95% CI = 0.55–0.87). Side-effects included hand–foot skin reaction, diarrhea and fatigue, but sorafenib was not found to be toxic to the liver.[109] Similar findings were reported in a subsequent Asia–Pacific RCT.[110]

Based on the results of these RCT, sorafenib has become the first-line therapy for advanced HCC worldwide. Some Japanese experts for HCC are claiming low response rates, although the survival was significantly prolonged compared with placebo. This phenomenon could be explained by a longer period with stable disease with sorafenib than with placebo, or the necrotic change in the tumor is present without size reduction.

In Japan, sorafenib was approved for the treatment of HCC on 20 May 2009. In the consensus meeting held in June, 35% of the Japanese experts agreed that sorafenib should be selected as the first-line therapy for advanced HCC considered unsuitable for resection, RFA or TACE. A further 36% of the experts were undecided because they did not have enough experience with using sorafenib.

Informative Statement 4. Sorafenib is the first-line therapy for advanced HCC with major vascular invasion and/or extrahepatic spread and good liver function. However, further studies are needed to compare the overall efficacy of HAIC and sorafenib.

TREATMENT ALGORITHM

TO TREAT HCC, the most appropriate therapeutic option needs to be selected among the available treatment modalities, including resection, percutaneous ablation, TACE and transplantation, but few evidence-based guidelines have been developed to aid decision-making.[1,28,29,88,89,111] Recently, two treatment algorithms for HCC have been proposed in the Japanese guidelines. The profile of these algorithms is briefly described here, in addition to the results of two questions and answers at the JSH Consensus Meeting for HCC at Kobe.

Evidence-based treatment algorithm

The Clinical Practice Guidelines for HCC was established in 2005 based on evidence-based methodology, and covers six topics including prevention, diagnosis, surgery, chemotherapy, TACE and percutaneous ablation. To develop these guidelines, a systematic review of the English medical published work was performed and a total of 7118 articles on HCC were identified, mainly from MEDLINE (1966–2002), of which 334 were selected based on the evidence level to form 58 pairs of clinical questions and recommendations.[1,88] For convenience in clinical use, two algorithms were created for the surveillance and treatment of HCC. A full English version was uploaded to the website of the JSH (www.jsh.or.jp/) in 2006.

The treatment algorithm for HCC was made on the basis of three independent factors: degree of liver damage, tumor number and tumor size. For the resulting six patients' subgroups, the first- and second-line therapies were recommended as objectively as possible (Fig. 1). The degree of liver damage is a modified system based on the Child–Pugh classification: "encephalopathy" was replaced by $ICGR_{15}$, to provide an accurate evaluation of liver functional reserve, particularly in surgical candidates.

Patients with mild (class A) or moderate (class B) liver damage are subject to the following recommendations: (i) in patients with a single tumor, liver resection is recommended, irrespective of the tumor size (percutaneous ablation may be performed if liver damage is of class B and the tumor is no more than 2 cm in size); (ii) for patients with two or three tumors smaller than 3 cm, resection or ablation are recommended; (iii) for patients with two or three tumors larger than 3 cm, resection or TACE are recommended; and (iv) for patients with more than four tumors, TACE or HAIC is recommended. The recommendations for patients with severe (class C) liver damage are as follows: (v) in patients with tumor(s) meeting the Milan criteria, liver transplantation is recommended; and (vi) for patients with more than four tumors, palliative treatment is recommended. For patients with extrahepatic metastasis, chemotherapy may be performed.

The rationale for selecting resection or ablation in patients with class A or B liver damage is based on the outcome of the largest multicenter study involving 12 888 patients in Japan.[59] The recommendation for TACE is based on the findings of two RCT showing a significant improvement in the survival of patients with multiple tumors and class A or B liver damage.[84,85] The indication for liver transplantation is derived from a prospective cohort study using the Milan criteria,[71] and a nationwide survey of Japan justifying the criteria in living donor transplantation.[74]

Consensus-based treatment algorithm

An expert panel of the JSH established a consensus-based treatment algorithm based on the therapeutic policies that are widely used in Japan.[89,111] This algorithm categories the patients on five clinical variables (extrahepatic spread, liver function, vascular invasion, tumor number and tumor size), and it divides the treatment options into resection, ablation, TACE, HAIC, liver

transplantation and palliative treatment (Fig. 3).[89,111] Because of the recent introduction of sorafenib in Japan, this consensus-based treatment algorithm was further revised and approved by the experts at the consensus meeting.[111,112]

Essentially, the consensus-based algorithm follows the evidence-based algorithm, but the treatments widely used in Japan were included by consensus, even though the evidence may be weak. The major differences in the consensus-based algorithm include: (i) ablation is sometimes performed in patients with a single, hypovascular early HCC; (ii) sorafenib is recommended for use in Child–Pugh A patients with vascular invasion, TACE failure or extrahepatic spread of HCC;[109,112] and (iii) liver transplantation is recommended, even for Child–Pugh A/B patients, if the Milan criteria are met.

