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Synergistic growth inhibition of human hepatocellular carcinoma cells by acyclic retinoid and GW4064, a farnesoid X receptor ligand

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ABSTRACT

Abnormalities in the expression and function of retinoid X receptor (RXR), a master regulator of the nuclear receptor superfamily, are associated with the development of hepatocellular carcinoma (HCC). Dysfunction of farnesoid X receptor (FXR), one of the nuclear receptors that forms a heterodimer with RXR, also plays a role in liver carcinogenesis. In the present study, we examined the effects of acyclic retinoid (ACR), a synthetic retinoid targeting RXRα, plus GW4064, a ligand for FXR, on the growth of human HCC cells. We found that ACR and GW4064 preferentially inhibited the growth of HLE, HLF, and Huh7 human HCC cells in comparison with Hc normal hepatocytes. The combination of 1 μM ACR plus 1 μM GW4064 synergistically inhibited the growth of HLE cells by inducing apoptosis. The combined treatment with these agents acted cooperatively to induce cell cycle arrest in the G_0/G_1 phase and inhibit the phosphorylation of RXRα, which is regarded as a critical factor for liver carcinogenesis, through inhibition of ERK and Stat3 phosphorylation. This combination also increased the expression levels of $p21^{CIP1}$ and SHP mRNA, while decreasing the levels of *c-myc* and cyclin D1 mRNA in HLE cells. In addition, a reporter assay indicated that the FXRE promoter activity was significantly increased by treatment with ACR plus GW4064. Our results suggest that ACR and GW4064 cooperatively inhibit RXRα phosphorylation, modulate the expression of FXR-regulated genes, thus resulting in the induction of apoptosis and the inhibition of growth in HCC cells. This combination might therefore be effective for the chemoprevention and chemotherapy of HCC.

1. Introduction

Nuclear receptors are ligand-dependent transcription factors that are involved in various physiological processes. Retinoid X receptors (RXRs) are regarded as master regulators of nuclear receptors because they play an essential role in controlling normal cell proliferation and metabolism by forming a heterodimer with other nuclear receptors [1,2]. Therefore, abnormalities in the expression and function of RXRs are closely associated with the development of various disorders, including cancer, whereas using a retinoid might be an effective strategy for the prevention and treatment of human malignancies [3]. A malfunction of RXRα, one of the subtypes of RXR, due to phosphorylation by the Ras/MAPK signaling pathway is profoundly associated with liver carcinogenesis [4–8]. On the other hand, administration of acyclic retinoid (ACR), a synthetic retinoid which targets RXRα, reduced the incidence of post-therapeutic recurrence of hepatocellular carcinoma (HCC) and improved the survival rate of patients with this malignancy [9,10]. ACR also inhibits the growth of HCC-derived cells by inducing apoptosis and cell cycle arrest in the G_0/G_1 phase [11,12]. These findings suggest that nuclear receptors, especially RXRα, are critical targets for the prevention and treatment of HCC.

Farnesoid X receptor (FXR), which has been characterized as a bile acid receptor, is also a member of the nuclear receptor superfamily of ligand-dependent transcription factors that form heterodimers with RXR [13]. FXR has been shown to be essential in controlling bile acid, lipid, and glucose homeostasis [13]. It also plays a critical role in normal liver regeneration and promotes liver repair after injury by mediating its related signaling pathways [14].

Abbreviations: ACR, acyclic retinoid; CI, combination index; DAPI, 4',6-diamidino-2-phenylindole; ERK, extracellular signal-regulated kinase; FXR, farnesoid X receptor; FXRE, farnesoid X receptor response element; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; IFN, interferon; MAPK, mitogen-activated protein kinase; PARP, poly (ADP-ribose) polymerase; RAR, retinoic acid receptor; RARE, retinoic acid response element; RTK, receptor tyrosine kinase; RT-PCR, reverse transcription PCR; RXR, retinoid X receptor; SHP, small heterodimer partner; Stat3, signal transducer and activator of transcription 3; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.

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In addition, recent studies have revealed that aberrations in FXR are involved in liver carcinogenesis. FXR deficiency in mice leads to the development of neoplasms in the liver, including hepatic adenoma, HCC, and hepatocholangiocellular carcinoma [15,16]. A significant decrease in FXR expression and activity is also observed in human HCC samples [17]. Therefore, targeting FXR and improving its function might be a promising strategy for the prevention and treatment of HCC.

Recently, combination therapy and prevention have garnered much interest in the cancer field because they can synergistically inhibit growth and induce apoptosis in cancer cells. In human HCC-derived cells, ACR acts synergistically with other agents, such as interferon (IFN)-β, OSI-461, vitamin K_2, valproic acid, and trastuzumab, in suppressing growth and inducing apoptosis [11,18–21]. The agents that inhibit RXRα phosphorylation are among the most promising agents to use in combination with ACR [11,20,21]. In addition, the induction of nuclear receptors that dimerize with RXR, such as retinoic acid receptor (RAR)-β, and activation of these receptors by their ligands may also lead to synergistic growth inhibition in HCC cells when combined with ACR [11,19]. GW4064, a synthetic ligand for FXR, is known to induce the expression of genes involved in the transport of bile acids in the liver and intestines [22,23]. GW4064 also inhibits the growth of breast and prostate cancer cell lines [24–26], whereas the anticancer effects of this agent on HCC cells have not been evaluated. In the present study, we examined the effects of GW4064 on the growth of human HCC cells. We also investigated whether the combination of ACR plus GW4064 exerts synergistic growth inhibitory effects on HCC cells and examined the possible mechanisms responsible for such synergy.

2. Materials and methods

2.1. Materials

ACR (NIK-333) was supplied by Kowa Pharmaceutical Co. Ltd., (Tokyo, Japan). GW4064 was purchased from Sigma–Aldrich (St. Louis, MO, USA). The anti-RXRα antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibodies for ERK, phosphorylated ERK (p-ERK), Stat3, phosphorylated Stat3 (p-Stat3), PARP, and GAPDH were from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell lines and cell culture conditions

HLE, HLF, and Huh7 human HCC cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). HLE and HLF cells were maintained in DMEM and Huh7 cells were in RPMI1640 media, respectively. All media were supplemented with 10% FCS and 1% Penicillin/Streptomycin. Hc human normal hepatocyte cell line was purchased from Cell Systems (Kirkland, WA, USA) and maintained in a CS-S complete medium (Cell Systems). These cells were cultured in an incubator with humidified air with 5% CO_2 at 37 °C.

2.3. Cell proliferation assays

One thousand of HCC (HLE, HLF, and Huh7) or Hc cells were seeded on 96-well plates. The following day, the medium was changed to serum free medium and the cells were treated with the indicated concentrations of ACR or GW4064 for 48 h. Cell proliferation assays were performed using a MTS assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. To determine whether the combined effects of ACR plus GW4064 were synergistic, HCC cells were treated with combinations of the indicated concentrations of ACR and GW4064 for 48 h and the combination index (CI)-isobologram was calculated. Variable ratios of drug concentrations were used in the studies, and mutually exclusive equations were used to determine the CIs. Each CI was calculated from the mean affected fraction at each drug ratio concentration (triplicate), as described previously [11,19,27].

2.4. Apoptosis assays

TUNEL, caspase-3 activity, and Annexin V assays are conducted to evaluate apoptosis. For TUNEL assay, HLE cells (1×10^6) were treated with 1 μM ACR alone, 1 μM GW4064 alone, or the combination of these agents for 48 h on glass bottom culture dishes. The cells were then fixed with 4% paraformaldehyde at room temperature for 10 min, permeabilized with 0.3% Triton X-100 in TBS (pH 7.4), and stained with both 4',6-diamidino-2-phenylindole (DAPI) and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) methods using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Mannheim, Germany) [11].

Caspase-3 activity and Annexin V assays were performed using HLE cells that were treated with the same concentration of test drugs for 72 h. The cell lysates were prepared and the caspase-3 activity assay was done using the Apoalert Caspase Fluorescent Assay Kit (Clontech Laboratories, Mountain View, CA, USA). The Annexin V-binding capacity of treated cells was investigated by flow cytometry using the Annexin V-FITC apoptosis detection kit I (BD, Franklin Lakes, NJ, USA). Cultured cells were washed with cold phosphate-buffered saline before incubation with Annexin V-FITC in a buffer containing propidium iodine (PI). Stained cells were analyzed by flow cytometry using the FACScan (BD). Annexin V-FITC-positive and PI negative cells were considered to be populations undergoing apoptosis.

