the expression of NK activating molecules on MC38 cells. Importantly, the administration of this low dose α-GalCer did not cause liver injury. The antitumor effect of the combination therapy of α-GalCer and 5-FU against MC38 and Colon26 liver tumors was stronger than that of 5-FU alone or α-GalCer alone. The antitumor effect of the combination therapy of low dose (0.4 μg/mouse) α-GalCer and 5-FU was equal to that of the combination therapy of high dose (2 μg/mouse) α-GalCer and 5-FU (Aketa *et al.*, unpublished data). Our results might offer new chemo-immunotherapy strategies, especially for those patients with advanced stages of cancer.

In this study, we demonstrated that 5-FU treatment enhanced the expression of both NKG2D ligands (Rae-1 and H60) and DNAM1 ligands (CD112 and CD155) on MC38 cells. In contrast, 5-FU treatment did not affect the activating molecules on NK cells. Both pathways involving NKG2D and DNAM1 play critical roles in the activation of NK cells and have been implicated in tumor surveillance.[19] The expression of NKG2D ligands has been associated with a good prognosis in patients with colon cancer.[20] Thus, these results suggest that the upregulation of NKG2D ligand expression might improve the prognosis of patients with colon cancer. Gasser *et al.*[21] previously reported that DNA-damaging agents and DNA-synthesis inhibitors including 5-FU could induce the expression of NKG2D ligands on tumor cells. We also demonstrated that 5-FU treatment could induce the expression of NK activating molecules in MC38 liver tumor tissues but not in nontumor tissues, which was consistent with the *in vitro* results. Our present results suggest that 5-FU treatment might have strong immune-editing potential to enhance the NK sensitivity of colon cancer cells by regulating DNAM1 and NKG2D ligands.

In this study, we demonstrated that 5-FU treatment enhanced the susceptibility of MC38 cells to the cytolytic activity of liver MNCs *via* the NKG2D-NKG2D ligand pathway. Because the blocking antibody of the DNAM1-DNAM1 ligand is not commercially available, we could not evaluate the involvement of this pathway. We have previously demonstrated that membrane-bound MICA, an activating molecule of NK cells, on HCC cells is essential in the NK sensitivity of HCC cells.[12,13] The addition of both epirubicin and sorafenib enhanced the NK sensitivity of HCC cells by increasing the membrane-bound MICA.[12,13] This finding is consistent with this study of a colon cancer model. Interestingly, the expression of death receptors, such as FAS and TRAIL receptors, on MC38 cells was significantly increased by 5-FU treatment (Aketa *et al.*, unpublished data). This result may also explain the enhancement of the susceptibility of MC38 cells to the cytolytic activity of liver MNCs. We previously demonstrated that α-GalCer administration resulted in rapid and strong activation of liver NK cells and that the cytolytic activity of liver MNCs early after α-GalCer administration mainly depended primarily on liver NK cells and not on NKT or T cells.[8,10] Taken together, these results suggest that the addition of 5-FU enhanced the NK sensitivity of MC38 cells by increasing the

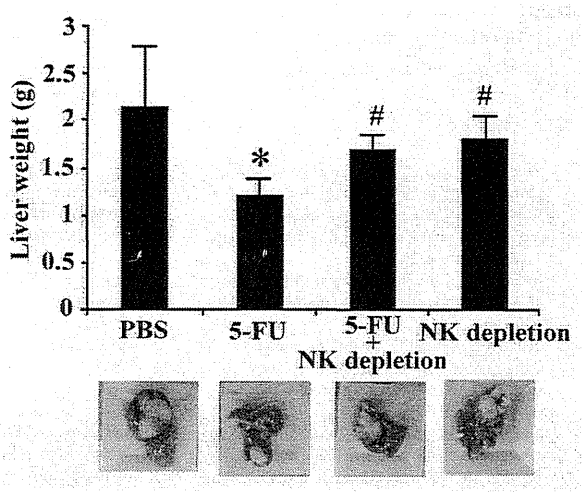

Figure 6. The antitumor effect of 5-FU against MC38 liver tumors in NK-depleted mice. To evaluate the involvement of NK cells in the antitumor effect of 5-FU, mice were injected with an anti-ASGM1 Ab. NK-depleted mice were treated with or without 5-FU (10 mg/kg body weight) for 5 consecutive days. Two weeks after the tumor injection, the livers of the treated mice were removed, and the weight was measured to examine the intrahepatic tumor growth. $*p < 0.05$ *versus* PBS group, $\#p < 0.05$ *versus* 5-FU group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

expression of Rae-1 or H60 on MC38 cells. Therefore, 5-FU treatment might be expected to enhance the susceptibility of MC38 cells to the cytolytic activity of NK cells by modifying the expression of NKG2D and DNAM1 ligands.

NK depletion decreased the antitumor effect of 5-FU against MC38 liver tumors, demonstrating that the antitumor effect of 5-FU depends on NK activity in addition to direct cytotoxicity. We also examined the antitumor effect of 5-FU against the Colon26 liver tumor model, derived from BALB/c colon cancer. The liver weights of 5-FU-treated mice were significantly lower than those of vehicle-treated mice. The depletion of NK cells also significantly inhibited the antitumor efficacy of 5-FU against Colon26 liver tumors in BALB/c mice. A significant upregulation of Rae-1, H60, CD112, and CD155 could also be observed in 5-FU-treated Colon26 cells derived from BALB/c mice (Aketa *et al.*, unpublished data). These results were consistent with the results of C57BL/6 mice and suggest that the antitumor effect of 5-FU may always depend on NK activity in the liver. The liver contains abundant NK cells. In cancer tissues that are rich in NK cells, the combination therapy of α-GalCer and 5-FU might have a potential as a new chemo-immunotherapeutic strategy.

The liver is the most common site of metastasis of gastrointestinal cancers (*i.e.*, colorectal, gastric, and pancreatic cancers). Thus, new therapeutic approaches of cancer immunotherapy for metastatic liver cancer need to be developed. We have shown here that 5-FU can enhance the NK sensitivity of cancer cells by inducing the expression of NK activating molecules in

addition to the direct cytotoxicity of 5-FU to the cancer cells. In addition, the combination therapy of α-GalCer and 5-FU showed sufficient antitumor effects against MC38 liver tumors. These findings indicate that this new combination chemo-immunotherapy might represent a particularly promising approach for patients with metastatic liver cancer.

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Valine, the branched-chain amino acid, suppresses hepatitis C virus RNA replication but promotes infectious particle formation

Hisashi Ishida [a], Takanobu Kato [b], Kenji Takehana [c], Tomohide Tatsumi [a], Atsushi Hosui [a], Takatoshi Nawa [a], Takahiro Kodama [a], Satoshi Shimizu [a], Hayato Hikita [a], Naoki Hiramatsu [a], Tatsuya Kanto [a], Norio Hayashi [d], Tetsuo Takehara [a,*]

[a] Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka, Japan
[b] Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan
[c] Exploratory Research Laboratories, Research Center, Ajinomoto Pharmaceuticals, Co, Ltd., Kanagawa, Japan
[d] Kansai Rosai Hospital, Amagasaki, Hyogo, Japan

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ABSTRACT

Background & aims: Concentrations of the branched-chain amino acid (BCAA) in the serum of patients with liver cirrhosis correlate with their liver function. Oral administration of BCAA can ameliorate hypoalbuminemia and hepatic encephalopathy. In this study, we aim to clarify the role of BCAA in regulating the replication of the hepatitis C virus (HCV).
Methods: HCV sub-genomic replicon cells, genome-length replicon cells, and cells infected with cell culture-infectious HCV (HCVcc) were cultured in media supplemented with various concentrations of BCAA, followed by evaluation of the replicon or HCV abundance.
Results: BCAA was capable of suppressing the HCV replicon in a dose-dependent manner and the effect was independent of the mTOR pathway. Of the three BCAAs, valine was identified as being responsible for suppressing the HCV replicon. Surprisingly, an abundance of HJ3-5(YH/QL), an HCVcc, in Huh7 cells was augmented by BCAA supplementation. In contrast, BCAA suppressed an abundance of HJ3-5(wild), an HCVcc that cannot assemble virus particle in Huh7 cells. Internal ribosome entry site of HCV was shown to be a target of BCAA. Single-cycle virus production assays using Huh7-25 cells, which lacked CD81 expression, revealed that BCAA, especially valine, promoted infectious virus particle formation with minimal effect on virus secretion. Thus, BCAA was found to have two opposing effects on HCV production: suppression of the HCV genome RNA replication and promotion of infectious virus formation.
Conclusions: BCAA accelerates HCV production through promotion of infectious virus formation in infected cells despite its suppressive effect on HCV genome replication.

1. Introduction

Persistent infection of hepatitis C virus (HCV) causes progressive liver disease in humans. Chronic inflammation in the liver leads to the accumulation of fibrosis and an eventual progression to liver cirrhosis. In patients with decompensated liver cirrhosis, a change in plasma amino acid composition is frequently observed. In particular, the ratio of branched-chain amino acid (BCAA) to aromatic amino acid (AAA), known as Fischer's ratio, decreases as the liver function deteriorates [1]. In such cirrhotic patients, hypoalbuminemia occurs, and it has been shown that oral administration of BCAA can ameliorate hypoalbuminemia and hepatic encephalopathy.

Three amino acids valine, leucine, and isoleucine are BCAAs, which are considered to be essential for protein anabolism. In addition to the role of acting as nutrient substrates, recent studies have demonstrated that BCAA also serve as physiologically active substances. BCAA have been shown to have pharmacological effects, such as induction of protein synthesis [2] and glucose metabolism [3]. In rat primary hepatocytes, albumin synthesis is significantly increased by BCAA administration, which is dependent on activation of the mammalian target of rapamycin (mTOR), mainly induced by leucine [4].

HCV replication is controlled by intracellular signaling pathways. In addition to the interferon (IFN)-induced JAK/STAT pathway, which activates interferon-stimulated genes, leading to strong anti-viral activity, activation of ERK [5], PI3 kinase/Akt [6,7], smad [8], PKC [9], and p38 [10], have been shown to be capable of regulating HCV replication. mTOR, one of the downstream molecules of Akt, phosphorylates the two substrates p70 S6 kinase and eukaryotic translation initiation factor 4E binding protein 1

* Corresponding author. Address: Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 3629.
E-mail address: takehara@gh.med.osaka-u.ac.jp (T. Takehara).