The consensus-based algorithm based on the consensus of a large number of specialists, and a treatment strategy for management of HCC in Japan is important, and should be revised based on prospective trials for aspects of the algorithm lacking sufficient evidence.[111,112]

Informative statement 5. RFA might be recommended as a first-line treatment option in patients with a single, hypervascular HCC of less than 2 cm in size and with preserved liver function (Child–Pugh A or Liver Damage Class A). However, there was a discrepancy between surgeons and non-surgeons for this statement. This statement is strongly supported by non-surgeons (68%), whereas 80% of the surgeons favor resection rather than RFA. Recommendation 17. Resection should be considered as the first-line treatment option for patients with a single, hypervascular HCC of 3 cm or more in size and with preserved liver function (Child–Pugh A or Liver Damage Class A).

The revised version of the consensus-based treatment algorithm for HCC proposed by the JSH (Fig. 3) should aid decision-making at every stage in clinical practice. By sharing the information contained within the treatment algorithm chart, the physicians can offer recommended treatment options to the patient who can then choose one based on their preference (Fig. 3).

CONCLUSIONS

THIS CONSENSUS STATEMENT is a conclusion of the consensus meeting of HCC, which was held at the 45th JSH meeting, Kobe, Japan on 4–5 June 2009 (Congress President: Professor Masatoshi Kudo). This manuscript and recommendations largely reflect the daily practice in the real world carried out throughout Japan. The biggest difference of Japan's HCC practice from Western countries are pathological assessment issue, prognostic staging system, surveillance and diagnostic strategy, treatment strategy including role of HAIC, and method of RFA procedure, and treatment algorithm shown in Figure 3.

We believe every reader of this manuscript will well understand the real Japanese HCC practice much better than the other already published arterial articles. It is needless to say that consensus statements like this article should be regularly revised every 3–4 years because solid evidence or new diagnostic and treatment tool/drug or concept will be published and then established in clinical practice every year.

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Special Report

Response Evaluation Criteria in Cancer of the Liver (RECICL) proposed by the Liver Cancer Study Group of Japan (2009 Revised Version)

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The World Health Organization (WHO) criteria and Response Evaluation Criteria in Solid Tumors (RECIST) are inappropriate to assess the direct effects of treatment on the hepatocellular carcinoma (HCC) by locoreginal therapies such as radiofrequency ablation (RFA) and transcatheter arterial chemoembolization (TACE). Therefore, establishment of response evaluation criteria solely devoted for HCC is needed urgently in the clinical practice as well as in the clinical trials of HCC treatment, such as molecular targeted therapies, which cause necrosis of the tumor. Response Evaluation Criteria in Cancer of the Liver (RECICL) was revised in 2009 by Liver Cancer Study Group of Japan based on the 2004 version of RECICL, which was commonly used in Japan. Major revised points of the RECICL 2009 is to provide TE4a (Complete response with enough ablative margin) and TE4b (complete response without enough ablative margin) for local ablation therapy. Second revised point is that setting the timing at which the overall treatment effects are assessed. Third point is that emergence of new lesion in the liver is regarded as progressive disease, different from 2004 version. Finally, 3 tumor markers including alpha-fetoprotein (AFP) and AFP-L3 and des-gamma-carboxy protein (DCP) were also added for the overall treatment response. We hope this new treatment response criteria, RECICL, proposed by Liver Cancer Study Group of Japan will benefit the HCC treatment response evaluation in the setting of the daily clinical practice and clinical trials as well not only in Japan, but also internationally.

Key words: Response Evaluation Criteria, hepatocellular carcinoma, WHO criteria, RECIST, Liver Cancer, Liver Cancer Study Group of Japan

INTRODUCTION

THE WORLD HEALTH Organization (WHO) criteria[1] and Response Evaluation Criteria in Solid Tumors (RECIST),[2] which are response evaluation criteria for solid tumors after chemotherapy, are commonly used for the evaluation of liver cancer treatment in Western countries. However, it is well known and obvious that both the WHO criteria and RECIST are inappropriate to assess the direct effects of treatment on the liver cancer lesions by ablative treatment and transcatheter arterial chemoembolization (TACE). Although effective treatments may exhibit a necrotizing effect on hepatocellular carcinoma (HCC) with deprivation of its blood flow, the WHO criteria and RECIST do not consider such necrotizing effects to be "effective"; instead, both criteria use only tumor size reduction as measures of effect. It has been shown that the tumor size reduction rate according to the WHO criteria and RECIST following TACE with lipiodol (Lip-TACE) is not correlated with the pathological necrosis rate.[3] When lipiodol is accumulated densely within the tumor, the early arterial staining is masked, and tumor size is not increased, the tumor is completely necrotized as confirmed by histology.[3] Even though the tumor is completely necrotized, it takes a long time to result in reduction of size. The nodule with complete necrosis after Lip-TACE can be seen for several years as a lipiodol more densely

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accumulated nodules than 2 weeks after the intervention. In case of radiofrequency ablation (RFA), the phenomenon is the same with Lip-TACE, though lipiodol accumulation is not seen.