2.5. Cell cycle assays

HLE cells were treated with 1 μM ACR alone, 1 μM GW4064 alone, or the combination of these agents for 72 h in DMEM medium with 1% FCS. The harvested cells were stained with PI using Cell Cycle Phase Determination Kit (Cayman, Ann Arbor, MI, USA), and the samples were then analyzed for DNA histograms and cell cycle phase distribution using a FACScan flow cytometer. The data were analyzed by using the CellQuest computer program (BD) [11].

2.6. Protein extraction and Western blot analysis

Equivalent amounts of extracted protein were examined by a Western blot analysis using specific antibodies [21]. To detect the expression level of phosphorylated RXRα (p-RXRα) protein, total phosphoprotein was affinity-purified from the total cell extracts using a PhosphoProtein Purification Column (QIAGEN, Valencia, CA, USA) and then was subjected to the Western blot analyses using an anti-RXRα antibody. GAPDH expression served as a loading control. The intensities of protein bands were quantified using NIH image software version 1.45.

2.7. RNA extraction and quantitative RT-PCR analysis

Total RNA was isolated from the HLE cells using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX, USA) and cDNA was amplified from 0.2 μg of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) [28]. Quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed using specific primers that amplify the *c-myc*, small heterodimer partner (SHP), $p21^{CIP1}$, cyclin D1, and β-actin genes. The specific primer sets for $p21^{CIP1}$, cyclin D1, and β-actin were used as described elsewhere [12,29]. The sequences for *c-myc*- and SHP-specific primers were as follows: FMYC (5'-CCC TGA GCG ATT CAG ATG AT-3') and RMYC (5'-GCT CCA GGA TGT TGT GGT TT-3'), and FSHP (5'-GCT GTC TGG AGT CCT TCT GG-3') and RSHP (5'-ACC TGA GCA AAA GCA TGT CC-3'), respectively.

2.8. FXRE reporter assays

HLE cells were transfected with FXR response element (FXRE) reporter plasmids (100 ng/well in 96-well dish), which were kindly provided by Dr. T. Nishimaki-Mogami (National Institute of Health Sciences, Tokyo, Japan), along with p*RL*-CMV (*Renilla* luciferase, 10 ng/well in 96-well dish; Promega) as an internal standard to normalize the transfection efficiency. Transfections were done using Lipofectamine LTX Reagent (Invitrogen). After exposure of the cells to the transfection mixture for 24 h, the cells were treated with 1 μM ACR alone, 1 μM GW4064 alone, or the combination of these agents for 24 h. The cell lysates were then prepared, and the luciferase activity of each cell lysate was determined using a dual-luciferase reporter assay system (Promega) [11].

2.9. Statistical analysis

The data are expressed as the means ± SD. Statistical significance of the differences in the mean values was assessed with a one-way ANOVA, followed by Tukey–Kramer's multiple comparison tests. Values of $P < 0.05$ were considered to be significant.

3. Results

3.1. ACR and GW4064 cause preferential inhibition of the growth of human HCC cells in comparison with Hc normal hepatocytes

In our initial study, we examined the growth inhibitory effect of ACR and GW4064 on HLE, HLF, and Huh7 human HCC cells and on Hc hepatocytes. ACR inhibited the growth of HCC cells with an IC_{50} value of less than 4 μM. The HLF cells were most susceptible to ACR

because the IC_{50} value with this agent was 2 μM (Fig. 1A). GW4064 also inhibited the growth of this series of HCC cells with an IC_{50} value of about 1.4 μM (Fig. 1B). On the other hand, Hc cells were resistant to these agents up to 5 μM (Fig. 1). These results suggest that ACR and GW4064 preferentially inhibit the growth of HCC cells compared with that of normal hepatocytes.

3.2. ACR plus GW4064 cause synergistic inhibition of the growth of HCC cells

Next, the effects of combined treatment were examined with a range of concentrations of ACR plus GW4064 to determine whether they synergistically inhibited the growth of HLE (Fig. 2A), HLF (Fig. 2B), and Huh7 (Fig. 2C) HCC cells. We found that the CI indices for less than 1 μM ACR (0.5 or 1 μM) plus less than 0.5 μM GW4064 (0.1 or 0.5 μM) were 1+(slight synergism), 2+(moderate synergism), or 3+(synergism), respectively, in this series of HCC cells (Fig. 2D and Table 1). These findings suggest that ACR plus GW4064 might be an effective combination for the inhibition of HCC cell growth due to their synergistic activity. The combination of 1 μM ACR (about IC_{25} value) and 1 μM GW4064 (about IC_{30} value) in HLE cells (Fig. 2A and D, and Table 1) was used for the following experiments because a CI-isobologram analysis gave this combination a CI index of 1+(0.88), indicating slight synergism.

3.3. ACR plus GW4064 cooperatively induce apoptosis in HLE cells

We next examined whether the synergistic growth inhibition in HLE cells induced by treatment with ACR plus GW4064 might be associated with the induction of apoptosis. In TUNEL assays, the treatment of HLE cells with either 1 μM ACR or 1 μM GW4064 alone induced TUNEL-positive cells in approximately 19.3% or 11.9% of the total viable cells, respectively. However, the combination of these agents markedly enhanced the induction of apoptosis, with 51.6% of the total viable cells being TUNEL-positive (Fig. 3A). Similar results were also observed in the Western blot analysis for PARP expression; the combination of ACR plus GW4064 markedly enhanced PARP cleavage, indicating the induction of apoptosis (Fig. 3B). We also found an increase in the levels of caspase-3 activity in ACR alone- and GW4064 alone-treated cells, and this was significantly enhanced when the cells were treated with a combination of these agents (Fig. 3C). In addition, the percentage of Annexin V-positive cells, which was increased by treatment with GW4064 alone, was substantially increased by the combined treatment with ACR plus GW4064 (Fig. 3D). These findings suggest that the combination with ACR plus GW4064 synergistically inhibited growth of HLE human HCC cells, mainly, through the induction of apoptosis.

3.4. ACR plus GW4064 cooperatively induce G_0/G_1 cell cycle arrest in HLE cells

A cell cycle analysis was performed using DNA flow cytometry to determine whether the synergistic effects on growth inhibition caused by combined treatment with ACR plus GW4064 were associated with specific changes in cell cycle distribution. As shown in Fig. 4, the combined treatment with 1 μM ACR plus 1 μM GW4064 significantly increased the percentage of cells in the G_0/G_1 phase in comparison to that of untreated cells (76.1 ± 4.3% vs. 57.3 ± 5.8%, $P < 0.05$), whereas the population of cells in this phase was not significantly increased by treatment with ACR alone (63.6 ± 3.0%) or GW4064 alone (65.3 ± 4.5%). These findings suggest that the combination of ACR plus GW4064 cooperatively induced G_0/G_1 phase cell cycle arrest in HLE human HCC cells.

3.5. ACR plus GW4064 additively suppress the phosphorylation of RXRα, ERK, and Stat3 proteins in HLE cells

RXRα phosphorylation plays a critical role in the development of HCC and might be a promising target for HCC chemoprevention [4–8]. Therefore, the effects of the combination of ACR plus GW4064 on the phosphorylation of this nuclear receptor and related signaling molecules were investigated in HLE cells. As shown in Fig. 5, when the cells were treated with 1 μM ACR, there was a marked decrease in the expression levels of p-RXRα and p-Stat3 proteins. Treatment with 1 μM GW4064 alone also decreased the expression levels of p-ERK and p-Stat3 protein. Moreover, the expression levels of p-RXRα, p-ERK and p-Stat3 proteins were markedly decreased when the cells were treated with the combination of these agents.

3.6. ACR plus GW4064 cooperatively affect the expression levels of $p21^{CIP1}$, c-myc, cyclin D1, and SHP mRNA in HLE cells

We next examined the combined effects of ACR plus GW4064 on the expression levels of $p21^{CIP1}$, c-*myc*, and cyclin D1 mRNA in HLE cells because these genes control cell proliferation and cell cycle progression. The quantitative RT-PCR analyses revealed that treatment with neither 1 μM ACR nor 1 μM GW4064 alone had any apparent effect on the expression levels of $p21^{CIP1}$, c-*myc*, and cyclin D1 mRNA. However, when the cells were treated with the combination of these agents, there was a significant increase

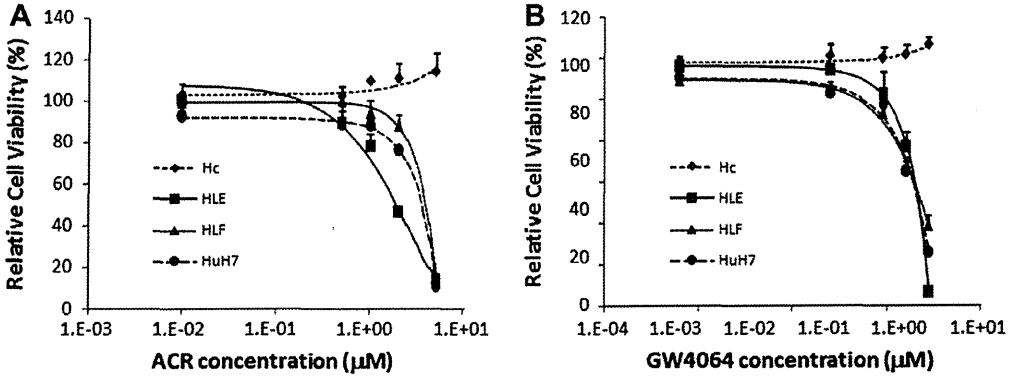

Fig. 1. Inhibition of cell growth by ACR and GW4064 in HLE, HLF, and Huh7 human HCC cells and Hc normal hepatocytes. HLE, HLF, Huh7, and Hc cells were treated with the indicated concentrations of ACR (A) or GW4064 (B) for 48 h. Cell viability was determined by MTS assay and was expressed as a percentage of the control value. *Error Bars*, SD of triplicate assays.