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(4EBP1). p70 S6 kinase phosphorylates ribosomal S6 protein, resulting in an increase of the protein synthesis complex. Phosphorylated 4EBP1 results in its dissociation from the eukaryotic translation initiation factor 4E (eIF4E), which consequently enables eIF4E to regulate the translation initiation. Thus, together, p70 S6 kinase and 4EBP1 are responsible for the mTOR-dependent regulation of cellular translation. Moreover, both have been demonstrated to be involved in the regulation of HCV replication [6].

The finding that BCAA, per se, can activate signaling pathways suggests that they may affect HCV replication, presumably via the activation of the mTOR pathway. However, to date, no detailed investigation has been reported. Therefore, we attempt to clarify whether BCAA have a role in regulating HCV replication by using the HCV replicon system and cell culture of infectious-HCV (HCVcc). The present study reveals that although BCAA, especially valine, suppresses HCV genome replication, they eventually promote total HCV production by accelerating viral formation.

2. Methods

2.1. Cells

The hepatoma-derived cell line Huh7 and its derivatives, Huh7.5 and Huh7-25 [11], were maintained in DMEM supplemented with 10% FCS. The HCV subgenomic replicon cell line Huh-RepSI [10], and the HCV genome-length replicon cell line 2–3 [12], both harboring the HCV-N strain (genotype 1b), were previously described. The molar ratio of the BCAA mixture was adjusted to Leu:Ile:Val = 2.0:1.0:1.2 according to data from previous studies [13]. For assays to examine the role of BCAA, cells were cultured in BCAA-deficient DMEM with 10% FCS supplemented with BCAA mixtures of various concentrations (0–2 mM).

2.2. Cell culture-infectious HCV

JFH-1 is a cell culture-infectious virus of genotype 2a as previously described [14]. HJ3-5(YH/QL) is a chimeric cell culture-infectious virus with a genome consisting of the core to NS2 sequence of genotype 1a (H77) virus placed within the background of the genotype 2a JFH-1 virus. This virus contained compensatory mutations in E1 (Y361H) and NS3 (Q1251L) [15]. These two mutations rendered the chimeric RNA highly infectious.

2.3. In Vitro transcription and transfection of synthetic RNA

Plasmid DNAs encoding HJ3-5(wild) and HJ3-5(YH/QL), a wild-type chimeric virus and a chimeric virus carrying two mutations, respectively, were linearized by *XbaI* prior to transcription. RNA was synthesized with the T7 RiboMAX Express Large Scale RNA Production System (Promega, Madison, WI, USA) following the

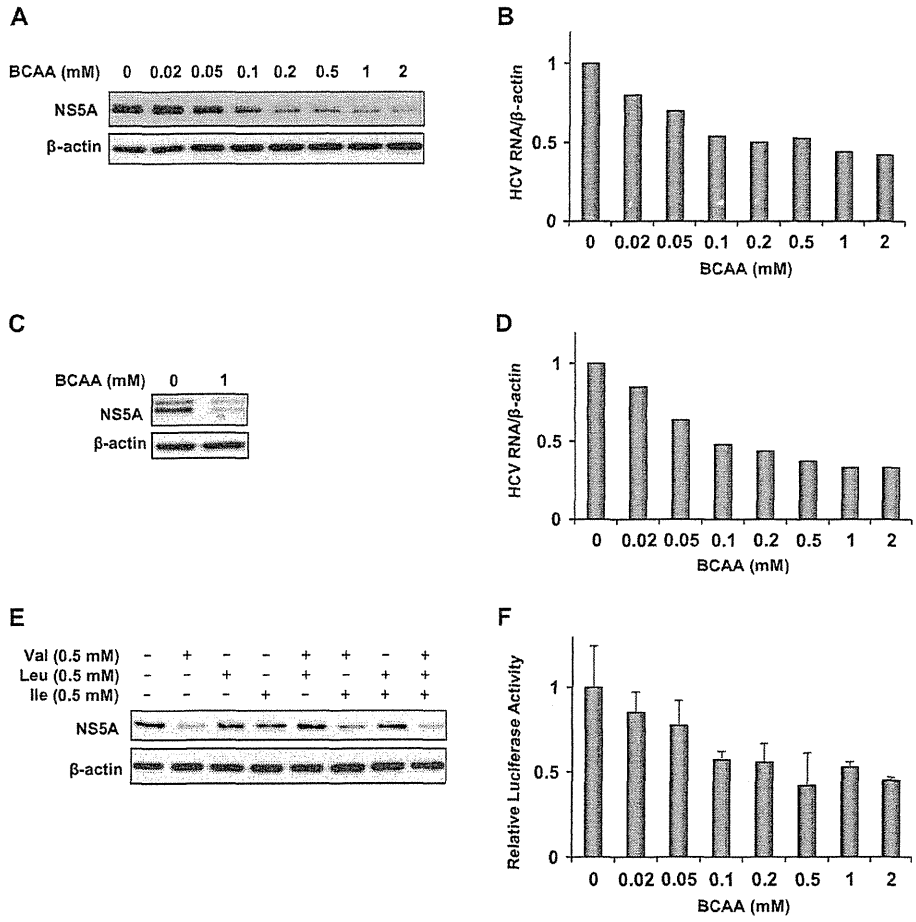

Fig. 1. BCAA limits the abundance of HCV replicon in HCV replicon cells. (A–D). Huh-RepSI (A and B) and 2–3 (C and D) cells were cultured in media for 2 days, with BCAA supplemented at concentrations of 0–2 mM. Total protein or total RNA was recovered and assayed for immunoblot (A and C) or real-time RT-PCR (B and D), respectively. (E) Three BCAAs (0.5 mM each) were added to BCAA-free culture medium of Huh-RepSI. After incubation for 2 days, immunoblot analysis of NS5A and beta-actin were performed. (F) Huh-RepSI cells were transfected with pRLHL, cultured in media with various BCAA concentrations between 0 and 2 mM. After incubation for 2 days, a dual luciferase assay was performed. The ratio of firefly luciferase activity to renilla luciferase activity was then calculated.

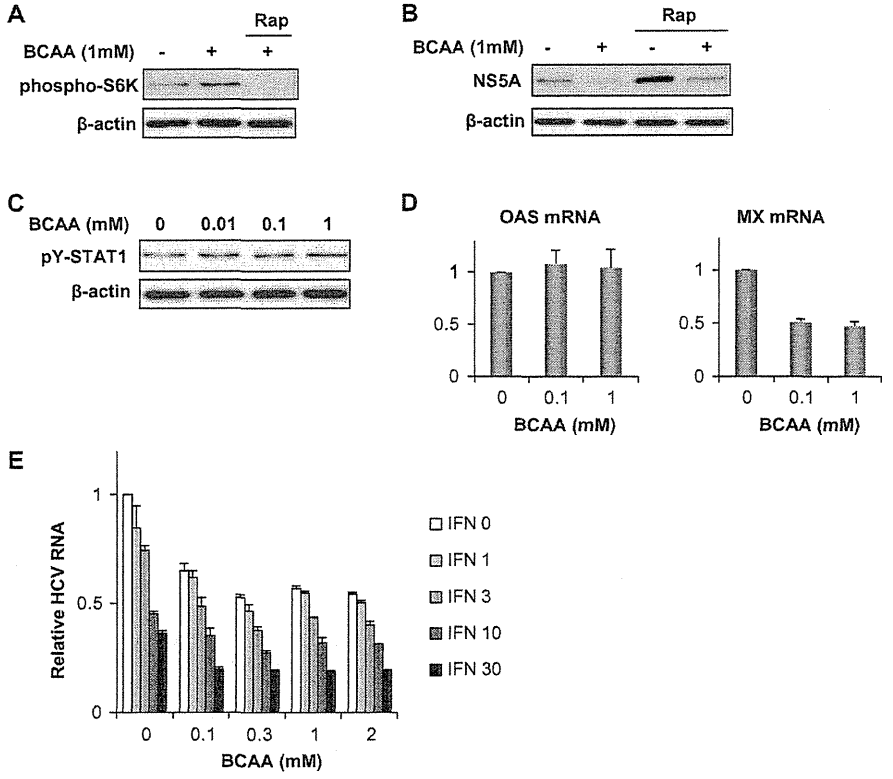

Fig. 2. BCAA-induced suppression of HCV replicon is independent of mTOR or JAK/STAT signaling. (A) Immunoblot of phosphorylated p70 S6 kinase and beta-actin in Huh-RepSI cells cultured in a medium with or without BCAA (1 mM). Rapamycin was added at 100 nM to the BCAA-containing medium. (B) Immunoblot analysis of NS5A and beta-actin in Huh-RepSI cells cultured in a medium with 1 mM BCAA or rapamycin (100 nM). (C) Huh-RepSI cells were incubated in media with various BCAA concentrations (0, 0.01, 0.1, 1 mM), and then, immunoblot analyses of phosphorylated STAT1 (Tyr701) and beta-actin were performed. (D) Huh-RepSI cells were incubated in media with various BCAA concentrations (0, 0.1, 1 mM), and then, a real-time RT-PCR analysis, for expression of OAS and MX, was performed. (E) Huh-RepSI cells were incubated in culture media with various BCAA concentrations (0–2 mM) and IFN-α (0–30 U/ml). HCV RNA abundance was normalized with beta-actin allowing the relative HCV RNA levels to be calculated, setting the HCV RNA level of 0 U/ml IFN-α and 0 mM BCAA as 1. Rap: rapamycin.

manufacturer's suggested protocol. For electroporation, Huh7 cells were washed twice with ice cold phosphate-buffered saline (PBS) and resuspended at a concentration of 10^7 cells/ml in PBS. Subsequently, 10 μg of RNA was mixed with 500 μl of the cell suspension in a cuvette, with a gap width of 0.2 cm (GenePulser II System; Bio-Rad, Hercules, CA, USA). The mixture was immediately subjected to two pulses of current with the intensities of 1.2 kV, 25 μF, and maximum resistance. Following a 10-min incubation at room temperature, the cells were transferred into growth medium.