Moreover, the WHO criteria are originally based on bi-dimensional measurement, which was changed to a uni-dimensional measurement in RECIST. Even if tumor necrosis is considered in the response evaluation criteria, uni-dimensional measurement is inappropriate for assessment of the direct treatment effect. Therefore, establishment of response evaluation criteria solely devoted for HCC is needed urgently in the clinical practice as well as in the clinical trials of HCC. The current report describes the newly established response evaluation criteria for HCC by revising the previously existing criteria established by the Liver Cancer Study Group of Japan.

CONCEPT OF THE RESPONSE EVALUATION CRITERIA IN CANCER OF THE LIVER (RECICL)

THE FIRST EDITION of Criteria for the Evaluation of Direct Treatment Effects in Hepatocellular Carcinoma was published in 1994.[4] The revised edition was published in 2004,[5] and is commonly used in Japan, but several problems remained in the revised criteria. Thus, a third revision was carried out before publishing the English edition of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer edited by the Liver Cancer Study Group of Japan (third edition).

Current response evaluation criteria focuses on the following points: (i) development of simple criteria that are sufficiently applicable in routine clinical practice centering on local treatment (ethanol injection therapy, microwave coagulation therapy, RFA) and transcatheter arterial therapy, radiotherapy and systemic chemotherapy can also be included; (ii) assessment of direct treatment effects on intrahepatic target lesions and overall effects are described separately; and (iii) the criteria follow the fifth edition of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer edited by the Liver Cancer Study Group of Japan.[6]

Considering the biological characteristics of HCC, high frequencies of "intrahepatic metastatic recurrence" and "multicentric carcinogenesis", it may not necessarily be appropriate for liver cancer to be indiscriminately diagnosed as "progressive disease" based on the appearance of "a new lesion" alone because such "a new lesion" has not been treated by ablation or TACE when the recurrent nodule exists outside of the treated area. Thus, evaluation of the direct effects of treatment on target lesions should focus on the direct therapeutic effect on the target lesions, and the overall evaluation should be investigated with close association with the prognosis.

Although the chemotherapeutic agent permeates through the liver in chemotherapy, the therapeutic effect of TACE and ablative treatments is limited only to the target lesion or the area fed by embolized artery with the tumor. Treatment is not done for the new lesions appearing outside the area where the ablation or TACE are performed. After the same treatment is carried out on the targeted new lesion, a similar treatment effect may be expected on the formerly treated lesion. Accordingly, when "a new lesion" appears in a region outside the treatment area, the new lesion (intrahepatic metastasis or multicentric carcinogenesis) may not directly indicate the prognosis. The basic concept of the 2004 version of the Japanese response evaluation criteria[5] was to exclude the new lesions from the evaluation of treatment effect on the formerly treated lesions. In other words, the emergence of a new lesion is regarded as out of the evaluation of the treatment effect for the former lesions, which is the most marked difference from the WHO criteria or RECIST.

Therefore, these criteria established by the Liver Cancer Study Group of Japan are exclusively specified for the Evaluation of Therapeutic Effects on Liver Cancer, and differ from other evaluation criteria for solid tumor regarding the various points described above.

The 2004 version of the Criteria for the Evaluation of Direct Treatment Effects in Hepatocellular Carcinoma are superior to the WHO criteria or RECIST because it considers the biological characteristics of HCC as follows: (i) tumor necrosis is regarded as a direct effect of treatment on the target lesion as well as tumor size reduction even though it is minimal; (ii) tumors are measured in two dimensions; (iii) the dense accumulation of lipiodol is regarded as necrosis;[3] and (iv) the emergence of a new lesion is not regarded as a "progressive disease" in evaluation of the treated nodule.