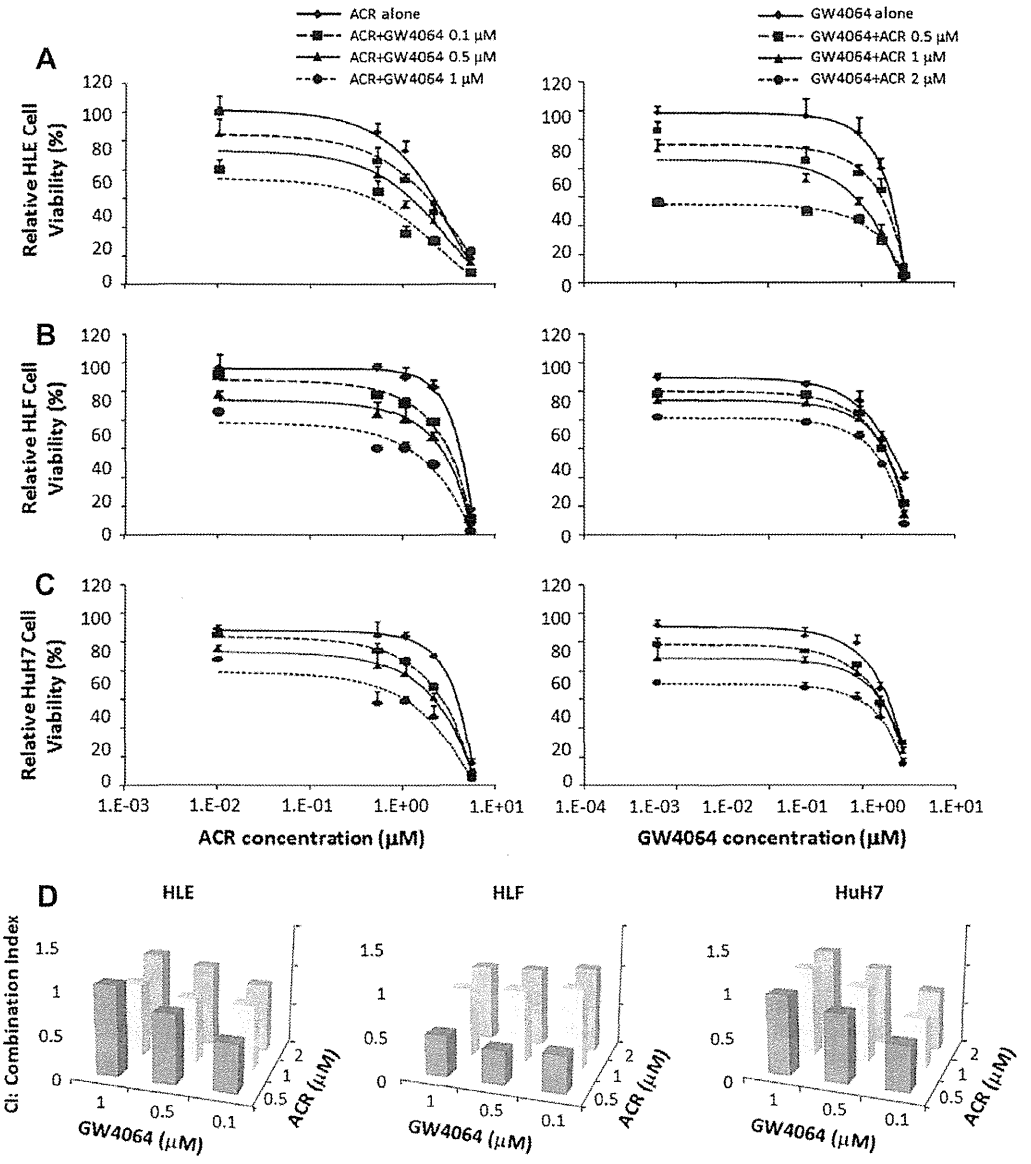

Fig. 2. Inhibition of cell growth by ACR alone, GW4064 alone, and various combinations of these agents in HCC cells. HLE (A), HLF (B), and Huh7 (C) cells were treated with the indicated concentrations of ACR alone, GW4064 alone, and various combinations of these agents for 48 h. Cell viability was determined by MTS assay and expressed as a percentage of the control value. *Error Bars*, SD of triplicate assays. (D) The data obtained in (A), (B), and (C) was used to calculate the combination index.

Table 1
Combined effects of ACR and GW4064 on HCC cells.

	HLE ACR concentration (μM)			HLF ACR concentration (μM)			HuH7 ACR concentration (μM)		
GW4064 concentration (μM)	0.5	1	2	0.5	1	2	0.5	1	2
0.1	+++	++	+	+++	+	±	+++	++	++
0.5	++	++	±	+++	++	±	++	+	±
1	±	+	±	+++	+	±	±	±	±

Note: "±", CI 0.9–1.1 additive effect; " + ", CI 0.8–0.9 slight synergism; "++", CI 0.6–0.8 moderate synergism; "+++", CI 0.4–0.6 synergism;
Abbreviations: CI, combination index; ACR, acyclic retinoid.