2.4. Titration of HCV infectivity

Huh-7.5.1 cells were seeded in 96-well plates at a density of 1×10^4 cells per well 24 h prior to culture media inoculation of the HCV infected cells. Three days after infection, HCV-positive cells were detected with mouse monoclonal antibody that recognized core proteins stained with an Alexa Fluor 488 anti-mouse secondary antibody (Invitrogen, Carlsbad, CA, USA). The infectivity titer was expressed as focus-forming units per mL of supernatant (ffu/mL), expressing the mean number of HCV core-positive foci. The intracellular infectivity and specific intracellular infectivity titer were determined as described previously [16].

3. Results

3.1. BCAA suppresses the amount of HCV replicon

To investigate the role of BCAA in HCV replication, we first examined the effect of BCAA on the HCV replicon. An HCV subgenomic replicon cell line, Huh-RepSI, was incubated in culture medium that contained various concentrations of BCAA (0–2 mM) for 2 days. HCV replicon RNA, as well as the amount of protein, was suppressed by adding BCAA in a dose-dependent manner (Fig. 1A and B). To confirm the effect of BCAA, another replicon cell line, 2-3, carrying a genome-length HCV replicon, was used. In this experiment, suppression of the replicon by BCAA was observed, which is in agreement with the Huh-RepSI assay (Fig. 1C and D). This activity suggested that BCAA possessed the ability to suppress HCV replication.

Three BCAAs exist: valine, leucine, and isoleucine. As previously demonstrated, leucine contains the biological activity to activate mTOR. In addition, we showed that mTOR, which is activated by PI3 kinase/Akt, was able to suppress HCV replication [6]. Therefore, it is possible that the BCAA-mediated suppression of HCV replication was due to leucine. To test this hypothesis, the three amino acids were added independently to BCAA-deficient medium while monitoring the HCV replication level. Unexpectedly, the result refuted the hypothesis (Fig. 1E). Compared to the cells cultured in BCAA-deficient medium, supplementation with only valine efficiently suppressed the HCV replicon, whereas leucine did not; instead, it caused a slight increase. This result showed that BCAA, especially valine, but not leucine, have a suppressive effect on HCV replication.

3.2. BCAA suppresses HCV IRES activity

HCV replication can be controlled by HCV specific translation regulated by IRES, the 5′ UTR region of HCV. Therefore, we next

investigated the effect of BCAA on HCV IRES activity. To do this, we utilized a dicistronic vector, pRLHL, which consists of firefly luciferase driven by HCV IRES and renilla luciferase, translated in a cap-dependent manner (Sup. Fig. 1). Relative HCV IRES activity was evaluated using the ratio of IRES-specific luciferase activity to the cap-dependent luciferase activity. As shown in Fig. 1F, HCV IRES activity was suppressed by BCAA in a dose-dependent manner, which is similar to the result of the replicon abundance (Fig. 1A and B). Thus, the BCAA-mediated suppression of HCV replication is likely due to the inhibition of HCV IRES activity.

3.3. BCAA-mediated suppression of HCV replicon is independent of the mTOR and JAK/STAT pathways

Previous reports have demonstrated that BCAA is capable of activating mTOR [4], and we have reported that mTOR suppresses HCV replication [6]. Therefore, we examined the contribution of mTOR activation on BCAA-mediated suppression of the HCV replicon. Administration of BCAA efficiently phosphorylated p70 S6 kinase, whereas rapamycin completely inhibited its phosphorylation (Fig. 2A). Despite rapamycin enhancing the amount of HCV replicon, BCAA could efficiently suppress it, even in rapamycin-containing medium (Fig. 2B), suggesting that the suppression of the HCV replicon by BCAA is independent of mTOR activation.

The IFN-JAK/STAT signal is known to be an anti-virus pathway, induced under the condition of virus infection. HCV replication is efficiently inhibited by interferon. Therefore, we examined whether BCAA could modify the IFN signal. First, we performed an immunoblot analysis and evaluated the status of STAT1 activation, in the presence or absence of BCAA. However, the phosphorylated STAT1 level was not altered by BCAA in Huh-RepSI cells, and ISG induction was not observed; instead, the expression level of Mx was suppressed by BCAA (Fig. 2C and D). A previous study showed that rapamycin diminished the suppressive effect of IFN-α toward HCV replication via the suppression of ISG induction [17]. Subsequently, we examined the HCV replicon abundance in cells that were cultured in media with various concentrations of BCAA and IFN-α stimuli. Even with the depletion of BCAA, IFN-α efficiently and dose-dependently suppressed HCV replicon abundance. However, IFN-α-induced anti-HCV activity was not augmented by BCAA supplementation (for example, the replicon RNA level decreased to approximately 30% in both BCAA-depleted medium and 2 mM BCAA-supplemented medium) (Fig. 2E). Consequently, BCAA did not influence JAK/STAT activation, and therefore, the suppression of HCV replicon by BCAA may have been independent of the IFN-α-induced signaling pathway.

3.4. BCAA enhances HCVcc production

Next, we examined the impact of BCAA on HCVcc, a system retaining the entire HCV life cycle in a cultured cell. Here, we used HJ3-5(YH/QL), a chimeric HCV of genotype 1a (H77) and 2a (JFH-1). Surprisingly, the results of HJ3-5(YH/QL) were opposite to that of the HCV replicon: HCV abundance was upregulated in a BCAA dose-dependent manner (Fig. 3A). The HCV replicon contains NS3 to NS5B proteins, which are required for HCV RNA genome replication, but not core, E1 and E2 proteins, which are structural proteins required for viral particle formation. The discrepancy in the results between HCV replicon cells and HCVcc-infected cells might be due to differences in virus particle production.

To investigate this discrepancy, we used the wild-type HJ3-5, designated as HJ3-5(wild). As described in the Methods section, HJ3-5(YH/QL) or the HCVcc used in this study, carries two amino acid substitutions at amino acid 361 and amino acid 1251, within E1 and NS3, respectively. These two mutations render the chimeric RNA highly infectious [15]. However, without these mutations, virus particle assembly and consequent virion release from the cells to the medium would not occur. This process is thought to be due to impaired association of the HCV proteins originating from different genotypes, whereas there is no apparent change in the HCV RNA replication level in the cells [15].

We introduced the *in vitro* transcribed genome RNA of HJ3-5(wild) or HJ3-5(YH/QL) into Huh7 cells with electroporation, and then, we examined the effect of BCAA on the cell line. Normally, synthesized HCV RNA introduced into cells executes replication by utilizing HCV proteins encoded in the genome and host factors, resulting in a robust increase that is detectable after 2–3 days. BCAA decreased the abundance of HJ3-5(wild), which was similar to their effect on the HCV replicon (Fig. 3B and C). Thus, HJ3-5(wild), a virus that is defective in virus particle formation, revealed the opposite reaction to BCAA compared to the virus HJ3-5(YH/QL), a virus that is competent in virus particle formation. Together, these findings revealed that although BCAA had the ability to suppress the HCV genome replication, they promoted viral production by enhancing other steps, which included virus assembly, virus particle release and cell re-infection.

3.5. BCAA promotes infectious HCV particle formation, not virus secretion

To further assess the BCAA intracellular mechanisms that influence the HCV life cycle, we adopted a single-cycle virus production assay [18]. We used Huh7-25 cells due to the lack of surface expression of one of the cellular HCV receptors, CD81, thus being non-permissive to HCV infection. Because HCV genome replication or virus production is intact in Huh7-25, we can evaluate viral replication and secretion without the influence of re-infection.

First, we studied the replication levels of the infectious virus, JFH-1, in Huh7-25 cells. The full length of the JFH-1 genome RNA

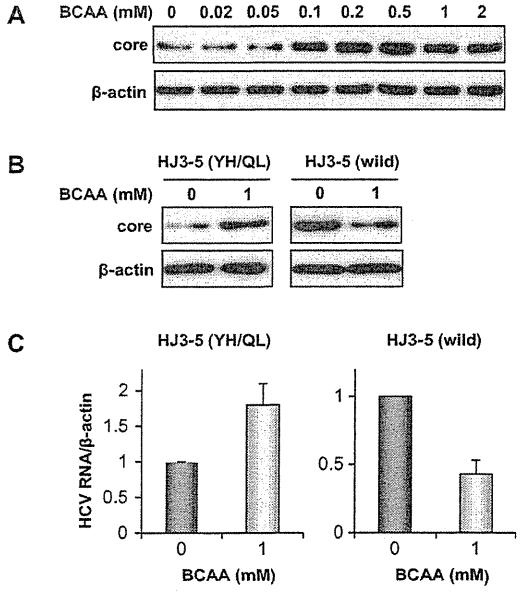

Fig. 3. HCVcc abundance was increased by BCAA. (A) HCVcc-infected Huh7 cells were cultured in media with various BCAA concentrations (0–2 mM). After incubation for 2 days, and an immunoblot analysis of core and beta-actin was performed. (B and C) Synthesized HCV genome RNA of HJ3-5 (YH/QL) or HJ3-5 (wild) was transfected into Huh7 cells via electroporation. After incubation for 24 h, cells were split into 6-well plates and incubated for 2 days in a culture medium with or without 1 mM BCAA. After the cells were harvested, immunoblot analysis of core and beta-actin (B) and real-time RT-PCR analysis (C) were performed.