However, several problems remained in the 2004 version: (i) assessment of direct treatment effects was performed at 3 months, while the overall evaluation was performed at 6 months; and (ii) even though the direct effects on target nodules varies among treatment methods, the timing of assessment was not described. To overcome these limitations, some minor changes were made in this 2009 revised version. These criteria may be suitable mainly for local treatment and transcatheter arterial therapy, but are also applicable for radiotherapy and chemotherapy in combination with

the WHO criteria and RECIST. Whether or not some criteria are superior to others will be investigated in future studies. We expect that the 2009 revised edition of Response Evaluation Criteria in Cancer of the Liver (RECICL), will be widely used in clinical practice as well as in the clinical trial settings, not only in our country but also worldwide, as the criteria are clearer and may be more suitable in response evaluation for liver cancer than WHO criteria or RECIST.

MAJOR REVISED POINTS OF THE RESPONSE EVALUATION CRITERIA IN THE 2009 VERSION

FIRST, WE HAVE clarified the direct effect of local treatments on target nodules. When the non-stained low-density area in local ablation therapy such as ethanol injection therapy, microwave coagulation therapy and RFA covers all parts of the low-density area in the late phase of dynamic computed tomography (CT) scan before treatment, the lesion is regarded as 100% necrotized and described as treatment effect 4 (TE4), even though the size of the nodule does not decrease in the follow-up CT scan or multiple resonance imaging (MRI). However, when the non-stained low-density area does not cover the low-density area before the treatment, the risk of local recurrence is high.[7–9] Therefore, for ethanol injection therapy, microwave coagulation therapy and RFA, when the non-stained low-density area is slightly wider across the entire circumference than the low-density area in the late phase of dynamic CT scan before treatment, the lesion is regarded as 100% necrotized (TE4a). When only hypervascularity has disappeared without a slightly wider non-stained region than the low-density area on dynamic CT scan, the condition is judged as TE4b (Table 1).

Second, we have settled the timing at which the overall treatment effects are assessed: (i) the maximum response within 3 months is regarded as the overall treatment effect; (ii) for transcatheter arterial therapy with lipiodol, it is desirable to assess the effect after at least 1 month; (iii) local ablative treatment can be assessed immediately after the treatment; and (iv) for radiotherapy, the maximum response within 6 months may be regarded as the overall effect.

Third, regarding the criteria for "progressive disease" in the overall evaluation, the emergence of a new lesion is regarded as "progressive disease", similar to that advocated in the WHO criteria or RECIST, as shown in the Appendix. However, new lesions are separately described in consideration of the biological characteristics of HCC and the description may contribute to a future review of the criteria, particularly for: (i) intrahepatic solitary lesions (whether it is in the treated area or outside of the treated area by ablation or TACE); (ii) intrahepatic multiple lesions; and (iii) vascular invasion/extrahepatic spread.

Fourth, the RECIST and WHO criteria may be appropriate for radiotherapy and systemic chemotherapy including molecular targeted agents because these are currently used internationally,[10–13] however, we recommend evaluation using the RECICL criteria in combination with the WHO criteria or RECIST in order to clarify which criteria among the three are the most appropriate in future studies. This point is described in the detailed regulation section.

Fifth, in the detailed regulation section, the lowest levels of three tumor markers (α-fetoprotein [AFP], AFP-L3 and Protein induced by vitamine K absence or antagonist [PIVKA-II] or des-gamma-carboxy prothrombin [DCP]) should be measured and described within 3 months and considered with reference to the overall evaluation. It may be useful to prospectively investigate whether there is a difference in the prognosis between complete response (CR) based on imaging alone and CR on imaging in combination with response of tumor markers.

Finally, we include a comparison between the WHO criteria, RECIST[14,15] and RECICL established by the Liver Cancer Study Group of Japan.

Table 1 Treatment effect (TE) on the target nodule

TE4:	The tumor-necrotizing effect is 100% or the tumor size reduction rate is 100%.*
TE4a:	Necrotized area with larger ablated area than original nodule.*
TE4b:	Necrotized area of same size with original nodule.
TE3:	The tumor-necrotizing effect or tumor size reduction rate is between 50% and <100%.*
TE2:	Effects other than TE3 and TE1.
TE1:	The tumor enlarged by >25% regardless of the necrotizing effect.

*For ethanol injection therapy, microwave coagulation therapy, and radiofrequency ablation, when the non-stained low-density area is slightly wider across the entire circumference than the low-density area in the late phase of dynamic computed tomography (CT) scan before treatment, the lesion is regarded as 100% necrotized (TE4a). When only hypervascularity has disappeared without a slightly wider non-stained region than the low-density area on dynamic CT scan, the condition is judged as TE4b. In transcatheter arterial chemoembolization (TACE), the tendency of reduction of tumor size, without tumor staining by CT scan with contrast enhancement, and denser uniform accumulation of lipiodol than just after lipiodol TACE when lipiodol is used, are classified to be TE4.

