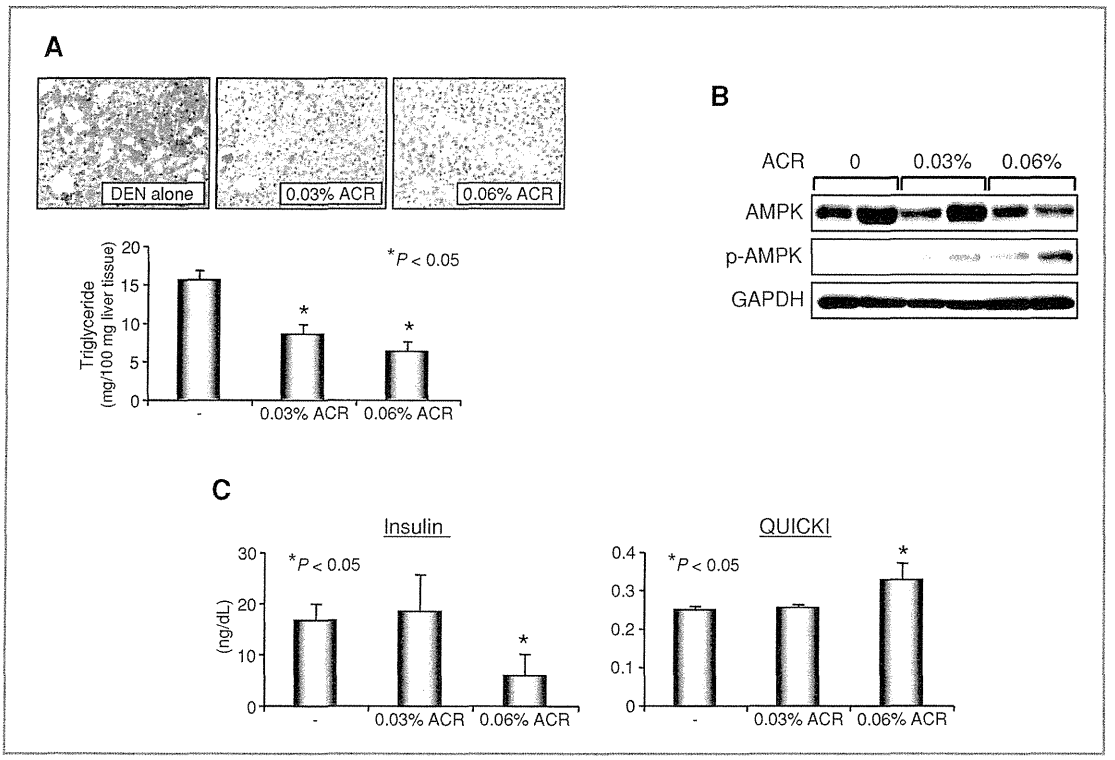

Figure 2. Effects of ACR on hepatic steatosis, the activation of the AMPK protein in the liver, and the levels of serum insulin and insulin sensitivity in DEN-treated *db/db* mice. A, frozen liver sections from DEN-exposed mice treated with or without ACR were stained with Sudan III to show steatosis (top). Hepatic lipids were extracted from the frozen livers of these mice, and the triglyceride levels were measured (bottom). B, the total proteins were extracted from the livers of DEN-treated mice, and the expression levels of the AMPK and p-AMPK proteins were examined by Western blot analysis, using the respective antibodies. A GAPDH antibody served as a loading control. C, the serum concentration of insulin was measured by enzyme immunoassay (left). The QUICKI value was calculated to evaluate insulin sensitivity (right). Values are the mean ± SD. *, $P < 0.05$ vs. ACR-untreated group.

prevents the development of obesity-related liver cell adenomas, and these effects are associated with improvement of hepatic steatosis and insulin resistance. Therefore, the findings of the present study, together with the results of previous studies using BCAA (3, 4), suggest that improvement of metabolic abnormalities by pharmaceutical or nutritional intervention might be an effective strategy for inhibiting obesity-related liver tumorigenesis.