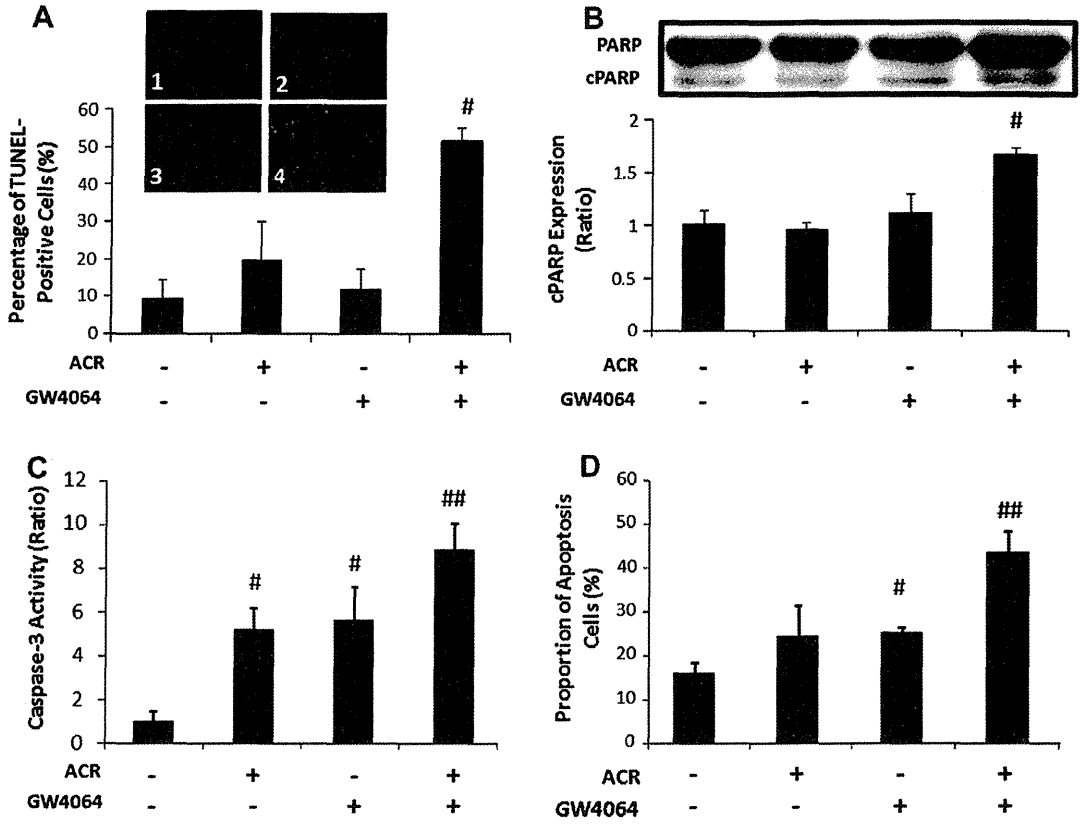

Fig. 3. Effects of the combination of ACR plus GW4064 on induction of apoptosis in HLE cells. The cells were treated with vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064 for 48 h or 72 h. TUNEL assays (A) and Western blot analysis using a PARP-specific antibody (B, upper panel) were performed using cells treated with test drugs for 48 h. Caspase-3 activity assays (C) and Annexin V assays (D) were performed using samples treated for 72 h. (A) TUNEL-positive cells were counted and examined as the percentage of the DAPI-positive cell number (500 cells were counted in each flask). (B) The intensities of the cleaved PARP (c-PARP) blots were quantified using densitometry. Columns and lines indicate mean and SD of triplicate assays (lower panel). (C) Caspase-3 activity was performed with a fluorometric system. (D) Cultured cells were incubated with Annexin V-FITC in a buffer containing propidium iodine (PI). Stained cells were then analyzed by flow cytometry. Annexin V-FITC-positive and PI-negative cells were counted as apoptotic cells. #: $P < 0.05$, compared with vehicle treated cells. ##: $P < 0.05$, compared with vehicle, ACR alone, or GW4064 alone treated cells.

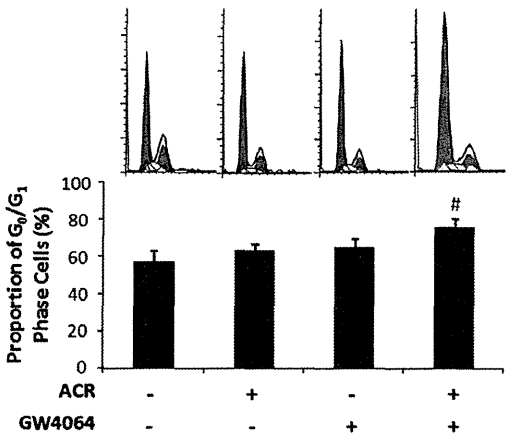

Fig. 4. Effects of the combination of ACR plus GW4064 on induction of the G_0/G_1 cell cycle arrest in HLE cells. HLE cells were treated with vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064 for 72 h. The cells were then stained with propidium iodide to analyze their cell cycle progression. The distributions of cells in the G_0/G_1 of the cell cycle were calculated using a FACScan. *Error Bars*, SE of triplicate assays. #: $P < 0.05$, compared with vehicle treated cells.

in the levels $p21^{CIP1}$, and a decrease in the levels of c-*myc* and cyclin D1 mRNA expression (Fig. 6A–C). In addition, the expression level of SHP mRNA, which is one of the target genes of FXR [17,25,30,31], was also significantly increased by the combination treatment with ACR plus GW4064 (Fig. 6D).

3.7. ACR enhances the induction of FXRE promoter activities by GW4064

FXR and RXRs modulate the expression of target genes by interacting with FXRE elements located in the promoter regions of such genes [13]. Therefore, we next examined whether ACR might enhance the transcriptional activity of the FXRE promoter induced by GW4064 using transient transfection luciferase reporter assays. As shown in Fig. 6E, 1 μM GW4064 significantly increased the FXRE reporter activity in comparison with control HLE cells which were not treated with either ACR or GW4064. Moreover, when the cells were treated with a combination of 1 μM GW4064 plus 1 μM ACR, there was a significant increase in the transcriptional activity of the FXRE reporter, thus suggesting that treatment with these agents might cooperatively enhance the FXRE reporter activity.

4. Discussion

The prognosis for patients with HCC is poor and more effective strategies for the chemoprevention and chemotherapy of this malignancy are urgently required. The present study provides the

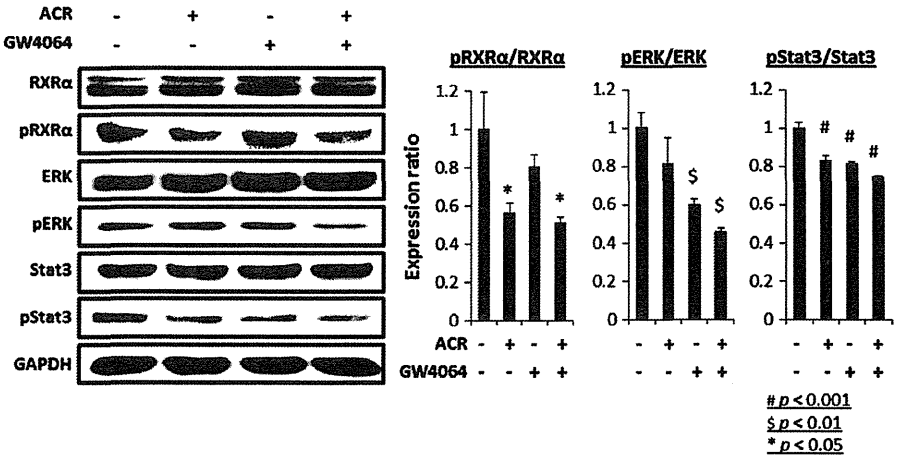

Fig. 5. Effects of the combination of ACR plus GW4064 on the phosphorylation of RXRα, ERK, and Stat3 proteins in HLE cells. HLE cells were treated with vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064 for 12 h. The extracted proteins were examined by a Western blot analysis using the respective antibodies (left panels). The intensities of the blots were quantified using densitometry. Columns and lines indicate means and SD of triplicate assays (right panels). Repeat Western blots gave similar results. *: $P < 0.05$, $: $P < 0.01$, #: $P < 0.001$, compared with vehicle treated cells.

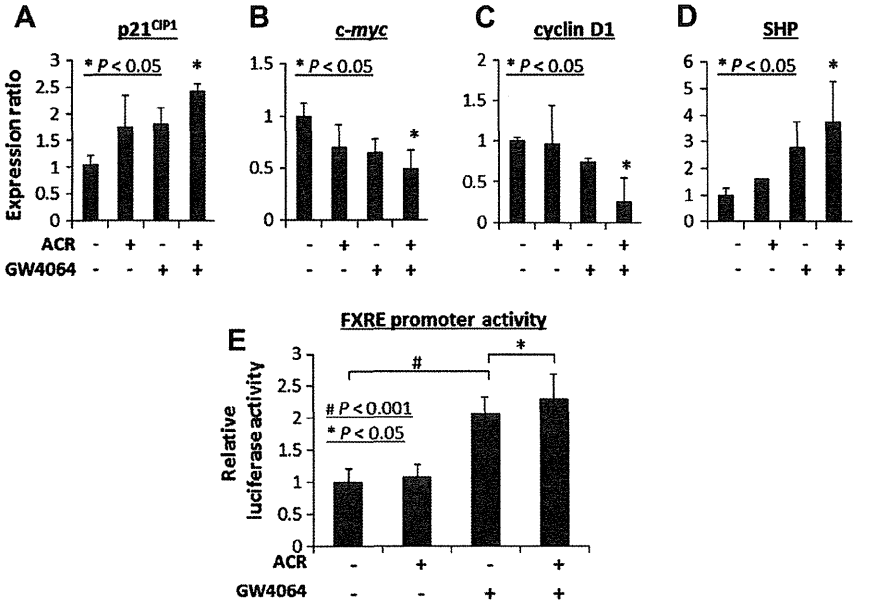

Fig. 6. Effects of the combination of ACR plus GW4064 on the expression of $p21^{CIP1}$, *c-myc*, cyclin D1, and SHP mRNA and on the transcriptional activity of the FXRE promoter in HLE cells. HLE cells were treated with vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064 for 24 h. The extracted mRNAs were examined by a quantitative real-time RT-PCR analysis using the $p21^{CIP1}$ (A), *c-myc* (B), cyclin D1 (C), and SHP (D) specific primers. The expression levels of each mRNA were normalized to the level of β-actin mRNA. Values represent the means ± SD of triplicate analyses. *: $P < 0.05$. (E) A transient transfection reporter assay was performed with the FXRE luciferase reporter in the presence of vehicle, 1 μM ACR alone, 1 μM GW4064 alone, or the combination of 1 μM ACR plus 1 μM GW4064. Relative luciferase activity was determined after 24 h. Columns and lines indicate the means and SD of triplicate assays. #: $P < 0.001$, **: $P < 0.05$.

first evidence that GW4064, a synthetic ligand for FXR, preferentially inhibits the growth of HCC cells compared with Hc normal hepatocytes. This study also clearly indicates that the combination of GW4064 plus ACR, which is expected as a HCC chemopreventive agent [8–10], cause a synergistic inhibition of growth in HLE human HCC cells and that this is associated with the induction of apoptosis. This combination also acted cooperatively to induce the arrest of the cell cycle in the G_0/G_1 phase and the expression of $p21^{CIP1}$ and SHP mRNA, but suppress the expression levels of c-*myc* and cyclin D1 mRNA.