DESCRIPTION OF RECICL PROPOSED BY LIVER CANCER STUDY GROUP OF JAPAN

Subjects

THE SUBJECTS ARE patients who are treated initially and for recurrence. Because responses to treatment are evaluated, as a rule, by dynamic CT, intrahepatic lesions with hypervascular tumors are the principle targets of the RECICL criteria. It is essential that tumors can be clearly visualized using an imaging technique.

Detailed description

Description of past medical history

1 Methods and date when definitive diagnosis of liver cancer was made.
2 Previous treatment modality (as described in "c. Description of treatment modalities").
3 Dates of initiation and completion of previous treatment.
4 Methods and date when recurrence was diagnosed.

Descriptions of liver cancer at the time of the initiation of treatment

These issues are based on the second English Edition of the General Rules for the Clinical and Pathological Study of Primary Liver Cancer (edited by the Liver Cancer Study Group of Japan).[16] The following items should be noted:

1 Tumor location.
2 Tumor size, number, and vascular invasion. The tumor size is presented as the major axis and maximum diameter crossing the major axis at a right angle.
3 Macroscopic types.[16,17]
4 Macroscopic staging. Even for tumors that are only assessable by imaging, staging should be described following the rules for surgical findings and the resected specimen.[16,17]
5 Histological grading when biopsy is performed.[16,17]

Description of treatment modalities

1 Name of treatment: transcatheter hepatic arterial therapy (transcatheter arterial infusion chemotherapy, transcatheter arterial embolization, TACE), local treatment (ethanol injection therapy, microwave coagulation therapy, RFA), radiotherapy such as Liniac, γ-knife, or proton beam line, systemic chemotherapy.
2 Details of treatment: for treatments using drugs, the name of drugs* (anticancer drugs, Lipiodol, etc.), route of administration, treatment interval and single dose, and the total number of administrations and total dose should be described. For other treatment methods, the details should be described appropriately. When the treatment is discontinued, the reason for discontinuation and the presence or absence of adverse effects should be described. (*In addition to the chemotherapeutic drugs, any drugs directly injected into the tumor to necrotize it, such as ethanol, and/or embolizing materials, should be described.)
3 Dates of initiation and completion or termination of treatment.

Assessment of direct treatment effect on target nodule

1 On assessment of the direct effect of treatment on the target nodule, the tumor-necrotizing effect and tumor size reduction rate are calculated based on the size reduction or disappearance of hypervascularity of the nodule on dynamic CT. Findings of dynamic MRI, and/or contrast-enhanced ultrasonography can substitute dynamic CT.
2 The necrotizing effect is assessed by imaging. The percent ratio of the necrotized area to the cross-sectional area of the tumor should be calculated.* (*When various cross-sections are obtained for a single tumor, the total sum of the necrotic area should be used; however, when the maximum cross-section represents the entire findings of the tumor, assessment may be made based on the maximum cross-sectional area.)
3 The size reduction rate is calculated using the equation below, after calculating the product of the major axis of the maximum cross-section by the maximum diameter crossing the major axis at a right angle: size reduction rate = ([product before treatment] – [product after treatment]) / (product before treatment) × 100.
4 Direct treatment effect (TE) on target nodule: effects on individual lesions are categorized into four degrees based on the tumor-necrotizing effect observed within a fixed term* after the initiation of treatment or the maximum tumor size reduction rate, as shown in Table 1. (*For local treatments [such as ethanol injection therapy, microwave coagulation therapy, RFA], the effects are assessed immediately after treatment. For transcatheter arterial chemotherapy using lipiodol, transcatheter arterial embolization and transcatheter arterial chemoembolization, it is desirable to assess the effect after at least 1 month. For radiotherapy, the effect assessed based on the maximum response within 6 months.)
5 When multiple lesions are present in the liver, TE is determined in individual lesions.

Table 2 Overall response evaluation (effect of treatment on all intrahepatic lesions at 3 months; radiotherapy can be evaluated at 6 months)

Overall evaluation of treatment effect	Effect of treatment on the tumor
CR (complete response)	100% tumor-necrotizing effect or 100% tumor size reduction rate
PR (partial response)	The tumor-necrotizing effect or tumor size reduction rate is between 50% and <100%
SD (stable disease)	Effects other than PR and PD
PD (progressive disease)	The tumor growth >25% regardless of the necrotizing effect, or emergence of a new lesion*

*With regard to the emergence of new lesions, the lesion should be classified as either: (i) intrahepatic solitary lesion (within or outside the treatment area); (ii) intrahepatic multiple lesions (within or outside the treatment area); or (iii) vascular invasion (the portal vein, hepatic vein, bile duct)/extrahepatic spread.