Several biological effects of ACR are relevant to the prevention of obesity-related hepatotumorigenesis. First, it should be noted that ACR inhibits RXRα phosphorylation by suppressing the Ras/ERK signaling pathway in the livers of DEN-treated *db/db* mice. These findings are consistent with those of previous *in vitro* studies (15, 23, 24), but this is the first *in vivo* experiment, and the results seem to be significant because RXRα malfunction due to the phosphorylation by Ras-ERK plays a role in liver carcinogenesis and phosphorylated RXRα is therefore a critical target for HCC chemoprevention (10, 21). ACR suppresses the growth of HCC cells by inhibiting RXRα phosphorylation and restoring its original function as a master regulator of nuclear receptors (15, 22–24). Therefore, the expression levels of the *RARβ*, *$p21^{CIP1}$*, *cyclin D1*, *c-Fos*, and *c-Jun* genes, which are ACR targets (12–15, 28), were notably regulated by treatment with this agent. Among these molecules, RARβ seems to be the most important with respect to the induction of apoptosis (36). The upregulation of $p21^{CIP1}$, which negatively modulates cell-cycle progression, also activates the promoter region of the *RARβ* gene (37). Because RARβ can form a heterodimer with RXRα and thus synergistically inhibit the growth of HCC cells (14, 15), its induction might also have played a role in preventing the development of liver tumors in the present study. In addition, $p21^{CIP1}$ induction, which might be caused by activation of transforming growth factor (TGF)-β, also contributes to prevent the development of liver neoplasms because TGF-β induces senescence and inhibits growth in HCC cells by upregulating $p21^{CIP1}$ and ACR can activate latent TGF-β in liver stellate cells (38, 39).

Next, the effects of ACR in improving hepatic steatosis and insulin resistance, both of which accelerate HCC development (7–9), are discussed. These effects might also

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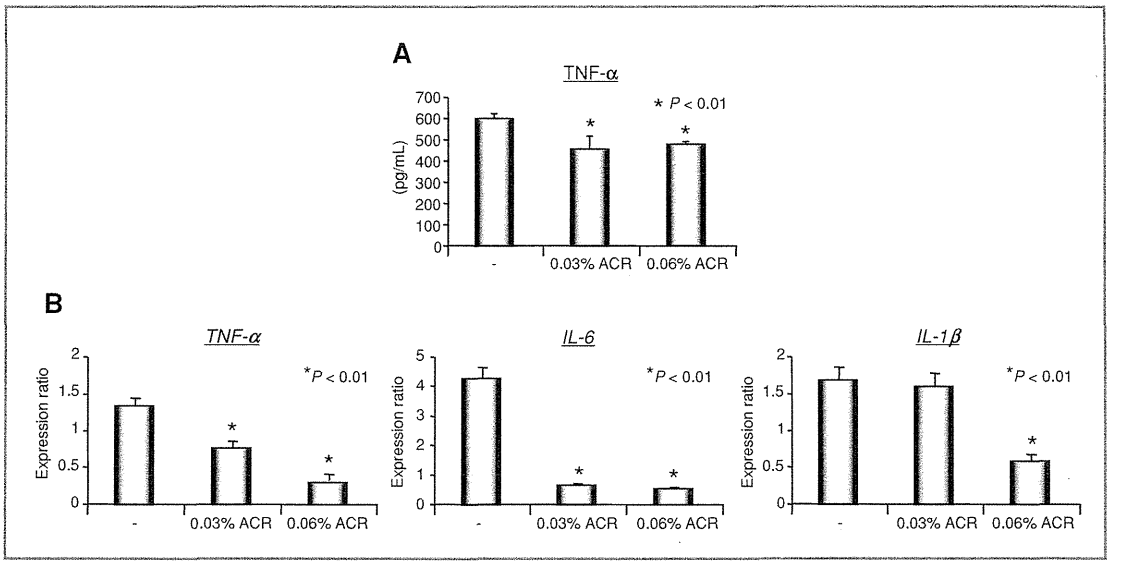