As illustrated in Fig. 7, we presume that the synergism generated by the combination of ACR plus GW4064 is mainly associated with the enhancement of the FXRE reporter activity. FXR regulates the expression of target genes by binding either as a monomer or as a heterodimer with RXR to the FXRE [13]. Therefore, in the present study, ACR and GW4064 cooperatively enhanced the binding of FXR to the FXRE promoter, thereby enhancing the expression of its target genes (Fig. 7, as indicated by A). Among the FXR target genes, SHP is considered to play a role in the inhibition of cell growth because it is a pivotal cell death receptor that targets the mitochondria, leading to the induction of apoptosis and inhibition of tumor growth [32]. GW4064 suppresses the growth of cancer cells through the activation of FXR targeted genes, including SHP [17,25,30,31]. SHP has also been shown to be a direct negative

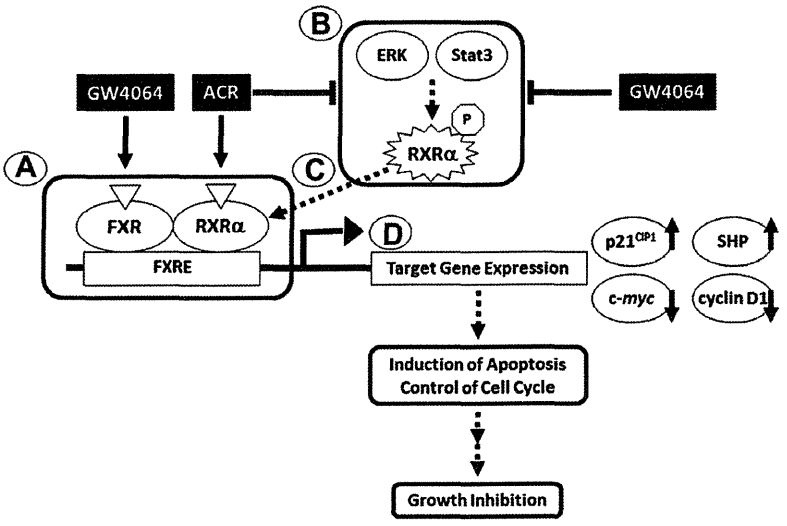

Fig. 7. A hypothetical schematic representation of the effects of the combination of ACR plus GW4064 on growth inhibition in HCC cells. ACR and GW4064 can bind to their receptors, RXRα and FXR, as ligands, and subsequently activate the FXRE promoter activity (A). ACR and GW4064 also inhibit RXRα phosphorylation, which is involved in liver carcinogenesis, by inhibiting ERK and Stat3 phosphorylation (B). The inhibition of RXRα phosphorylation by these agents might restore the function of this nuclear receptor as a heterodimeric partner for FXR (C), thus resulting in the activation of the FXRE promoter activity. Cooperative activation of this promoter activity by ACR and GW4064 regulates the expression of target genes, such as $p21^{CIP1}$, c-*myc*, cyclin D1, and SHP, which play a critical role in the induction of apoptosis, control of cell cycle progression, and inhibition of cancer cell growth (D). For additional details see Section 4.

regulator of cyclin D1 gene transcription [30]. Treatment with 1 μM GW4064 alone did not significantly increase the expression levels of SHP mRNA in the present study, whereas its expression was clearly increased by combined treatment with ACR plus GW4064. These findings may indicate that the concentration of 1 μM is insufficient to increase the levels of SHP mRNA in HCC cells and, therefore, an appropriate partner, such as ACR, is required for GW4064 to exert a synergistic effect on growth inhibition in HCC cells (Fig. 7).

In addition, recent studies have revealed that activating FXR suppresses the expression of cyclin D1 and c-*myc*, but induces the expression of $p21^{CIP1}$, by targeting the Wnt/β-catenin signaling pathway [17,31]. These findings seem to be significant because the Wnt/β-catenin pathway plays a critical role in liver carcinogenesis, and thus may be a promising target for the treatment of HCC [33]. In FXR knockout mice, sustained activation of this pathway was shown to be involved in the development of HCC [17]. On the other hand, ACR has been shown to exert growth inhibitory effects in HCC cells by targeting the Wnt/β-catenin pathway [12]. ACR also induces apoptosis and cell cycle arrest in the G_0/G_1 phase in HCC cells by regulating the expression of $p21^{CIP1}$, cyclin D1, and c-*myc* [12,19,34,35]. Therefore, the activation of FXR by GW4064 may act cooperatively with ACR to inhibit the activation of the Wnt/β-catenin pathway, subsequently decreasing the expression of cyclin D1 and c-*myc*, but increasing the expression of $p21^{CIP1}$, as was demonstrated in the present study.

In addition to chronic inflammation and subsequent cirrhosis of the liver induced by persistent infection with hepatitis virus, increased evidence has indicated that a malfunction of RXRα due to phosphorylation is profoundly involved in the development of HCC [4–8]. In HCC cells, the Ras/MAPK signaling pathway is highly activated, leading to phosphorylation of RXRα, which indicates that the Ras/MAPK pathway and p-RXRα are potential targets for inhibiting the growth of HCC cells [4–8]. Indeed, ACR dephosphorylates RXRα, ERK, and Stat3 proteins, and restores the function of RXRα, thus inhibiting the growth of HCC cells and suppressing liver tumorigenesis in obese mice [4,11,20,36]. The combinations of ACR plus valproic acid or vitamin K_2 also synergistically suppressed the growth of HCC cells by inhibiting RXRα phosphorylation [11,20]. Similar to these previous studies [11,20], in the present study, inhibition of RXRα phosphorylation by the combination of ACR plus GW4064 may also have restored the function of RXRα as a master regulator of nuclear receptors, thus contributing to synergistic growth inhibition in HCC cells (Fig. 7, as indicated by B). Dephosphorylation of RXRα by this combination treatment may play a role in the observed enhancement of the FXRE promoter activity because phosphorylation of RXRα abolishes its ability to form heterodimers with other nuclear receptors, but inhibition of this phosphorylation can restore its heterodimeric activity [7]. The combination of ACR plus GW4064 may also promote RXRs homodimerization and thus enhance the promoter activity of retinoid X response element, which is associated with the anticancer mechanisms of ACR [11,37].

One of the major questions that arose was how the combination of ACR plus GW4064 could inhibit the phosphorylation of ERK and Stat3 proteins. One of the mechanisms which might explain this phenomenon is that the effects of ACR and GW4064 inhibit the activation of specific receptor tyrosine kinases (RTKs). ACR has been shown to reduce HCC development and inhibit cancer growth by targeting growth factors and their corresponding RTKs, such as the epidermal growth factor (EGF) receptor (EGFR), and downstream signaling pathways, including the Ras/MAPK and Jak/Stat3 pathways [29,38]. The activation of FXR by its ligand also reduces the expression of HER2, a member of the EGFR family of RTKs, and inhibits EGF-mediated HER2 and ERK phosphorylation in human breast cancer cells [24]. Therefore, GW4064 may increase the inhibitory effects of ACR on certain types of RTKs by activating FXR, which results in the inhibition of ERK and Stat3 phosphorylation and subsequent RXRα phosphorylation. Future studies are required to clarify whether both ACR and GW4064 synergistically exert inhibitory effects on the activation of specific RTKs.

Finally, it should be noted that, in a clinical trial showing the chemopreventive effects of ACR on the recurrence of secondary HCC [9,10], the plasma concentration of this agent (which ranged from 1 to 5 μM) was approximately the same as the concentration used in the present study (1 μM). In phase II clinical trials, a FXR

ligand also ameliorated the increase in the alkaline phosphatase levels in patients with primary biliary cirrhosis and improved the insulin sensitivity in patients with diabetes and liver steatosis, although some unfavorable events that might be associated with FXRE reporter overactivity were observed [39]. The combination of ACR plus GW4064 may resolve such problems because this combination permits the administration of lower doses of both agents for treatment. Future pharmacokinetic studies are required to determine whether the dose of GW4064 used in this study is clinically relevant and pilot studies confirming are thus called for to clarify the safety of this agent.

In conclusion, the observation that a combination of appropriate concentrations of ACR plus GW4064 can inhibit the growth of human HCC cells without affecting the growth of normal hepatocytes should encourage further clinical studies using these agents to investigate their potential for HCC chemoprevention and chemotherapy. The results of our present study suggest that combining ACR with GW4064 might hold promise as a clinical modality for the prevention and treatment of HCC, due to their synergistic effects.