OVERALL EVALUATION OF THE TREATMENT RESPONSE

1 The overall evaluation is determined, based on the effect in the entire liver and its persistence, and categorized as CR, partial response (PR), stable disease (SD) and progressive disease (PD), as defined in Table 2.
2 To use this method to predict the prognosis, TE is determined and recorded at 3 months when retreatment is not performed after the initiation of treatment, as an overall response evaluation, except for radiotherapy, in which the overall evaluation is performed at 6 months.
3 When multiple lesions are present, but the assessment of all of the lesions is difficult, evaluation of the five largest lesions may be considered to represent the overall evaluation of the entire liver, but it is not regarded as CR. In addition, CR should not be given when the findings of the maximum cross-section is regarded to represent the entire tumor. Tumors may only be described as CR when all of the intrahepatic lesions are assessable as well as the effect shown in Table 2 (100% tumor-necrotizing effect or 100% tumor size reduction rate) is obtained.

DETAILED REGULATIONS

THE NECROTIZING EFFECT is assessed based on the response evaluation criteria of treatment on target nodules.

1 The presence, on dynamic CT with an i.v. bolus injection, of a non-stained low-density area after treatment is regarded as a necrotizing effect. A non-stained low-density area represents an apparently lower level than that in the surrounding liver parenchyma in the early and late phases* of dynamic CT with an i.v. bolus injection. Usually, the CT attenuation value of a non-stained low-density area does not increase on dynamic imaging. (*The early phase represents the arterial dominant phase of dynamic CT. The late phase represents the equilibrium phase of dynamic CT.)
2 When lipiodol is used, the presence of a region retaining lipiodol homogeneously and densely in the tumor shown on CT 1 month after therapy is regarded as a necrotizing effect. Dynamic MRI, Doppler ultrasonography and contrast-enhanced ultrasonography can be also used.
3 The effects of radiotherapy, systemic chemotherapy (including treatment with molecular targeted agents) and hepatic arterial chemotherapy should be described by both RECIST and present criteria, RECICL.
4 The lowest levels of three tumor markers (AFP, AFP-L3 fraction, PIVKA-II or DCP) should be recorded as reference values for the overall response evaluation.

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APPENDIX I

TOVERALL EVALUATION OF treatment effects on liver cancer: a comparison between the World Health Organization (WHO) criteria, Response Evaluation Criteria in Solid Tumors (RECIST) and Response Evaluation Criteria in Cancer of the Liver (RECICL)

	WHO criteria (after 4 weeks)	RECIST (after 4 weeks)	RECICL (after 3 months)
Lesion evaluated	All evaluable lesions	All measurable lesions, target lesions (five lesions, a maximum of 10 lesions when lesions are present over 2 or more organs)	Target lesions (a maximum of five lesions when more than 5 lesions are present)
Evaluation method	Bi-dimensional measurement (changes in the product of the major axis and the diameter crossing the major axis at a right angle). Sum of the all lesions.	Uni-dimensional measurement (changes in the sum of the major axis)	Bi-dimensional measurement (changes in the product of the major axis and the diameter crossing the major axis at a right angle, non-stained regions on dynamic CT and/or lipiodol-deposited regions are measured as necrosis). Sum of the all target lesions.
Overall evaluation			
CR (complete response)	Disappearance of all lesions	Disappearance of all target lesions	100% tumor-necrotizing effect or 100% tumor size reduction rate
PR (partial response)	50% or greater disappearance of all lesions	30% or greater reduction of target lesions	A tumor-necrotizing effect or tumor size reduction rate between 50% and <100%
SD (stable disease)	Effects other than PR and PD	Effects other than PR and PD	Effects other than PR and PD
PD (progressive disease)	≥25% enlargement of a lesion or appearance of a new lesion	≥20% increase or appearance of a new lesion	≥25% enlargement of the tumor regardless of the necrotizing effect or appearance of a new lesion (categorized into three groups: intrahepatic solitary lesion, intrahepatic multiple lesions, and vascular invasion/extrahepatic spread).

APPENDIX II

Example RECICL Evaluation Sheet

Patient Age Male/female ID

1. **Description of Liver Cancer**
   (1) Past medical history
      (i) Method and date of definite diagnosis of liver cancer
      (ii) Past treatment history (only patients treat for recurrence)
   (2) Condition of liver cancer
      Tumor location, number and size of lesions, vascular invasion, macroscopic classification, macroscopic staging, histological type or degree of differentiation

2. **Description of Treatment Method**
   (1) Initial treatment or treatment for recurrence
   (2) Name of treatment (describe all treatments when multiple treatments were performed)
   (3) Details of treatment, including the reason for the discontinuation and the presence or absence of an adverse event when treatment is discontinued
   (4) Dates of initiation and completion of treatment

3. **Treatment Effect on Target Nodule (TE1, 2, 3, 4)**[*1] _____

   (Describe TE4a or 4b for local ablation) Assessment results: Lesion 1 _____
   Lesion 2 _____
   Lesion 3 _____
   Lesion 4 _____
   Lesion 5 _____

4. **Overall Evaluation (CR, PR, SD, PD)**[*2] _____

   Assessment results: _____

When a new lesion appears in PD (new lesion: a, b, c)

Additional notes: tumor markers

Name of tumor marker	Before treatment	Lowest level within 3 months Time point ( )	6 months (only for radiotherapy)
AFP			
AFP-L3 fraction			
PIVKA-II (DCP)			

*1: Refer to Table 1. *2: Refer to Table 2.