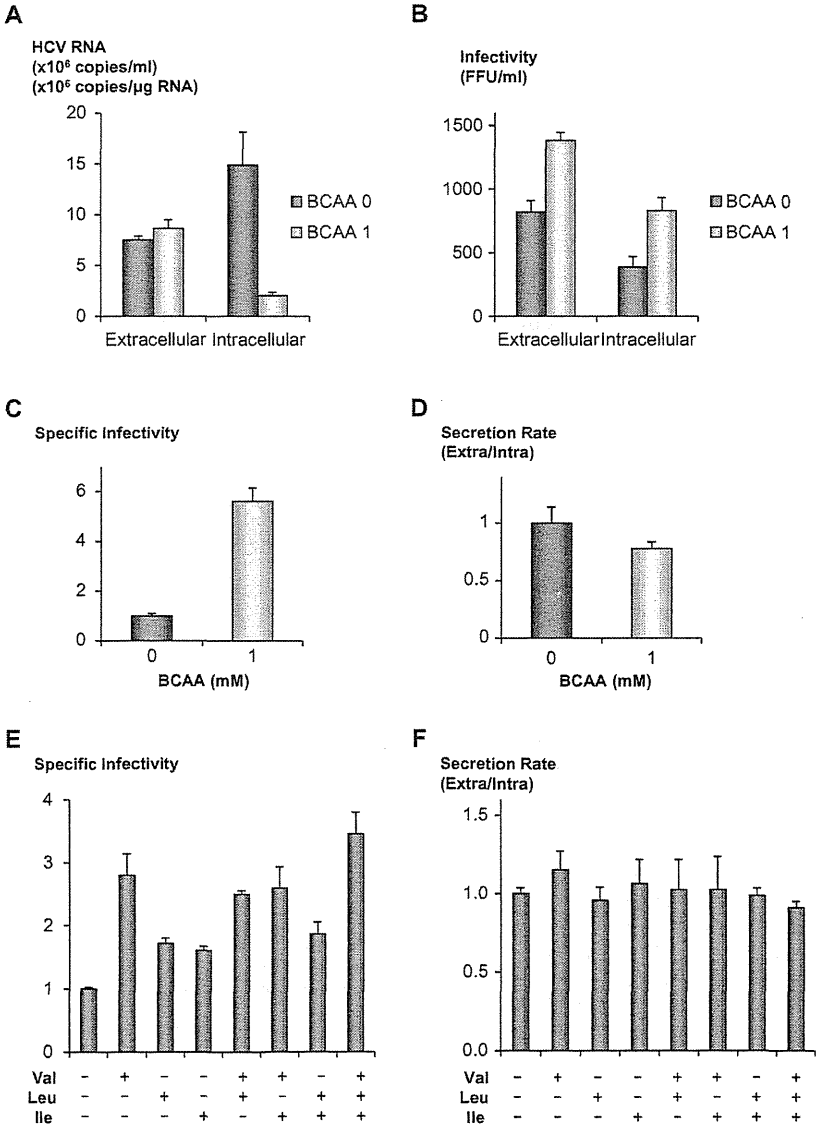

Fig. 4. Single-cycle virus production assay indicates a promoting effect of BCAA on virus formation. (A) Huh7-25 cells were transfected with *in vitro*-transcribed RNA of JFH-1, incubated in media with or without BCAA, followed by the RNA levels in the media or in the cells being calculated using the real-time quantitative RT-PCR method. (B) Infectivities in the media or in the cell lysates were measured. (C) Specific infectivities were calculated by dividing the infectivities by the HCV RNA amounts. (D) Secretion rates were calculated by dividing extracellular infectivities by intracellular infectivities. The data were presented as ratios defining the value of BCAA at 0 mM as 1. (E and F) Specific infectivities and secretion rates in the presence of valine (0.5 mM), leucine (0.5 mM), or isoleucine (0.5 mM). The data were presented as ratios defining the value with no BCAA as 1.

was translated *in vitro* and transfected into the Huh7-25 cells. The cells were cultured in media, with or without 1 mM of BCAA, with the RNA levels being monitored using quantitative RT-PCR. As observed in the experiment of replicon cells or virus particle formation-deficient viruses, the intracellular RNA level of HCV was suppressed by the presence of BCAA (Fig. 4A). However, the levels of extracellular HCV RNA were similar. Despite the suppression of intracellular HCV RNA levels by BCAA-containing medium, the infectivity titer of the intracellular virus in the cells treated with 1 mM BCAA was significantly higher than that of the cells with 0 mM BCAA (Fig. 4B). Extracellular infectivity titers were similar to those of intracellular infectivity. The specific infectivity of intracellular virus was calculated by dividing the infectivity titer by the HCV RNA level and this revealed that cultivation of the cells in a medium of 1 mM BCAA resulted in a 5.6-fold higher specific virus infectivity than that of 0 mM BCAA (Fig. 4C). Next, we measured virus secretion rates by dividing extracellular infectivity titers by intracellular infectivity titers. There was a minimal difference between infectious virus particle secretions (Fig. 4D). Thus, these results indicated that the infectious virion production was promoted in the BCAA-supplemented medium, although the virus RNA replication was suppressed.

In the study using replicon cells, valine was shown amino acid responsible for regulating HCV RNA replication (Fig. 1E). Finally, we assessed the effect of individual BCAA on virus production. HCV infected cells were cultured in media containing each amino acid at 0.5 mM or a combination of them and subsequently specific infectivity and secretion rate were examined (Fig. 4E and F). Among the three BCAAs, valine promoted infectious virus production most effectively, while leucine and isoleucine promoted infectious virus production modestly. Secretion rates did not show a significant difference.

4. Discussion

In the present study, we investigated the role of BCAA in the HCV life cycle and discovered that these amino acids suppress HCV genome replication but promote viral particle formation. Thus far, many reports have indicated that various cellular factors are involved in the regulation of HCV. In particular, intracellular signaling pathways are important modulators for HCV genome replication [5–10]. BCAA, specifically leucine, were demonstrated to have a role in activating the mTOR pathway, leading to protein synthesis such as upregulation of albumin [4] and HGF production [19]. Recently, mTOR was reported to be involved in IFN-α signaling [17]. IFN-α induced phosphorylation of STAT1 was diminished by rapamycin (but not by LY294002, a PI3 kinase inhibitor). Consequently, rapamycin inhibited the IFN-stimulated regulatory element. Although we demonstrated that BCAA can activate mTOR (Fig. 2A), the inhibition of mTOR revealed that it was not the main pathway for the BCAA suppression of HCV replication. BCAA supplementation did not change the STAT1 phosphorylation status, nor did it induce ISG expression, indicating that the JAK/STAT pathway was not relevant for the suppression of HCV replication. Considering that leucine, the factor required for mTOR activation, did not actually take part in regulation of the HCV replicon (Fig. 1E), it was not surprising that mTOR was shown to not be the responsible molecule.

Very recently, Honda et al. demonstrated that STAT1 phosphorylation was increased by BCAA in a dose-dependent manner [20]. They showed that BCAA increased the phosphorylation levels of STAT1, Foxo3a and p70 S6 kinase leading to downregulation of Socs3 expression and HCV replication. The range of BCAA concentration examined in the present study was between 0 and 2 mM. We ranged the concentration of BCAA between 0 and 2 mM because its concentration in blood is approximately 1.6 mM after oral administration of 5 g of BCAA. However, in the Honda et al. study, the BCAA concentration at which STAT1 was efficiently phosphorylated was more than 4 mM, whereas at 2 mM or less, no obvious increase in phospho-STAT1 was observed. Therefore, we may have detected no change in phospho-STAT1 due to BCAA levels used in this study. Thus, BCAA may be capable of suppressing HCV genome replication at a low concentration by inhibiting HCV IRES activity while decreasing virus replication by augmentation of IFN signaling at high concentrations.

Although BCAA suppressed replication of HCV replicon, they increased HCVcc production in infected cells. The life cycle of HCV has many steps that are required to achieve infection, such as attachment to the cell surface, endocytosis of the virus, uncoating and releasing genome RNA, RNA replication, polyprotein synthesis and processing, viral assembly, and release of progeny virus. Among these, the HCV replicon system only represents the steps of genome RNA replication and non-structural protein synthesis in the cells, and BCAA affects these by impairing protein synthesis via suppression of HCV IRES activity. However, HCVcc replication requires all of these steps. We assumed that the increase of HCVcc due to BCAA indicated that some step(s) must be upregulated by BCAA to the extent of overcoming the decreased genome replication. The study of particle formation-deficient viruses suggested that virus assembly or some downstream step in the virus life cycle was critical for the augmentation of HCVcc by BCAA. A single-cycle virus production assay indicated that the production of an infectious virus was prominent in the presence of BCAA, while virus secretion was not strongly affected. Although HCV genome replication was suppressed by BCAA, more infectious virus particles were secreted into the media, and they could have re-infected the Huh7 cells. We suggest that the abundant infectious HCV in BCAA-supplemented medium causes amplification of the virus during repetition of such re-infection, which leads to an accumulation of HCV in the cells, and thus, the abundance of HCV RNA in the cells with BCAA medium overcomes that without BCAA. Further investigation is needed on the detailed mechanisms defining how BCAA regulates HCV particle formation. Clarification of this process could contribute to new insights into HCV replication and could also suggest a basis for treatment of HCV patients.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.051.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Incidence of hepatocellular carcinoma in HCV-infected patients with normal alanine aminotransferase levels categorized by Japanese treatment guidelines

Naoki Harada · Naoki Hiramatsu · Tsugiko Oze · Ryoko Yamada · Mika Kurokawa · Masanori Miyazaki · Takayuki Yakushijin · Takuya Miyagi · Tomohide Tatsumi · Shinichi Kiso · Tatsuya Kanto · Akinori Kasahara · Masahide Oshita · Eiji Mita · Hideki Hagiwara · Yoshiaki Inui · Kazuhiro Katayama · Shinji Tamura · Harumasa Yoshihara · Yasuharu Imai · Atsuo Inoue · Norio Hayashi · Tetsuo Takehara

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Abstract
Background This study was conducted to evaluate Japanese treatment guidelines for patients with chronic hepatitis C virus (HCV) infection and normal alanine aminotransferase (N-ALT) levels from the viewpoint of the incidence of hepatocellular carcinoma (HCC).
Methods Four groups of patients with chronic HCV infection treated with pegylated interferon (Peg-IFN) plus ribavirin, and classified according to the N-ALT guidelines, were examined for HCC incidence: group A ($n = 353$), ALT $\leq$30 IU/L and platelet (PLT) $\geq 15 \times 10^4/mm^3$; group B ($n = 123$), ALT $\leq$30 IU/L and PLT $<15 \times 10^4/mm^3$; group C ($n = 233$), $30 <$ ALT ≤ 40 IU/L and PLT $\geq 15 \times 10^4/mm^3$; and group D ($n = 100$), $30 <$ ALT ≤ 40 IU/L and PLT $<15 \times 10^4/mm^3$. The mean observation period was 36.2 ± 16.5 months
Results In groups A and C, the HCC incidence was low even in patients with non-response (NR) (cumulative rates at 3 years, 0.0 and 2.9 %, respectively). In groups B and D, 14.5 and 5.3 % of NR patients had developed HCC at 3 years, but none of the patients with sustained virologic response (SVR) or relapse had developed HCC. In group B, no patients with mild fibrosis developed HCC irrespective of the antiviral effect of the treatment. Among patients with PLT $<15 \times 10^4/mm^3$ (group B plus group D), the HCC incidence was significantly lower in patients with SVR and relapse than in NR patients ($p < 0.001$, $p = 0.021$, respectively).
Conclusion These results suggest that N-ALT patients with PLT $<15 \times 10^4/mm^3$ could be candidates for early antiviral therapy.