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Special Report

Guidelines on nutritional management in Japanese patients with liver cirrhosis from the perspective of preventing hepatocellular carcinoma

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Aim: The Japanese Nutritional Study Group for Liver Cirrhosis (JNUS) was assembled in 2008 with the support of a Health Labor Sciences Research Grant from the Ministry of Health, Labor and Welfare of Japan. The goal of the study group was to propose new nutritional guidelines for Japanese patients with liver cirrhosis (LC), with the aim of preventing hepatocellular carcinoma.

Methods: Between 2008 and 2010, the member investigators of JNUS conducted various clinical and experimental studies on nutrition on LC. These included anthropometric studies, a questionnaire study on daily nutrient intake, clinical trials, experimental studies using animal models, re-evaluation of previous publications and patient education. Over this 3-year period, the group members regularly discussed the nutritional issues related to LC, and a proposal was finally produced.

Results: Based on the results of JNUS projects and discussions among the members, general recommendations were made on how Japanese patients with LC should be managed nutritionally. These recommendations were proposed with a specific regard to the prevention of hepatocarcinogenesis.

Conclusion: The new JNUS guidelines on nutritional management for Japanese patients with LC will be useful for the actual nutritional management of patients with LC. The JNUS members hope that these guidelines will form the basis for future discussions and provide some direction in nutritional studies in the field of hepatology.

Key words: hepatocellular carcinoma, liver cirrhosis, malnutrition, nutrition, protein-energy malnutrition

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INTRODUCTION

THE LIVER IS a major organ in nutritional metabolism. Therefore, metabolic abnormalities in nutritional elements are generally observed in the progression of chronic liver disease (CLD). Malnutrition, which is characterized by protein-energy malnutrition (PEM), is known as an essential complication in patients with liver cirrhosis (LC) and is closely associated with LC prognosis.[1–7] On the other hand, the major cause of CLD in Japan is infection by hepatitis B virus (HBV) and hepatitis C virus (HCV) and approximately 34 000 patients with CLD die annually due to hepatocellular carcinoma (HCC).[8–11] Importantly, 90% of HCC cases are associated with LC.[12,13] Therefore, standard therapeutic guidelines on the use of antiviral agents for CLD patients with HBV and HCV infection have been established to prevent the occurrence of HCC.[14,15] However, because the number of elderly CLD patients is increasing, patients are often unable to tolerate full antiviral therapy. CLD caused by non-alcoholic steatohepatitis (NASH), which is associated with overweight status, has also been increasing.[16–18] These findings indicate that total nutritional management, including both diet and nutritional supplements, is required in order to prevent the progression of CLD and onset of HCC.[19–22]

Japanese dietitians were consulted in preparing the guidelines of both the Japan Society of Metabolism and Clinical Nutrition (2003)[23] and the European Society of Parenteral and Enteral Nutrition (2006 and 2009).[24,25] In addition, nutritional recommendations for the treatment of LC in Japan were incorporated into guidelines in Japan in 2010.[26] However, specific and detailed guidelines for the nutritional management of patients with LC in Japan have been lacking.

From these perspectives, the Japanese Nutritional Study Group for Liver Cirrhosis (JNUS) was assembled between 2008 and 2010 in order to establish new nutritional guidelines for LC. The study group was supported by a Health Labor Sciences Research Grant from the Ministry of Health, Labor and Welfare of Japan (H20-Hepatitis-General-005). Here, we describe the guidelines on nutritional management of Japanese LC patients, with the aim of preventing HCC.

METHODS

THE JNUS GROUP performed the following projects: (i) investigation of clinical and anthropometric characteristics in Japanese patients with LC; (ii) evaluation of daily nutrient intake (total calories, and individual intake of protein, fat, carbohydrate, trace elements such as iron and zinc, and sodium) using a 3-day questionnaire in CLD patients; (iii) development of new biomarkers representing non-protein respiratory quotients (npRQ) measured by indirect calorimetry; (iv) development of a new analytical system to estimate iron status in the blood; (v) a prospective controlled trial to examine whether branched-chain amino acid (BCAA) granule supplementation prevents recurrence of HCC after primary HCC treatment; (vi) a prospective double-blind controlled study evaluating the effects of zinc supplementation on the ammonia metabolism in LC patients with hyperammonemia; (vii) a pilot study to evaluate the effects of late-evening snacks (LES) and a new treatment (α-glucosidase inhibitor) in LC patients with impaired glucose tolerance; (viii) an experimental study to estimate the effects of supplementation of BCAA granules on the development of HCC in a mouse model of NASH; (ix) education programs for nutritional management in both LC patients and the general Japanese population; and (x) re-evaluation of previous publications concerning nutritional therapies in LC patients. After repeated discussion of the results, we then proposed the new guidelines for nutritional management of Japanese LC patients with the aim of preventing HCC.

RESULTS

THE FOLLOWING FINDINGS were obtained: (i) approximately 30% of patients with LC are overweight (body mass index >25), with the incidence being higher in male LC patients due to NASH and alcohol; (ii) only 30% of LC patients have adequate dietary intake for both energy and protein; (iii) iron intake (mean value, 6.7 mg/day) does not differ among CLD patients; (iv) percent arm circumference, percent arm muscle circumference, and serum concentrations of free fatty acid, tumor necrosis factor (TNF)-α and soluble TNF receptors are significantly correlated with npRQ;[27–29] (v) serum non-transferrin-bound iron (NTBI) determined by a newly developed high-performance liquid chromatography system is elevated in LC patients,[30,31] although further study is necessary to clarify whether serum NTBI levels are associated with the development of HCC; (vi) plasma amino acid imbalance is closely associated with the numbers and functions of peripheral dendritic cells;[32] (vii) long-term zinc supplementation therapy in LC patients tends to decrease HCC occurrence; (viii) LES and administration of α-glucosidase inhibitor improve impaired glucose

Table 1 Recommendations for nutritional management of liver cirrhosis: part 1

I. Assessment before nutrition and diet therapy
(1) Evaluate clinical stage (compensated or decompensated liver cirrhosis) and the severity of liver damage (i.e. Child–Pugh classification) as well as presence of portal-systemic shunt.
(2) Perform SGA† and anthropometry.‡
(3) Evaluate impaired glucose tolerance, insulin resistance§ and postprandial hyperglycemia.
(4) Evaluate oxidative stress conditions.¶
(5) Examine dietary intake using a questionnaire.
(6) Perform indirect calorimetry†† and trace element measurement.

†Subjective global assessment (SGA) is an effective method in the screening of malnourished patients. It examines age, sex, height, bodyweight, changes in bodyweight, changes in food intake, the presence of gastrointestinal symptoms, intensity of activities of daily living (ADL), the condition of loss of subcutaneous fat and muscles, the presence of edema/ascites, hair condition, among other factors.
‡In addition to height, bodyweight and body mass index (BMI: bodyweight [kg]/height $[m]^2$), arm circumference (AC) and triceps skinfold thickness (TSF) are measured using an insert tape and adipometer. Moreover, arm muscle circumference (AMC) is calculated by AC – 3.14 × TSF. Data are evaluated using standard values for the physical measurements of a Japanese individual (Japanese Anthropometric Reference Data: JARD 2001).[39] This allows the calculation of basal energy expenditure, resting energy expenditure and protein (amino acid) requirements according to age, sex difference and physical measurements. More detailed body composition analysis methods have recently become available, and these are based on bioelectrical impedance analysis.
§Homeostatic Model of Assessment of Insulin Resistance (HOMA-IR = blood fasting insulin [μU/mL] × fasting blood glucose level [mg/dL] / 405) is used as an index for insulin resistance, with HOMA-IR ≥2.5 considered to indicate insulin resistance. However, this equation assumes that the fasting blood glucose levels are <140 mg/dL.
¶Although there are numerous biomarkers for evaluating oxidative stress, the measurement of serum ferritin levels should be used for the purpose of preventing hepatocellular carcinoma. In addition, the presence of anemia is examined using hemoglobin concentrations.
††Where indirect calorimeters are available, measurement of resting energy expenditure, non-protein respiratory quotient (npRQ) and oxidation rates for various nutrients (carbohydrate, fat, protein) after overnight fasting is useful in evaluating protein-energy malnutrition. Anthropometric values (%AC, %AMC) and the serum free fatty acid levels are useful indexes for npRQ during routine care; serum levels of tumor necrosis factor (TNF)-α and soluble TNF receptors and plasma ghrelin levels may also be used as references.

tolerance;[33–35] (ix) supplementation of BCAA granules and BCAA-enriched nutrients improve liver function and energy metabolism;[36,37] and (x) supplementation of BCAA granules inhibits carcinogenesis in a mouse model of NASH, possibly via improvement of insulin resistance.[38]

Based on these data and discussions among the members of JNUS, guidelines for nutritional management of Japanese LC patients were prepared and are shown in Tables 1 and 2. The guidelines consist of two parts. The first part (Table 1) describes essential nutritional assessments that should be performed before instituting nutritional and diet therapy. The second part (Table 2) describes the recommended dietary management for each nutrient, including energy, protein, fat, sodium chloride, iron and other nutrient requirements. Restriction of sodium chloride was decided based on the therapeutic guidelines for hypertension by the Japanese Society of Hypertension.[40] We also included supplemental descriptions in the tables in order to ensure that dietitians are able to perform nutritional assessment and therapy in accordance with these guidelines.

