genome-length HCV RNA was eliminated by IFN-α treatment (500 IU/ml for 2 weeks) without G418, as previously described (13).

Luciferase reporter assay. For the *Renilla* luciferase (RL) assay, approximately 1.0×10^4 to 1.5×10^4 OR6 cells (72-hour treatment) or 0.5×10^4 OR6 cells (120-hour treatment) were plated onto 24-well plates in triplicate and cultured for 24 h. The cells were treated with each nutrient or compound for 72 or 120 h. Then, the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and subjected to the RL assay according to the manufacturer's protocol.

Western blot analysis. For Western blot analysis, 4×10^4 to 4.5×10^4 OR6c cells harboring HCV-O/KE/EG (strain O of genotype 1b) (K. Abe, M. Ikeda, and N. Kato, unpublished data) were plated onto six-well plates and cultured for 24 h and then were treated with each nutrient or compound for 72 h. Preparation of the cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting were then performed as previously described (18). The antibodies used in this study were those specific to HCV core antigen (CP11; Institute of Immunology, Tokyo) and β-actin (Sigma). The epitope of CP11 was located within amino acid positions 21 to 40 of the core antigen. Immunocomplexes on the membranes were detected by enhanced chemiluminescence assay (Renaissance; Perkin Elmer Life Science, Wellesley, MA).

Cell viability. To examine the suppressive effects of nutrients on OR6 cell viability, approximately 4.5×10^4 to 5×10^4 OR6 cells (72-hour viability assay) or approximately 1×10^4 to 1.5×10^4 cells (120-hour viability assay) were plated onto six-well plates in triplicate and were cultured for 24 h. The cells were treated without nutrients or with each nutrient for 72 or 120 h, and then the number of viable cells was counted after trypan blue dye treatment as previously described (30).

Statistical analysis and synergistic statistics. Differences between the anti-HCV activities of the nutrients at each concentration and controls were tested using Student's *t* test. *P* values of less than 0.05 were considered statistically significant. Then, an isobologram method was used to evaluate the effects of a combination of nutrients or compounds on HCV RNA replication (21). OR6 cells were treated with each combination of nutrients or compounds at various concentrations for 72 h. The 50% effective concentration (EC_{50}) against HCV RNA replication in each combination treatment was analyzed by sigmoid regression, and isoboles of EC_{50} were plotted using the resulting data.

RESULTS

Effects of ordinary nutrients on HCV RNA replication. To date, information about the anti-HCV effects of ordinary nutrients has been limited to only a few studies, and in those studies, a plasmid (26), a subgenomic replicon (21), and recombinant HCV proteins (5, 8, 9) were used in the assays. We recently developed OR6 assay system by the selection after introducing genome-length ORN/C-5B/KE RNA (Fig. 1A) into HuH-7 cells. Our OR6 assay system renders it possible to carry out the prompt and precise evaluation of genome-length HCV RNA replication (13, 30). Therefore, we comprehensively analyzed 46 ordinary nutrients to determine their effects on HCV RNA replication using our novel OR6 assay system (Table 1). The effects of the preexistent nutrients in the medium on HCV RNA replication were under a significant level, because the concentrations of the nutrients already contained in the medium were less than a one-thousandth part of the minimum concentration in the treatment.

We first examined 8 liposoluble vitamins and 10 water-soluble vitamins to investigate their effects on HCV RNA replication. Among the liposoluble vitamins, VA (Fig. 2D), VE (Fig. 2E), and VK (Fig. 2F and G) significantly enhanced HCV RNA replication. However, BC and VD2 significantly inhibited HCV RNA replication in a dose-dependent manner (the mean EC_{50}s ± standard deviations [SDs] were 6.3 ± 0.7 μM and 3.8 ± 0.9 μM, respectively) (Fig. 2A and B). In contrast with VD2, VD3 apparently enhanced relative luciferase activity, but this promotive effect was thought to result from cell proliferation, since the amount of β-actin increased in a manner similar to that of HCV core antigen (Fig. 2H). Most of the water-soluble vitamins exerted no effect on HCV RNA replication (data not shown), while only VC moderately enhanced HCV RNA replication (Fig. 2I).