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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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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 *RARβ* 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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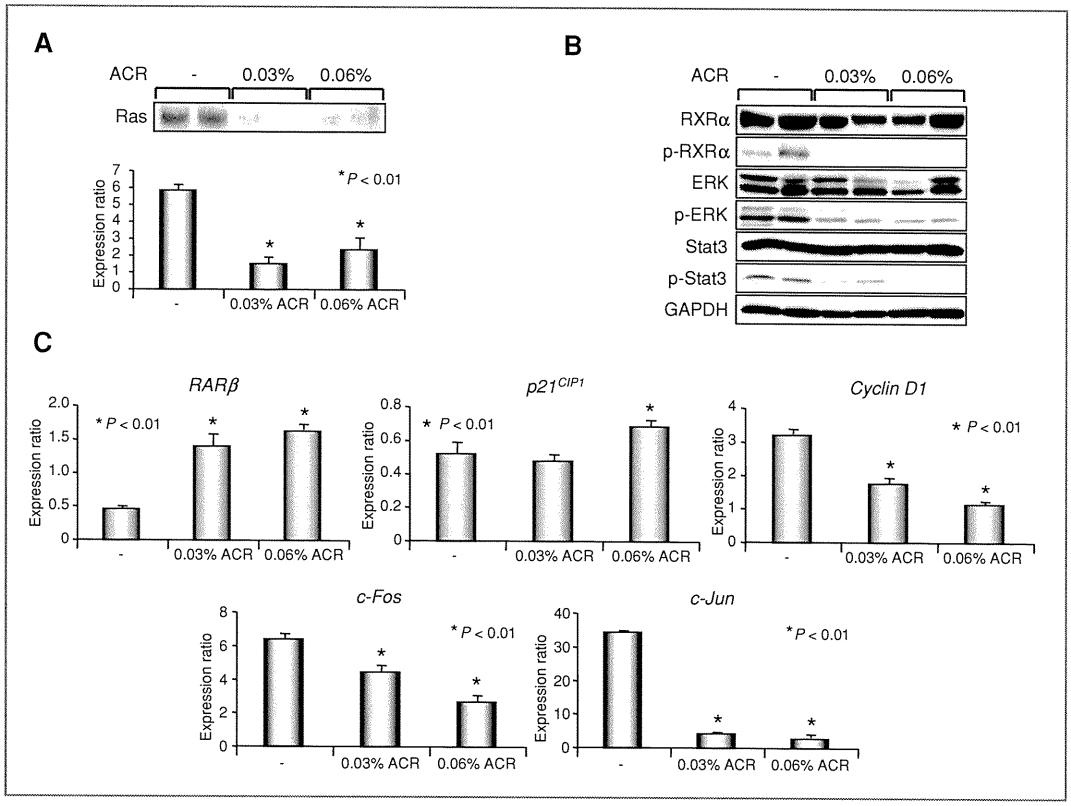