N. Harada · N. Hiramatsu (✉) · T. Oze · R. Yamada · M. Kurokawa · M. Miyazaki · T. Yakushijin · T. Miyagi · T. Tatsumi · S. Kiso · T. Kanto · A. Kasahara · T. Takehara
Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, 565-0871 Suita, Osaka, Japan
e-mail: hiramatsu@gh.med.osaka-u.ac.jp

M. Oshita
Osaka Police Hospital, Osaka, Japan

E. Mita
National Hospital Organization Osaka National Hospital, Osaka, Japan

H. Hagiwara · N. Hayashi
Kansai Rosai Hospital, Amagasaki, Japan

Y. Inui
Hyogo Prefectural Nishinomiya Hospital, Nishinomiya, Japan

K. Katayama
Osaka Medical Center for Cancer and Cardiovascular Disease, Osaka, Japan

S. Tamura
Minoh City Hospital, Minoh, Japan

H. Yoshihara
Osaka Rosai Hospital, Sakai, Japan

Y. Imai
Ikeda Municipal Hospital, Ikeda, Japan

A. Inoue
Osaka General Medical Center, Osaka, Japan

Keywords Hepatitis C virus · Normal alanine aminotransferase · Pegylated interferon plus ribavirin combination therapy · Cumulative carcinogenesis rate · Treatment guidelines

Introduction

Continuous hepatitis C virus (HCV) infection causes liver inflammation and can lead to liver fibrosis, which may progress to cirrhosis and hepatocellular carcinoma (HCC) [1–4]. Because HCV carriers with persistent normal alanine aminotransferase (PNALT) levels have minimal liver inflammation and the progression of liver fibrosis in such patients is slow, they are generally considered to be at low risk for carcinogenesis [5–7]. Moreover, patients with PNALT had not been considered as candidates for antiviral therapy in the era of interferon (IFN) monotherapy because of reports of ALT flare-up owing to antiviral therapy in some cases (47–67 %) [8–10].

However, in recent years, the antiviral efficacy of pegylated IFN (Peg-IFN) plus ribavirin combination therapy for patients with chronic HCV infection has been reported to be equivalent for patients with normal alanine aminotransferase (N-ALT) levels and those with elevated ALT levels [11–15]. In addition, for patients with PNALT, there have been fewer cases of ALT flare-up caused by Peg-IFN plus ribavirin combination therapy than with IFN monotherapy [12, 15]. Thus, patients with chronic HCV infection and N-ALT have come to be treated with Peg-IFN plus ribavirin combination therapy.

Treatment guidelines for patients with chronic HCV infection and N-ALT levels have been prepared by a Japanese group conducting "Research on Hepatitis" supported by Health and Labour Sciences Research Grants from the Japanese Government. In these guidelines, HCV carriers with N-ALT ($\leq$40 IU/L) are categorized into four groups according to their ALT levels ($\leq$30 or $\geq$31 IU/L) and platelet (PLT) counts ($\geq$15 or $<15 \times 10^4/mm^3$). Briefly, the therapeutic strategies are as follows: patients with ALT levels of more than 31 IU/L are candidates for antiviral treatment, but observation is recommended for patients with ALT levels of <30 IU/L. However, the goal of antiviral treatment is to improve the long-term prognosis, including inhibition of HCC. Therefore, the indication of antiviral therapy for patients with chronic HCV infection and N-ALT should be decided based on whether or not Peg-IFN plus ribavirin combination therapy can suppress the cumulative rate of HCC incidence and improve prognosis. It is thus very important to examine the effect of inhibition of HCC induced by antiviral therapy in patients with chronic HCV infection and N-ALT.

In the present study, we evaluated the treatment guidelines for patients with chronic HCV infection and N-ALT from the viewpoint of HCC inhibition by analyzing the differences in the cumulative rates of HCC incidence among the above four groups. The treatment guidelines also recommend that if patients with ALT $\leq$30 IU/L and PLT $<15 \times 10^4/mm^3$ have moderate to severe liver fibrosis (F2–4), they should receive antiviral therapy. We also evaluated the effect of Peg-IFN plus ribavirin on HCC incidence according to the degree of fibrosis in this group.

Patients and methods

This retrospective study was conducted by Osaka University and institutions participating in the Osaka Liver Forum. Among patients with chronic HCV infection who had received Peg-IFN plus ribavirin combination therapy from December 2004 to December 2009, four groups of patients, classified according to the N-ALT guidelines, who had not suffered from HCC, were examined for their HCC incidence: group A ($n = 353$), ALT $\leq$30 IU/L and PLT $\geq 15 \times 10^4/mm^3$; group B ($n = 123$), ALT $\leq$30 IU/L and PLT $<15 \times 10^4/mm^3$; group C ($n = 233$), $30 <$ ALT ≤ 40 IU/L and PLT $\geq 15 \times 10^4/mm^3$; and group D ($n = 100$), $30 <$ ALT ≤ 40 IU/L and PLT $<15 \times 10^4/mm^3$. The Kaplan–Meier method was used to examine the cumulative rates of HCC incidence in the four groups. Excluded from this study were patients who developed HCC within 12 months from the start of Peg-IFN plus ribavirin combination therapy, patients with co-infection with hepatitis B or human immunodeficiency virus, patients with drug-induced or alcoholic liver disorders, and patients with autoimmune hepatitis. The protocol was performed after obtaining informed consent from each patient before treatment in accordance with the ethical guidelines of the Declaration of Helsinki amended in 2008. This study was approved by the Institutional Review Board and registered in the Universal Hospital Medical Information Network (UMIN) Clinical Trials Registry (UMIN unique trial number, C000000197).

Treatment protocol

All patients received Peg-IFN alpha-2b (PEGINTRON; Merck & Co., Whitehouse Station, NJ, USA) plus ribavirin (REBETOL; MSD) for the duration of the study. Peg-IFN alpha-2b was given subcutaneously once weekly at a dosage of 60–150 μg/kg based on body weight (body weight 35–45 kg, 60 μg; 46–60 kg, 80 μg; 61–75 kg, 100 μg; 76–90 kg, 120 μg; 91–120 kg, 150 μg) and ribavirin was given orally twice a day at a total dose of 600–1000 mg/day based on body weight (body weight $\leq$60 kg, 600 mg;

60–80 kg, 800 mg; >80 kg, 1000 mg), according to the standard treatment protocol for Japanese patients. Dose modification according to the intensity of the hematological adverse effects followed, as a rule, the manufacturer's drug information. The dose of Peg-IFN alpha-2b was reduced to 50 % of the assigned dose if the white blood cell (WBC) count declined to $<1500/mm^3$, the neutrophil count declined to $<750/mm^3$, or the PLT count declined to $<8 \times 10^4/mm^3$, and was discontinued if the WBC count declined to $<1000/mm^3$, the neutrophil count declined to $<500/mm^3$, or the PLT count declined to $<5 \times 10^4/mm^3$. Ribavirin was also reduced, from 1000 to 600 mg, or 800 to 600 mg, or 600 to 400 mg, if the hemoglobin (Hb) level decreased to <10 g/dL, and was discontinued if the Hb level decreased to <8.5 g/dL. Both Peg-IFN alpha-2b and ribavirin had to be discontinued if there was a need to discontinue one of the drugs. During this therapy, no medicine containing iron or hematopoetic growth factors, such as erythropoietin alpha, or granulocyte–macrophage colony-stimulating factor, was administered. The serum HCV RNA levels were qualitatively analyzed using the COBAS AMPLICOR HCV Test, version 2.0 (lower limit of detection 50 IU/mL; Roche Diagnostics, Branchburg, NJ, USA), and the COBAS AMPLICOR HCV MONITOR test, version 2.0 (detection range 6–5000 KIU/ml). In the patients with HCV genotype 1, as a rule, treatment duration was 48 weeks, but the patients with detectable HCV RNA (≥50 IU/mL) at week 12 and undetectable HCV RNA (<50 IU/mL) at week 24 were treated for 72 weeks. Patients with HCV genotype 2 were treated for 24 weeks.

Definition of virologic response

A sustained virologic response (SVR) was defined as undetectable HCV RNA at the end of treatment and at 24 weeks after completion of treatment. A relapse was defined as undetectable HCV RNA at the end of treatment but detectable HCV RNA at 24 weeks after completion of treatment. A non-response (NR) was defined as detectable HCV RNA at the end of treatment.

Histological evaluation

Liver biopsy was performed immediately before initiation of the Peg-IFN plus ribavirin combination therapy. Liver biopsy specimens were scored using the METAVIR system, and the grade of activity and stage of fibrosis were evaluated [16].

HCC surveillance

Ultrasonography or computed tomography (CT) was carried out before the initiation of the Peg-IFN plus ribavirin combination therapy and every 3–6 months during the follow-up period. New space-occupying lesions detected or suspected at the time of ultrasonography were further examined by CT or hepatic angiography. HCC was diagnosed by the presence of typical hypervascular characteristics on angiography, in addition to the findings from CT. If no typical image of HCC was observed, fine-needle aspiration biopsy was carried out, with the patient's consent, or the patient was carefully followed until a diagnosis was possible with a definite observation by CT or angiography.

End point

The observation period was defined as the period from the start of Peg-IFN plus ribavirin combination therapy. Patients who developed HCC and patients whose treatments were switched to other types of IFN therapy were defined as censored cases at that point in time.