At this point, it is not clear whether supplementation with BCAA granules has any preventive effects on HCC recurrence after primary treatment for HCC, as the number of enrolled patients is small. A double-blind controlled study for zinc supplementation in LC patients with hyperammonemia is also still on-going. The final results of this study are expected to be available by the end of 2012.

DISCUSSION

IN ORDER TO establish new guidelines on nutritional management in LC patients, it is important to consider hepatocarcinogenesis. In this article, based on the results of JNUS projects between 2008 and 2010 and re-evaluation of previous publications concerning nutritional therapies in LC patients with or without HCC, we proposed new guidelines for nutritional management of Japanese LC patients, with the aim of preventing HCC.

We hope these guidelines will form a basis for future discussions on nutritional management of LC by specialists such as hepatologists and dietitians.

Table 2 Recommendations for the nutritional management of liver cirrhosis: part 2

II. Nutrition and diet therapy
(1) Energy requirements[a]
25–35 kcal/kg (ideal bodyweight) per day, based on *Standards for Dietary Intake* (2010 Edition, Recommended Dietary Allowance According to Intensity of Daily Activity).
If any abnormalities are seen in glucose tolerance, intake should be 25 kcal/kg (ideal bodyweight) per day.
(2) Required protein intake[b]
If there is no protein intolerance: 1.0–1.5 g/kg/day (including oral BCAA granules).[c]
If there is protein intolerance: 0.5–0.7 g/kg per day + BCAA-enriched enteral nutrient mixture.[d]
(3) Required fat intake:[e] lipid energy ratio 20–25%.
(4) Sodium chloride:[f] ≤6 g/day and <5 g/day if there are ascites and/or edema, respectively
(5) Iron:[g] ≤7 mg/day if serum ferritin levels are above the upper limit of the reference interval.
(6) Others: zinc supplementation,[h] adequate intake of vitamins and dietary fiber (e.g. vegetables, fruits).
(7) LES as a divided meal (4 times/day) (amounts to 200 kcal).[i]

[a]Resting energy expenditure is often accelerated in liver cirrhosis patients and protein-energy malnutrition (PEM) is observed in approximately 80–90% of patients. However, approximately 30% of patients are obese, with a body mass index (BMI) of ≥25. Moreover, in cases of hepatitis C, there is a high frequency of insulin resistance exhibited. It is important to determine the required amount of energy by taking into account such nutritional conditions.
[b]Required protein intake includes the protein content of branched-chain amino acid (BCAA) formulation (BCAA granules or BCAA-enriched nutrient mixture for chronic liver failure). The majority of patients with decompensated liver cirrhosis (LC) often have protein intolerance, which is determined by referring to the blood ammonia levels.
[c]Patients in the decompensated state, including cases with hyperammonemia, are judged as having protein intolerance. The administration of BCAA granules (e.g. Livact Granules) is essential for the patient with serum albumin <3.5 g/dL, Fischer's ratio <1.8 and/or BTR < 3.5, and is usually administrated by dividing the dosage of 3 packs/day (12 g) into 3 administrations, but there is also a method whereby 2 packs are administrated (before sleep). Prevention of hepatocellular carcinoma (HCC) is expected in male hepatitis C patients with BMI >25 due to long-term administration of this formula. Improvement of the amino acid imbalance is also useful in recovering decreased dendritic cell functions.
[d]When administrating BCAA-enriched enteral mixtures (e.g. Aminoleban EN and Hepan ED), the amount of energy and protein present in this nutrient should be included in the total intake of energy and protein for the day. BCAA-enriched enteral mixtures should be the first choice in patients with PEM, regardless of the presence of protein intolerance.
[e]Ideal ratio of fatty acid composition for the inhibition of HCC has not been clarified, but a decline in n-6 and n-3 polyunsaturated fatty acids has been observed in patients with LC.
[f]Even patients who are not physically observed to have edema/ascites have a tendency for water retention, so fundamentally salt should be restricted.
[g]Excess deposition of iron in the liver causes oxidative stress and promotes hepatocarcinogenesis; thus, unless severe anemia is observed, an iron-restricted diet should be standard. Moreover, although the standard value of serum ferritin level differs with sex, phlebotomy in small amounts should be considered for patients with values ≥150 ng/mL.
[h]Zinc supplementation improves hyperammonemia and may suppress the occurrence of HCC in patients with LC over long-term administration.
[i]Lifestyle and eating habits of patients should examine. Late-evening snack (LES) is also useful for managing the blood glucose level in patients with impaired glucose tolerance, and combined use with α-glucosidase inhibitor enhances this effect. Usually, snacks such as rice balls (*onigiri*) are provided, but with the recommendation of using enteral nutrients, food products rich in BCAA are also used.
Fischer's ratio: BCAA/tyrosine + phenylalanine.
BTR: molar ratio of BCAA and tyrosine.

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Special Report

Guideline on the use of new anticancer drugs for the treatment of Hepatocellular Carcinoma 2010 update

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The "*Guideline on the Use of New Anticancer Drugs for the Treatment of Hepatocellular Carcinoma*" was prepared by the Study Group on New Liver Cancer Therapies established by the "*Research Project on Emergency Measures to Overcome Hepatitis*" under the auspices of the Health and Labour Sciences Research Grant. The Guideline brings together data collected by the Study Group on the use and incidence of adverse events in 264 patients with advanced hepatocellular carcinoma (HCC) treated using sorafenib and in 535 patients with advanced HCC treated using miriplatin at 16 participating institutions up until 22 December 2010, as well as referring to the published studies, academic presentations, and reports from the private sector. The aim of this Guideline is to facilitate understanding and current thinking regarding the proper usage of new anticancer drugs towards actual use in therapy. In terms of the format, the Guideline presents "clinical questions" on issues pertaining to medical care, makes "recommendations" on diagnosis and treatment in response to each of these clinical questions, and provides a rationale for these recommendations in the form of "scientific statements".

Key words: hepatic arterial infusion, hepatocellular carcinoma, miriplatin, molecular targeting therapy, sorafenib

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INTRODUCTION

THE MOLECULAR-TARGETED agent sorafenib has been found to significantly prolong survival in patients with hepatocellular carcinoma (HCC).[1,2] In May 2009, sorafenib was approved in Japan for unresectable

HCC. Furthermore, miriplatin was approved in Japan for the treatment of HCC in January 2010, and clinical trials are also currently underway on a number of other promising new anticancer agents. Treatment of HCC is thus undergoing a period of major transition, but the role of these anticancer drugs and conventional therapies remains unclear, leading to concerns about the risk of serious adverse events (SAEs).

The Study Group on New Liver Cancer Therapies (the Study Group) was formed as part of the "Research Project on Emergency Measures to Overcome Hepatitis" sponsored by the Health and Labour Sciences Research Grant, with the overall purpose of formulating a guideline to facilitate understanding on the practical usage of new anticancer drugs.

The Study Group collected information on the use of new anticancer drugs, sorafenib and miriplatin at 16 affiliated institutions and compiled current opinions regarding the proper use of these drugs based on published studies, academic conference papers and reports from the private sector. These results have now been compiled in the form of a guideline.

However, of note is that this guideline is provisional and has been prepared to expedite the provision of proper information because information on these new anticancer drugs is constantly being updated.

STUDY METHODS, SUBJECTS AND PARTICIPATING INSTITUTIONS

Basic statistics

THE STUDY GROUP'S "New Liver Cancer Therapies" (NLCT) study was based on data from patients with advanced HCC treated using sorafenib or miriplatin up until 22 December 2010 at the participating institutions. Clinical data were recorded by each institution in case report files (CRFs) created by the Study Group. Of the patients enrolled in this study, 264 were treated with sorafenib and 535 were treated with miriplatin. Any input variables that were unclear were excluded from the analyzed data. After analyzing collecting data on the use of these drugs, the Study Group compiled current opinions on proper use based on published papers, academic conference papers and reports from the private sector. The Study Group proposed a series of "clinical questions" (CQ) on issues pertaining to practical medical care and summarized the current evidence in response to each of these CQ in the form of "scientific statements", as well as making "recommendations".