Figure 3. Effects of ACR on the serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice. A, the serum concentration of TNF-α was measured by enzyme immunoassay. B, the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA were examined by quantitative real-time RT-PCR using specific primers. The expression levels of these mRNAs were normalized to the level of the β-*actin* mRNA. Values are the mean ± SD. *, $P < 0.01$ vs. ACR-untreated group.

be associated with RXRα dephosphorylation, as RXR can control insulin sensitization and lipid metabolism by forming a heterodimer with peroxisome proliferator-activated receptor (PPAR), an important molecule in the regulation of lipid homeostasis and energy metabolism (40). This speculation is interesting because the inhibition of RXRα phosphorylation and the activation of the RXR/PPAR heterodimer are also activities that cooperatively inhibit the growth of cancer cells (41). In addition, ACR might improve these metabolic abnormalities by activating AMPK, which increases glucose uptake and fatty acid oxidation but decreases fatty acid synthesis (33). This is another positive finding with regard to the prevention of hepatotumorigenesis because decreased AMPK activation is implicated in tumor development and therefore may be a promising target for cancer chemoprevention (42, 43). For instance, a human study suggests that metformin, an AMPK activator used to treat type 2 diabetes mellitus, reduces the cancer risk in diabetic patients (44). Dietary energy restriction suppresses mammary tumorigenesis in rats by increasing the levels of activated AMPK (45). Pitavastatin, a lipophilic statin, was found to prevent obesity- and diabetes-related colon carcinogenesis in mice by activating AMPK in the colonic mucosa (29). These reports suggest the possibility that activation of AMPK by ACR aided in suppressing the development of obesity-related liver cells adenomas, as observed in the present study.

Insulin resistance and lipid accumulation in the liver produce inflammatory changes in the liver (7–9). ACR might decrease the serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of experimental mice by improving hepatic steatosis and insulin resistance. These findings are significant because obesity-related HCC development clearly depends on enhanced production of TNF-α and IL-6, which cause hepatic inflammation and activate ERK and Stat3 (34). TNF-α, which lies at the core of the association between obesity and insulin resistance (46), contributes to obesity-induced IL-6 production and hepatocarcinogenesis (34). IL-6 is a major Stat3 activator in the liver, and the activation of the IL-6–Stat3 axis plays a critical role in HCC development (47, 48). In addition, uncontrolled activation of the Ras/ERK and Jak/Stat pathways is essential for HCC development (49). In the present study, ubiquitous activation of Ras-ERK signaling presumably caused accumulation of the p-RXRα protein in the liver of the obese mice. Our findings indicate that the effects of ACR in improving the inflammatory response and inhibiting Ras-ERK and Stat3 activation are crucial to prevent the development of obesity-related liver tumors.

Finally, it should be emphasized again that prevention of HCC by targeting hepatic steatosis, insulin resistance, and the state of chronic inflammation, which are caused by dysregulation of energy homeostasis, might be one of the promising strategies for the treatment of obese individuals who are at an increased risk of developing HCC (3, 4). ACR seems to be potentially effective and critical candidate for this purpose because it can improve hepatic steatosis and insulin resistance while also attenuating chronic inflammation. It inhibits RXRα phosphorylation induced by

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Ras-ERK activation, which might be associated with excess adipose tissue, and this effect is also important for preventing obesity-related liver tumorigenesis. The findings of the present study, together with the results of previous clinical trials indicating that ACR can significantly prevent the development of HCC in patients with viral cirrhosis without causing serious adverse effects (17–19), encourage the clinical usage of this agent for cirrhotic patients with obesity and diabetes. On the other hand, careful observation is required to apply a retinoid in clinical practice because of its potential toxicity. For instance, ACR may worsen hypertriglyceridemia in obese and diabetic subjects, which is a side effect observed in previous clinical trial (17), limiting the application of ACR to such subjects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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