Figure 1. Effects of ACR on Ras activity; phosphorylation of RXRα, ERK, and Stat3 proteins; and the expression of target genes in the livers of DEN-treated *db/db* mice. The total proteins and mRNAs were extracted from the livers of DEN-treated mice. A, the Ras activities were determined using a Ras 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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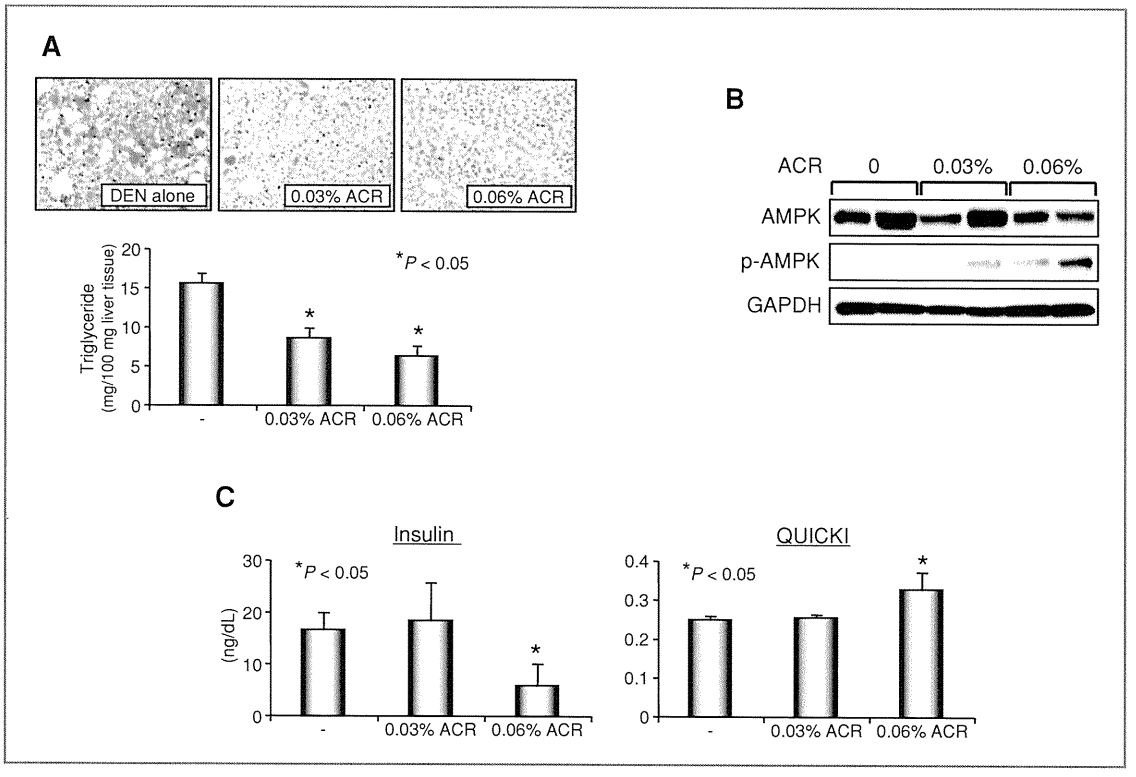

Figure 2. Effects of ACR on hepatic steatosis, the activation of the AMPK protein in the liver, and the levels of serum insulin and insulin sensitivity in DEN-treated *db/db* mice. A, frozen liver sections from DEN-exposed mice treated with or without ACR were stained with Sudan III to show steatosis (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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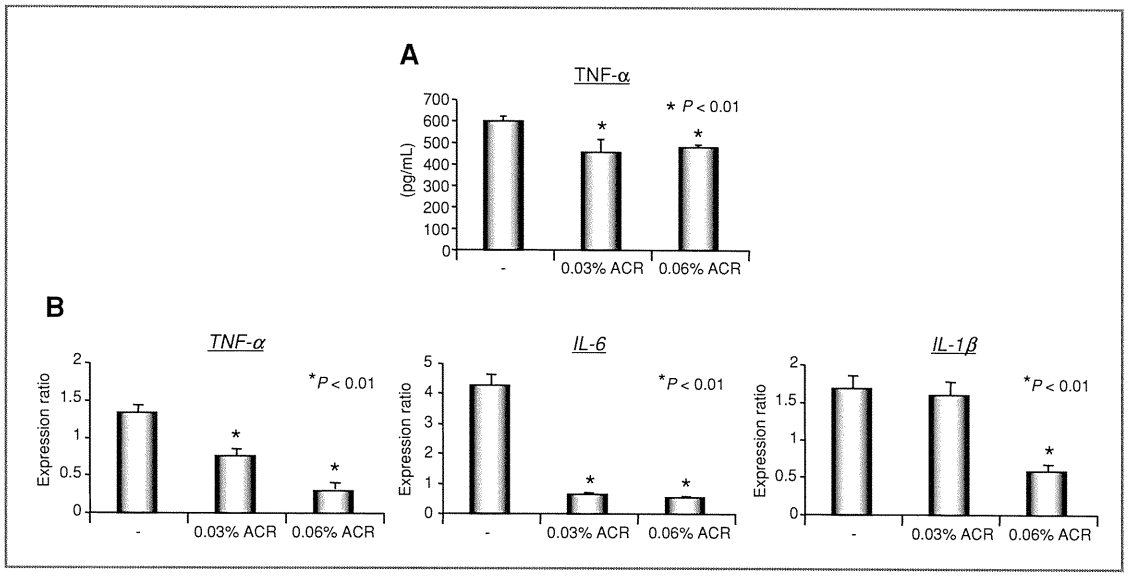

Figure 3. Effects of ACR on the serum levels of TNF-α and the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA in the livers of DEN-treated *db/db* mice. A, the serum concentration of TNF-α was measured by enzyme immunoassay. B, the expression levels of *TNF-α*, *IL-6*, and *IL-1β* mRNA were examined 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