Statistical analysis

Baseline data for various demographic, biochemical, and virologic characteristics of the patients were expressed as means ± SD. To analyze differences between baseline data among the four groups, analysis of variance or the χ^2 test was performed. The Kaplan–Meier method was used to calculate the cumulative incidence of HCC. The prognostic relevance of clinical variables and HCC incidence was evaluated by univariate analysis with the log-rank test. A value of $p < 0.05$ (two-tailed) was considered to indicate significance. The statistical software used for this analysis was IBM SPSS for Windows v. 19.0.0 (SPSS, Armonk, NY, USA).

Results

Baseline characteristics of patients categorized by the treatment guidelines

The baseline clinical features of the patients are shown in Table 1. There were significant differences in age; sex; body mass index (BMI); HCV genotype; past history of IFN therapy; grade and stage of liver histology; WBC, neutrophil, and PLT counts; Hb levels; and virologic response among the four groups. The mean ages of the patients in groups B and D were significantly higher than those of the patients in groups A and C. The proportion of males was lowest in group A (26 %) and highest in group C (41 %). The proportion of patients with progression of liver fibrosis (F3–4) diagnosed by the METAVIR score was 7.8 % among all patients tested and highest in group D (22.5 %). In groups B and D, peripheral blood cell counts (WBC, neutrophils, Hb, PLT) were significantly lower and the

Table 1 Baseline characteristics of the patients with chronic HCV infection and normal ALT levels

	Group A ALT ≤30 IU/L PLT count ≥15 × 10^4/mm^3	Group B ALT ≤30 IU/L PLT count <15 × 10^4/mm^3	Group C 30 < ALT ≤ 40 IU/L PLT count ≥15 × 10^4/mm^3	Group D 30 < ALT ≤ 40 IU/L PLT count <15 × 10^4/mm^3	*p* value
Number of patients	353	123	233	100	
Age (years)	55.6 ± 11.3	60.3 ± 8.4	54.6 ± 11.8	60.7 ± 8.6	<0.001
Sex: male/female	95/258	44/79	95/138	35/65	0.005
BMI (kg/m^2)	22.6 ± 3.3	22.1 ± 3.0	23.2 ± 3.4	22.3 ± 2.6	0.029
HCV genotype: 1/2	203/144	86/35	180/52	81/16	<0.001
HCV RNA (KIU/mL), mean ± SD	2333 ± 1664	2276 ± 1478	2261 ± 1599	2354 ± 1644	0.998
Past IFN therapy: naïve/experienced[a]	266/81	79/41	173/52	63/33	0.018
Histology[b]: activity: A0/A1/A2/A3	32/179/48/1	6/64/23/0	20/105/36/1	0/46/24/1	0.026
Fibrosis: F0/F1/F2/F3/F4	41/169/40/9/1	4/49/29/7/5	16/107/31/7/1	0/34/21/13/3	<0.001
White blood cell count (/mm^3)	5543 ± 1606	4405 ± 1211	5601 ± 1638	4677 ± 1337	<0.001
Neutrophil count (/mm^3)	3008 ± 1213	2332 ± 948	2999 ± 1243	2578 ± 1026	<0.001
Hemoglobin (g/dL)	13.3 ± 1.3	13.3 ± 1.4	13.9 ± 1.4	13.3 ± 1.3	<0.001
Platelet count (×10^4/mm^3)	21.1 ± 4.7	12.2 ± 2.1	21.3 ± 4.8	12.1 ± 2.2	<0.001
ALT (IU/L)	22.8 ± 5.2	23.5 ± 5.4	35.4 ± 2.9	35.8 ± 2.9	<0.001
Virologic response: SVR/relapse/NR	218/82/53	59/32/32	133/51/49	44/26/30	0.005

BMI body mass index, *ALT* alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon, *SVR* sustained virologic response, *NR* non-response, PLT platelet

[a] Virologic response to previous treatment was unknown for 22 patients

[b] Fibrosis stages are evaluated on a scale of 0–4 and activity grades are evaluated on a scale of 0–3 according to the METAVIR histological score. Fibrosis data were not available for 222 patients. Activity data were not available for 223 patients

numbers of patients with progression of liver fibrosis were significantly higher than in groups A and C. The mean duration of the observation period was 36.2 ± 16.5 months.

Antiviral efficacy of Peg-IFN plus ribavirin combination therapy

In genotype 1 patients, the rates of SVR, relapse, and NR were 50.7, 25.1, and 24.1 %, respectively, in group A; 39.5, 24.4, and 36.0 % in group B; 52.2, 23.9, and 23.9 % in group C; and 39.5, 25.1, and 35.2 % in group D. Although there was no significant difference in the treatment effect among the four groups, the SVR rate was significantly higher in groups A and C than that in groups B and D (groups A and C: SVR 51.4 %, relapse 24.5 %, NR 24.0 %; groups B and D: SVR 39.5 %, relapse 25.1 %, NR 35.2 %, $p = 0.012$). In genotype 2 patients, the rates of SVR, relapse, and NR were 77.8, 20.1, and 2.1 %, respectively, in group A; 65.7, 31.4, and 2.9 % in group B; 75.0, 15.4, and 9.6 % in group C; and 62.5, 31.3, and 6.3 % in group D. Although there was no significant difference in the treatment effect among the four groups, the SVR rate tended to be higher in groups A and C than that in groups B and D (groups A and C: SVR 77.0 %, relapse 18.9 %, NR 4.1 %; groups B and D: SVR 64.7 %, relapse 31.4 %, NR 8.9 %, $p = 0.152$).

Cumulative rate of HCC incidence according to the treatment effect of Peg-IFN plus ribavirin combination therapy

Eleven patients developed HCC during the observation period, and all were infected with HCV genotype 1. Figure 1 shows the cumulative rates of HCC incidence according to the treatment effect in the four groups.

In group A, no patients developed HCC during the 3 years of observation, regardless of the effect of Peg-IFN plus ribavirin combination therapy. Moreover, among those with SVR and relapse, no patients developed HCC during the 3-year observation period, while in NR patients the cumulative rate of HCC incidence at 5 years was 4.0 %. No significant difference in HCC incidence was found among the patients with SVR, relapse, and NR ($p = 0.071$) (Fig. 1a). In group C, no significant difference in HCC incidence was found among the patients with SVR, relapse, and NR (cumulative rates of HCC at 3 years, 2.2, 0.0, and 2.9 %, respectively; at 5 years, 3.7, 0.0, and 2.9 %, respectively, $p = 0.631$) (Fig. 1c). In group B, a marginally significant difference was found in HCC incidence among patients with SVR, relapse, and NR ($p = 0.054$), and patients with SVR had a significantly lower rate of HCC incidence than that of patients with NR (SVR vs. relapse, $p = 0.346$, SVR vs. NR, $p = 0.013$, relapse vs.

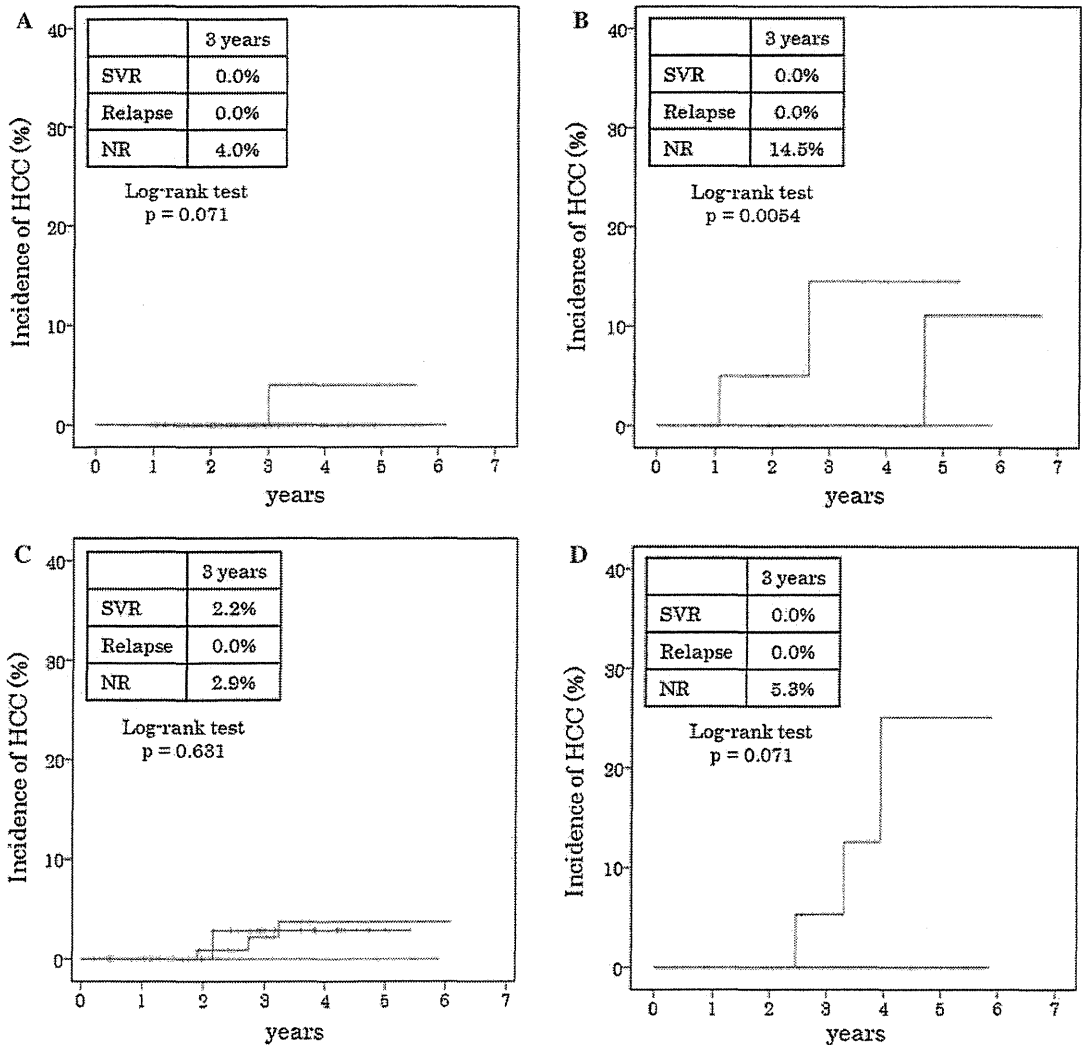

Fig. 1 Cumulative rates of hepatocellular carcinoma (*HCC*) incidence in groups A, B, C, and D, categorized according to the treatment effect of pegylated interferon (Peg-IFN) plus ribavirin combination therapy. **a** Group A (patients with alanine aminotransferase [ALT] level ≤30 IU/L and platelet [PLT] count ≥15 × 10^4/mm^3), **b** group B (patients with ALT ≤30 IU/L and PLT <15 × 10^4/mm^3), **c** group C (patients with 30 < ALT ≤ 40 IU/L and PLT ≥15 × 10^4/mm^3), **d** group D (patients with 30 < ALT ≤ 40 IU/L and PLT < 15 × 10^4/mm^3). *Blue line* patients with sustained virologic response (*SVR*), *green line* patients with relapse, *red line* patients with non-response (*NR*)