Participating institutions

The 16 institutions that participated in this study were: Kinki University; Chiba University; Yamaguchi University; Kurume University; Kyorin University; Showa University; Ehime University; Okayama University; Kyoundo Hospital; Tohoku University; Osaka University; Gifu University; Hyogo College of Medicine; Toranomon Hospital; Saitama Medical University; and Kanazawa University.

RESULTS

Sorafenib therapy

Indications

CQ1-1 For which patients with HCC is sorafenib therapy indicated?

Recommendation Sorafenib therapy is indicated in HCC patients with good performance status (PS) and Child–Pugh class A for whom surgical resection, local ablation therapy (LAT), and transcatheter arterial chemoembolization (TACE) are not possible or not indicated.

The safety and efficacy of sorafenib has not been established in Child–Pugh class B/C patients.

Furthermore, the usefulness of sorafenib as adjuvant chemotherapy after resection, LAT, or TACE of HCC has not been demonstrated.

Scientific statement Two randomized, placebo-controlled trials demonstrating the usefulness of sorafenib were conducted on patients in whom surgical resection, LAT and TACE were not indicated or who were unresponsive to TACE.[1,2]

The Japan Society of Hepatology provides the following definitions for impossible and refractory cases to TACE.[3]

Definition of "Impossible cases to TACE"

1 Deterioration of treated vessel resulting in inability to select catheter for insertion into the nutrient vessel;
2 Deterioration in hepatic function to Child–Pugh class C due to repeated treatment;
3 Patients with tumor thrombus in main trunk or first branch of portal vein;
4 Patients with large arterio-portal shunts.

Definition of "Refractory cases to TACE"

(1) Intrahepatic lesion(s)
    (i) Poor Lipiodol deposits (≤50%) observed on at least two consecutive occasions in computed tomography (CT) assessment of therapeutic response immediately after (>1 month) correctly performing TACE;

(ii) Multiple new lesions observed on at least two consecutive occasions in CT assessment of therapeutic response immediately after (>1 month) TACE;

(2) Appearance of vascular invasion;

(3) Appearance of distant metastasis;

(4) Tumor markers.

(i) Continued increase in tumor markers with transient decrease only immediately after TACE procedure.

In the present NLCT study, as many as 91% of patients underwent prior treatment, in whom 29% received hepatic arterial infusion chemotherapy (HAIC). Comparison of the characteristics of the remaining NLCT study patients with those of previous clinical trials[1,2,4–6] is presented in Table 1.

An adverse event (AE) report on all-patient special drug use surveillance (SDUS) conducted in Japan[7] contains analysis and reporting of AEs for 777 patients for whom CRFs were collected up until 19 December 2009.

That report compared the clinical characteristics for 51 of these 777 patients who died within 30 days of treatment ("early death group") and the 382 patients who survived for ≥61 days ("control survival group"). The data indicate that the prevalence of Eastern Cooperative Oncology Group (ECOG) PS grades ≥2 tended to be high among patients in the "early death group" at 5.9% compared with those in the "control survival group" at 0.5%, suggesting the need to carefully follow the course of patients with poor PS. In the NLCT study, 98% of patients had a PS score of 0–1.

In terms of hepatic function, two randomized, placebo-controlled trials demonstrating the usefulness of sorafenib were conducted on Child–Pugh class A patients.[1,2]

Meanwhile, in the NLCT study, 81% of evaluable patients were Child–Pugh class A, and 94% had a Child–Pugh score of ≤7. Comparison of treatment results of Child–Pugh class A and B patients did not reveal any difference in tumor control rates (46% vs. 50%; $P = 0.52$), but overall survival (OS) was inferior in Child–Pugh class B patients (median OS: 11.5 months vs. 5.2 months; $P < 0.01$).

In a Phase I trial conducted in Japan, no clear increase in toxicity was observed in Child–Pugh class B patients compared with Child–Pugh class A patients.[8] On the other hand, the aforementioned SDUS found that hepatic functional reserve was poor in the "early death group" compared to the "control survival group".[4]

A Phase II study of sorafenib therapy in HCC patients including those with Child–Pugh class B is currently underway in Japan (UMIN [University Hospital Medical Information Network] 000002972). Another study currently being conducted worldwide is the Global Investigation of therapeutic decisions in HCC and of its treatment with sorafenib (GIDEON); a large-scale prospective study on actual sorafenib therapy of patients with unresectable HCC. The GIDEON study is recruiting 3000 patients from over 400 sites in more than 40 countries in the Asia-Pacific region, Europe, USA, Latin America, and Japan.[9] The study's first interim analysis has been released and the findings of 511 recruited patients including those in Child–Pugh class B have been examined. No significant difference in grade 3 or 4 AEs was found to exist between Child–Pugh class A and B patients, at 31% and 38%, respectively.[10] Future GIDEON study analyses are expected to provide crucial information concerning the safety of sorafenib for Child–Pugh class B patients.

A Phase III study of post-TACE adjuvant sorafenib chemotherapy versus placebo conducted in Japan and South Korea failed to demonstrate the usefulness of sorafenib administration.[11] In addition, a Phase III placebo-controlled trial of adjuvant sorafenib chemotherapy following radical treatment (either surgical resection or LAT) of HCC (STORM Trial) is currently underway.[12]

The NLCT study did not include any patients treated with sorafenib as adjuvant chemotherapy.

Method of administration

CQ1-2 What is the optimal dosage regimen for sorafenib therapy?

Recommendation The standard dosage regimen for sorafenib therapy is 400 mg administered twice daily (800 mg/day).

The safety and efficacy of sorafenib therapy in combination with other anti-neoplastic agents or TACE have not been established.

Scientific statement In the two aforementioned randomized, placebo-controlled trials demonstrating the usefulness of sorafenib, a single 400 mg dose of sorafenib was administered twice daily (800 mg/day),[1,2] and usefulness was not observed at a reduced dosage. A high-fat diet reportedly lowers the plasma concentration of sorafenib so administration should be avoided from 1 h before to 2 h after meals.

Reduced dose regimen due to AEs was conducted in the abovementioned studies as follows:

Step-down dose (step 1): 400 mg once a day

Step-down dose (step 2): 400 mg every another day

Table 1 Characteristics of patients receiving sorafenib therapy

	NLCT Study ($n = 264$) %	SDUS[4,6] ($n = 777$) %	SHARP Trial[1] ($n = 299$) %	Asia-Pacific Trial[2] ($n = 150$) %	Sorafenib phase II[5] ($n = 137$) %
Age (years)					
Median	70		64.9 ± 11.2	51	69
Range	33–87		(mean ± SD)	23–86	28–86
Gender					
Male	79		87	84.7	71
PS					
0	83	69.5	54	25.3	50
1	15	26.5	38	69.3	50
Child–Pugh class					
A	81	88.2	95	97.3	72
B	19	9.9	5	2.7	28
HBs antigen					
Positive	20	24.6	19	70.7	17
HCV antibody					
Positive	62	52.2	29	10.7	48
Prior treatment					
Yes	91	91.2	49		
Resection	31		19		
LAT	47		15		
TACE	78		29		
HAIC	29				
Advanced vascular invasion					
Yes	18		36	36.0	
Extrapulmonary lesion(s)					
Yes	51	54.4	53	68.7	–
Lymph node(s)	22	15.4	30	52	–
Lung(s)	26	30.6	22	30.7	–
Maximum tumor size (mm)	34				
Range	7–170				
≥30 mm	59				
Stage	†	‡	§	§	‡
I	1	1.2			0
II	9	4.8			3
III	30	20.7	B: 18	B: NE	31
IV A	17	23	C: 82	C: 95.3	66
IV B	43	47.6			
T-Bil (mg/dL)					
Median	0.8		0.7		
Range	0–7.7		0.1–16.4		
Alb (g/dL)					
Median	3.5		3.9		
Range	1.7–4.8		2.7–5.3		
AFP (ng/mL)					
Median	218		44.3		
Range	0.8–252150		0–2080000		
≥10	84			77.3	76

†Japanese Classification of Liver Cancer.
‡UICC classification.
§BCLC classification.
AFP, α fetoprotein; Alb, albumin; HAIC, hepatic arterial infusion chemotherapy; HBs, Hepatitis B surface antigen; HCV, hepatitis C virus; LAT, local ablation therapy; NLCT, New Liver Cancer Therapies; PS, performance status; SD, standard deviation; SDUS, special drug use surveillance; SHARP, sorafenib hepatocellular carcinoma assessment randomized protocol; TACE, transcatheter arterial chemoembolization; T-Bil, total bilirubin.