We next examined three branched-chain amino acids and three aromatic amino acids for their effects on HCV RNA replication. We tested the six amino acids at concentrations of 0, 100, 500, and 1,000 μM, and only tryptophan exerted moderate promotive effects on HCV RNA replication (Fig. 2J).

We further examined four saturated fatty acids, three monounsaturated fatty acids, and four polyunsaturated fatty acids (PUFAs) to assess their effects on HCV RNA replication. As has been noted in previous reports (17, 21), all of the PUFAs, i.e., LA, AA, EPA, and DHA, inhibited HCV RNA replication in OR6 cells in a dose-dependent manner (the mean EC_{50}s ± SDs were 20.2 ± 4.8 μM, 22.1 ± 1.7 μM, 36.2 ± 2.5 μM, and 37.0 ± 3.6 μM, respectively). However, we found that with the exception of LA, treatment with 50 μM of PUFA resulted in

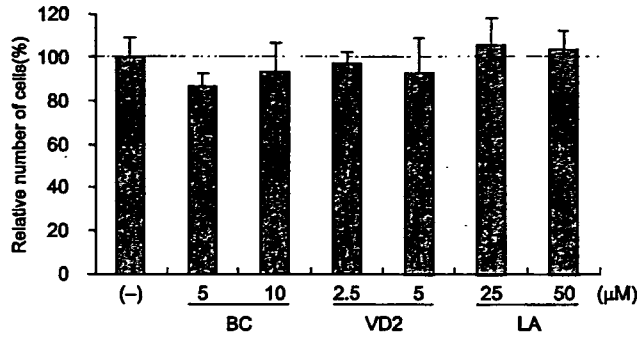


FIG. 3. The anti-HCV activities of three nutrients were not due to the suppression of cell growth. Cell viabilities after treatment with BC, VD2, and LA are shown. OR6 cells were cultured in control medium [(−)] and in the presence of BC (5 and 10 μM), VD2 (2.5 and 5 μM), and LA (25 and 50 μM) for 72 h, and then the number of viable cells was counted after trypan blue dye treatment. Shown here are the percent relative cell numbers calculated when the relative cell number of untreated cells was assigned as 100%. The data indicate means ± SDs of triplicates from at least two independent experiments.

FIG. 2. Effects of ordinary nutrients on HCV RNA-replicating cells. (A through K) Reporter assay and Western blot analysis of nutrient sensitivity of HCV RNA replication. OR6 cells were treated with each nutrient at a four-grade-modulated concentration in the medium. After 72 h of treatment, the RL assay was performed as described in Materials and Methods. Shown here are the percent relative luciferase activities calculated when the RL activity of untreated cells was assigned the value of 100%. The data indicate means ± SDs of triplicate samples from at least three independent experiments. Subsequently, OR6c cells, into which authentic HCV RNA was introduced, were treated with nutrients exhibiting either inhibitory effects, i.e., BC (A), VD2 (B), and LA (C), or promotive effects, i.e., VA (D), VE (E), VK1 (F), VK2 (G), VD3 (H), VC (I), tryptophan (J), and Se (K) at the same concentrations as those used in the OR6 assay (bar graphs). After 72 h of treatment, the production of HCV core antigen was analyzed by immunoblotting using antibody specific to HCV core antigen (upper lanes). β-Actin was used as a control for the amount of protein loaded per lane (lower lanes). *, $P < 0.01$; **, $P < 0.05$.