NR, $p = 0.250$). Of the NR patients, 14.5 % had developed HCC at 3 years, while none of the SVR or relapse patients had developed HCC at 3 years (Fig. 1b). In group D, there was a significant difference in HCC incidence among patients with SVR, relapse, and NR ($p = 0.006$), and patients with SVR or relapse had a significantly lower rate of HCC incidence than patients with NR (SVR vs. NR, $p = 0.012$, relapse vs. NR, $p = 0.047$). In the NR patients, 5.3 % had developed HCC at 3 years and 25.0 % had developed HCC at 5 years, but none of the SVR or relapse patients had developed HCC at 3 years (Fig. 1d).

In the analysis of the differences in the cumulative rates of HCC incidence in the patients with 30 < ALT ≤ 40 IU/L (group C plus group D), the p value for a significant difference was 0.059 among the patients with SVR, relapse, and NR (Fig. 2). In the analysis of the differences in the cumulative rates of HCC incidence among the patients with PLT counts of less than 15 × 10^4/mm^3 (group B plus group D), there was a significant difference in HCC incidence among patients with SVR, relapse, and NR ($p < 0.001$), and patients with SVR or relapse had a significantly lower rate of HCC incidence than patients with NR (cumulative rates of HCC incidence at

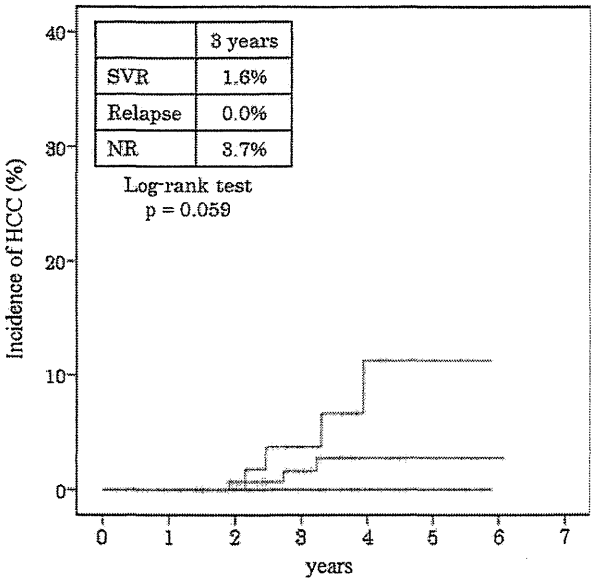

Fig. 2 Cumulative rates of HCC incidence according to ALT levels. Cumulative rates of HCC incidence in patients with ALT levels of 30 < ALT ≤ 40 IU/L (group C plus group D). *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

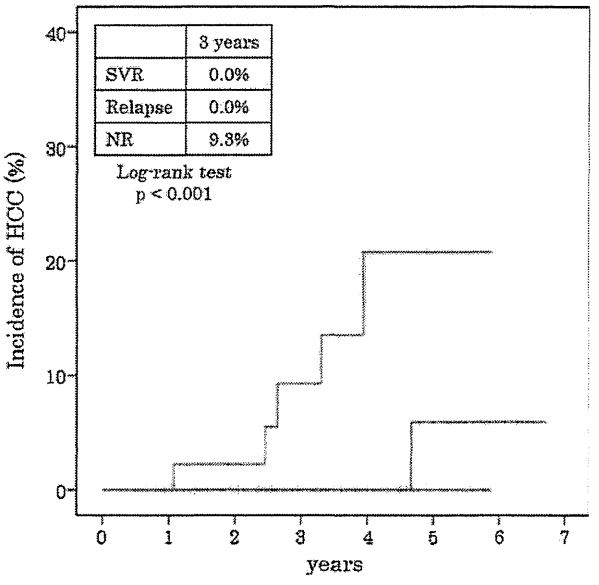

Fig. 3 Cumulative rates of HCC incidence according to PLT counts. Cumulative rates of HCC incidence in patients with PLT counts of $<15 \times 10^4/mm^3$ (group B plus group D). *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

3 years, 0.0, 0.0, and 9.3 %, respectively; at 5 years, 0.0, 11.1, and 20.8 %, respectively; SVR vs. NR, $p < 0.001$, relapse vs. NR, $p = 0.021$) (Fig. 3).

Cumulative rate of HCC incidence in group B according to the stage of liver fibrosis

Based on the pattern of the Japanese treatment guidelines, we categorized the patients in group B into two groups according to the stage of liver fibrosis (F0–1 or F2–4) and compared the cumulative rates of HCC incidence. Patients with no fibrosis or mild fibrosis (F0–1) showed no HCC development regardless of the virologic response (SVR, relapse, or NR). Of note, in those with moderate to severe fibrosis (F2–4) in group B, there was no significant difference in HCC incidence among patients with SVR, relapse, and NR ($p = 0.174$), although SVR patients tended have a lower rate of HCC incidence than NR patients (SVR vs. relapse, $p = 0.414$, SVR vs. NR, $p = 0.071$, relapse vs. NR, $p = 0.383$). No patient in the SVR or relapse groups developed HCC, while the cumulative rate of HCC incidence at 3 years for the NR group was 25.0 % (Fig. 4).

Discussion

Patients with chronic HCV infection and N-ALT have been reported to show the possibility of ALT flare-up during the natural course of the disease (22–27 %) [17, 18] and to develop moderate to severe progression of liver fibrosis (5–30 %) [18–21]. However, very low cumulative incidences of HCC have been reported among patients with average ALT integration values less than or equal to 20 IU/L (5-year, 0.0 %, 10-year, 3.6 %) [22]. Therefore, it remains controversial whether HCV eradication by antiviral therapy can reduce the incidence of HCC in patients with chronic HCV infection and N-ALT [23–26].

The definition of N-ALT remains unclear because its cutoff value is still under consideration [22, 27, 28]. In Japan, treatment guidelines for patients with chronic HCV infection and N-ALT define N-ALT as serum ALT levels of ≤40 U/L, and the therapeutic strategy is decided after categorizing patients into four groups according to ALT levels and PLT counts. However, the indication of antiviral therapy should be based on whether or not HCC incidence can be suppressed by the antiviral therapy. Therefore, we examined the treatment guidelines from the viewpoint of inhibiting HCC in patients with chronic HCV infection and N-ALT.

In the present study, the antiviral efficacy of Peg-IFN plus ribavirin combination therapy for patients with chronic HCV infection and N-ALT was almost equivalent to the efficacy in those with elevated ALT levels, as previously reported [11–15]. The SVR rate was significantly higher in groups A and C than in groups B and D for patients with genotype 1, and the same tendency was found

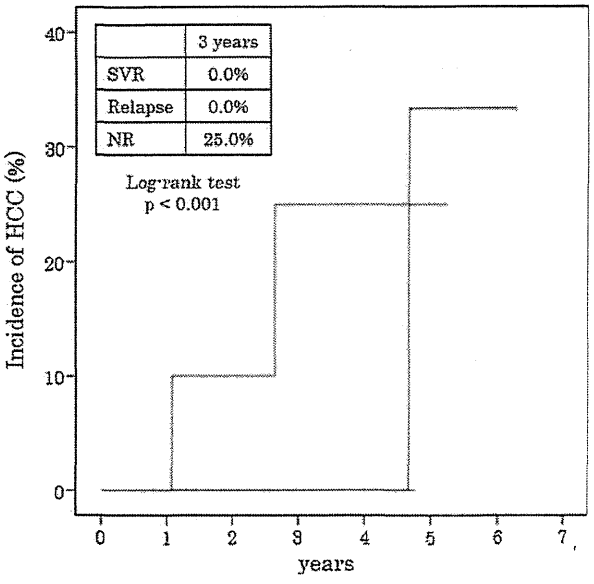

Fig. 4 Cumulative rates of HCC incidence in group B patients (ALT $\leq$ 30 IU/L and PLT $< 15 \times 10^4/mm^3$) with moderate to severe liver fibrosis (F2–4), according to the treatment effect of Peg-IFN plus ribavirin combination therapy. *Blue line* patients with sustained virologic response, *green line* patients with relapse, *red line* patients with non-response

for those with genotype 2. The reason for this was considered to be that groups B and D included many patients with moderate to severe liver fibrosis (F3–4, 17.0 %), which can lead to a lower SVR rate [23, 29, 30].

The present study revealed the cumulative rates of HCC incidence according to the treatment effect in the four groups. In group D, the cumulative rate of HCC incidence in the SVR and relapse patients was significantly lower than that for the NR patients. This result supports the recommendation by the treatment guidelines that patients in group D be managed in the same way as patients with chronic hepatitis C (CH-C) and elevated ALT levels.

In group B patients, the treatment guidelines recommend antiviral therapy for those who have moderate to severe liver fibrosis (F2–4). In our present study, patients with no fibrosis to mild fibrosis (F0–1) did not develop HCC, and in the patients with moderate to severe fibrosis (F2–4), the cumulative rate of HCC incidence tended to be lower in the SVR group than that in the NR group ($p = 0.071$). These results also indicate the appropriateness of the Japanese treatment guidelines. However, further study is needed because of the small number of cases studied here.

It appears that group A patients have time to wait for therapy with the next generation of direct antiviral agents (DAAs), such as Peg-IFN plus ribavirin plus a second-generation protease inhibitor, because none of the patients had developed HCC at 3 years. Even in group C, for which the treatment guidelines recommend antiviral therapy, there was no significant difference in the cumulative rate of HCC incidence among the SVR, relapse, and NR patients, with the incidence being below 5 % at 3 years. Accordingly, patients with PLT counts of more than $15 \times 10^4/mm^3$ (groups A or C) have time to wait until the next generation of DAAs becomes available, because patients with PLT counts of more than $15 \times 10^4/mm^3$ have a low 3-year carcinogenesis rate.

The Japanese treatment guidelines recommend antiviral therapy for patients with 30 < ALT $\leq$ 40 IU/L levels. However, in the present study, in the patients with 30 < ALT $\leq$ 40 IU/L levels, the p value for a significant difference in the cumulative rate of HCC incidence among the patients with SVR, relapse, and NR was 0.059. This result indicates that the patients with 30 < ALT $\leq$ 40 IU/L levels have the potential to be candidates for antiviral therapy, and further study is needed to clarify this. However, these patients may not be candidates for immediate antiviral therapy because the cumulative rates of HCC incidence at 3 years in the patients with SVR, relapse, and NR were low (cumulative rates of HCC at 3 years: 1.6, 0.0, and 3.7 %). On the other hand, as mentioned above, in the patients with PLT counts of $<15 \times 10^4/mm^3$, the cumulative rate of HCC incidence was significantly lower in the SVR and relapse patients than that in the NR patients (cumulative rates of HCC at 3 years: 0.0, 0.0, and 9.3 %; at 5 years: 0.0, 11.1, and 20.8 %; $p < 0.001$). This result suggests that patients with PLT counts of $<15 \times 10^4/mm^3$ may be candidates for antiviral therapy.

A limitation of this study was that the incidence of HCC was not compared between a treatment group and a non-treatment group. This study showed the suppressive effect of antiviral therapy on HCC incidence by comparing patients according to the treatment's antiviral effect. Peg-IFN plus ribavirin combination therapy has become acceptable for patients with chronic HCV infection and N-ALT levels. However, if there were no difference in HCC incidence between patients with SVR and non-SVR in the group receiving Peg-IFN plus ribavirin combination therapy, it would not be necessary for patients with chronic HCV infection and N-ALT to receive this therapy. In this study, we compared the incidence of HCC according to the treatment effect in HCV-infected patients with N-ALT levels categorized by the Japanese treatment guidelines. Indeed, although our results did not demonstrate that N-ALT patients should be treated, they indicated that it could be appropriate to treat N-ALT patients, because the incidence of HCC in these patients with SVR was suppressed compared with that in the NR patients.

In conclusion, in patients with N-ALT and PLT counts of $<15 \times 10^4/mm^3$ who received Peg-IFN plus ribavirin

combination therapy, the cumulative rate of HCC incidence was significantly lower in those with SVR or relapse than in those with NR. Therefore, HCV-infected patients with N-ALT and PLT counts of $<15 \times 10^4/mm^3$ could be candidates for early antiviral therapy for the purpose of reducing the risk of developing HCC.

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Original Article

Hepatocellular carcinoma risk assessment using gadoxetic acid-enhanced hepatocyte phase magnetic resonance imaging

Nobutoshi Komatsu,[1] Utaroh Motosugi,[2] Shinya Maekawa,[1] Kuniaki Shindo,[1] Minoru Sakamoto,[1] Mitsuaki Sato,[1] Akihisa Tatsumi,[1] Mika Miura,[1] Fumitake Amemiya,[1] Yasuhiro Nakayama,[1] Taisuke Inoue,[1] Mitsuharu Fukasawa,[1] Tomoyoshi Uetake,[1] Masahiko Ohtaka,[1] Tadashi Sato,[1] Yasuhiro Asahina,[3] Masayuki Kurosaki,[4] Namiki Izumi,[4] Tomoaki Ichikawa,[2] Tsutomu Araki[2] and Nobuyuki Enomoto[1]

[1]*First Department of Internal Medicine,* [2]*Department of Radiology, University of Yamanashi, Yamanashi,* [3]*Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, and* [4]*Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan*

Aim: To investigate whether the patients with hypovascular liver nodules determined on the arterial phase and hypointensity on the hepatocyte phase gadoxetic acid-enhanced magnetic resonance imaging (hypovascular hypointense nodules) are at increased risk of hepatocarcinogenesis, we assessed subsequent typical hepatocellular carcinoma (HCC) development at any sites of the liver with and without such nodules.

Methods: One hundred and twenty-seven patients with chronic hepatitis B or C and without a history of HCC, including 68 with liver cirrhosis, were divided into those with (non-clean liver group, $n = 18$) and without (clean liver group, $n = 109$) hypovascular hypointense nodules. All the patients were followed up for 3 years, and HCC development rates and risk factors were analyzed with the Kaplan–Meier method and the Cox proportional hazard model, respectively.

Results: A total of 17 patients (10 in the non-clean liver group and seven in the clean liver group) developed typical HCC. Cumulative 3-year rates of HCC development were 55.5% in the non-clean liver group and 6.4% in the clean liver group ($P < 0.001$), and those at the different sites from the initial nodules was also higher in the non-clean liver group (22.2%) than the clean liver group (6.4%) ($P = 0.003$). Multivariate analysis identified older age ($P = 0.024$), low platelet counts ($P = 0.017$) and a non-clean liver ($P < 0.001$) as independent risk factors for subsequent HCC development.

Conclusion: Patients with hypovascular hypointense liver nodules are at a higher risk for HCC development at any sites of the liver than those without such nodules.

Key words: gadoxetic acid, hepatocellular carcinoma, hepatocyte phase, magnetic resonance imaging, risk assessment

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most common cancers worldwide and is a major cause of death in patients with chronic viral liver disease. Despite many advances in multidisciplinary treatment, complete curative treatment of early stage HCC remains the only possible therapeutic choice for long-term survival. Therefore, surveillance programs for patients at a high risk for HCC that include imaging-based evaluations are crucial for the detection and treatment of early stage HCC.

The newly introduced magnetic resonance imaging (MRI) contrast agent, gadolinium ethoxybenzyl

Correspondence: Dr Nobuyuki Enomoto, First Department of Internal Medicine, University of Yamanashi, 1110 Shimokato, Chuo, Yamanashi 409-3898, Japan. Email: enomoto@yamanashi.ac.jp

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diethylenetriamine pentaacetic acid (gadoxetic acid), has enabled concurrent assessment of tumor vascularity and unique hepatocyte-specific contrast (hepatocyte phase).[1–3] This has led to the frequent identification of hypovascular nodules determined on the arterial phase with hypointensity on the hepatocyte phase (hypovascular hypointense nodules),[4–8] while many of these nodules are difficult to be detected by ultrasonography (US) or computed tomography (CT). Recently, the natural history of hypovascular hypointense nodules themselves were reported in several studies,[9–12] revealing the high risk of subsequent progress to typical HCC from these nodules. However, it is not well known whether patients with such nodules have a higher risk of developing typical HCC at any sites of the liver, including at the different sites from initial nodules, compared to those without such nodules.

If patients with these nodules may have a high risk of developing typical HCC not only at the same sites but also at the different sites from initial nodules, a significant proportion of these nodules are precancerous lesions or early stage HCC as reported,[13–15] and more importantly, the liver with these nodules may reflect a higher potential for hepatocarcinogenesis or the presence of undetectable precursor lesions in other sites of the liver. Conversely, the absence of these nodules potentially identifies the patients at a low risk for subsequent typical HCC development at any sites. The purpose of this study was to assess the risk of subsequent typical HCC development at any sites of the liver with and without hypovascular hypointense nodules on gadoxetic acid-enhanced MRI.

METHODS

Ethical review

THE PROTOCOL OF this retrospective study was approved by the ethics committee of Yamanashi University Hospital, which waived the requirement for written informed consent because the study was a retrospective data analysis, with appropriate consideration given to patient risk, privacy, welfare and rights.

Patients

We recruited 559 consecutive outpatients with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection who underwent gadoxetic acid-enhanced MRI at Yamanashi University Hospital between January 2008 and December 2010. The exclusion criteria were as follows: (i) presence or history of typical HCC ($n = 420$), because intrahepatic metastasis does not always develop through the usual multistep hepatocarcinogenesis process, skipping the early pathological stage with hypovascularity to an advanced pathological stage even when the size is small;[16,17] (ii) Child–Pugh class C disease ($n = 9$), because the hepatocyte phase findings are not reliable in patients with this condition because of reduced gadoxetic acid uptake in the liver;[18] and (iii) patients who dropped out during the 3-year follow-up period ($n = 3$).

After excluding 432 patients, 127 patients were included in this retrospective cohort study. They were divided into groups with hypovascular nodules determined on the arterial phase and hypointensity on the hepatocyte phase (non-clean liver group; $n = 18$ patients) and without such nodules (clean liver group; $n = 109$ patients) as shown in Figure 1. In this study, we divided cases into two groups according to the presence or absence of these nodules at the baseline, even when such nodules were initially detected during the follow-up period; we assigned these patients to the clean liver group.

Follow up and diagnosis of HCC

All 127 patients were followed up at the liver disease outpatient clinic of our institution with blood tests, including those for tumor markers and diagnostic imaging modality (US, CT or MRI). The development of typical HCC that required treatment as proposed by the American Association for the Study of Liver Diseases (AASLD) guidelines[19] and that was diagnosed according to imaging criteria, showing arterial hypervascularity and venous phase washout, or based on histological examination of liver biopsies from hypovascular nodules that grew to more than 10 mm during follow up. Biopsies were obtained using a 21-G core needle. Two patients each had a liver nodule of more than 10 mm in diameter on initial MRI (12 mm and 13 mm), which were diagnosed on the basis of the biopsy as dysplastic nodules.

The end-point of this study was the development of typical HCC not only from the hypovascular hypointense nodules observed initially but also from other areas without these nodules ("de novo HCC"). Dynamic CT and/or MRI were also performed in cases with hepatic nodules detected by US, liver cirrhosis, a tendency of tumor marker elevation and difficult evaluation of the liver parenchyma by US. All 127 patients were followed up for 3 years after the initial gadoxetic acid-enhanced MRI examination. When imaging