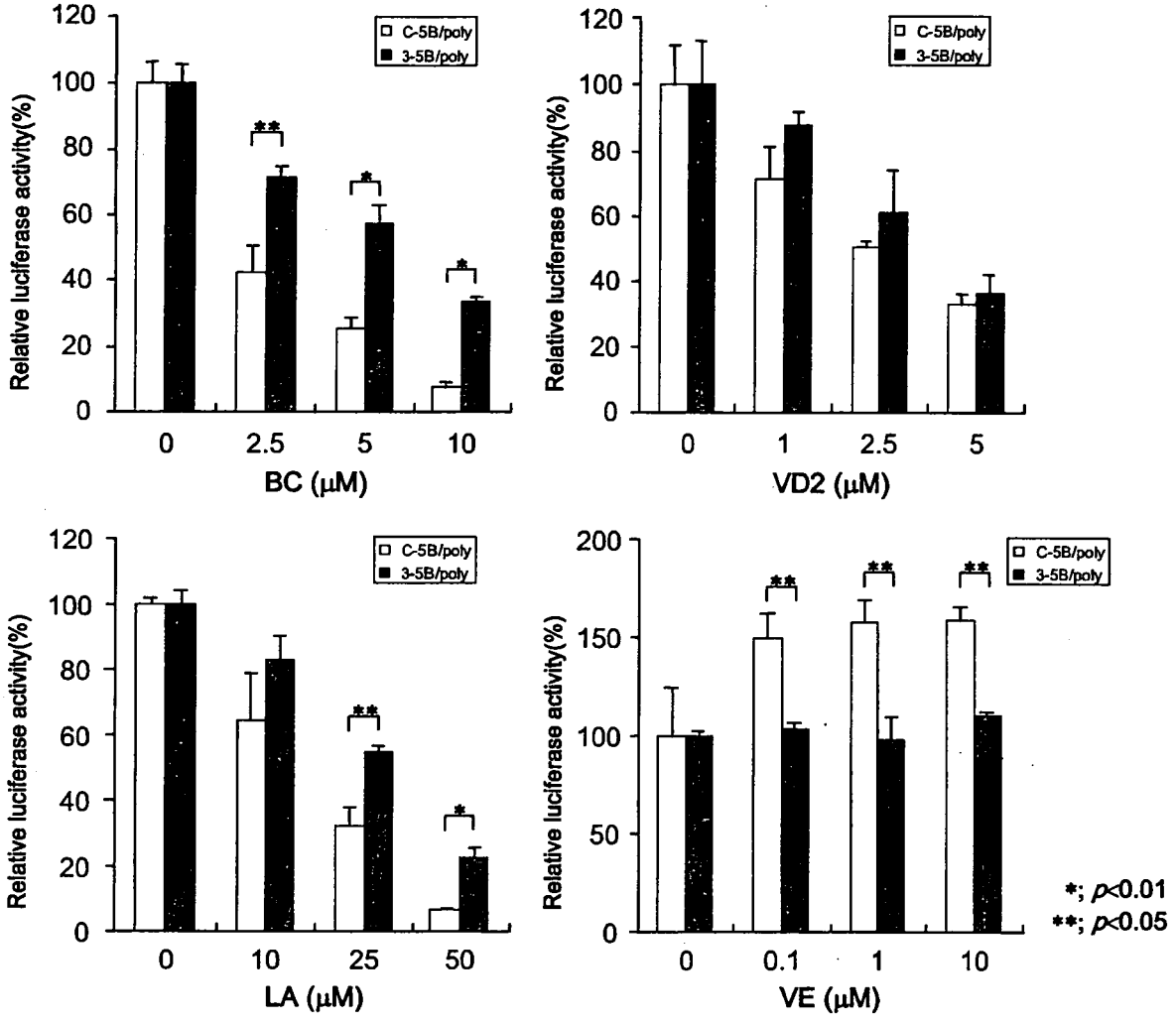


FIG. 4. The effects of BC, VD2, LA, and VE on polyclonal genome-length and subgenomic HCV RNA replication. Both polyclonal genome-length HCV RNA-replicating cells (ORN/C-5B/KE/poly) and subgenomic replicon cells (ORN/3-5B/KE/poly) were treated with BC, VD2, LA, or VE according to the same protocol as that used for the OR6 assay. The RL assay was performed at 72 h postapplication, and then RL activity was calculated as described in the legend to Fig. 2.

the suppression of cell growth due to cytotoxicity (data not shown). These data indicate that among PUFAs, only LA exhibited a significant inhibitory effect on HCV RNA replication without concomitant cytotoxicity (Fig. 2C and 3).

Finally, we examined 11 salts in order to assess their effects on HCV RNA replication. Iron [Fe(II) in the form of $FeSO_4$ and Fe(III) in the form of $Fe(NO_3)_3$] and zinc (in the form of $ZnCl_2$) exhibited anti-HCV effects without cytotoxicity at concentrations of up to 50% inhibition, but beyond 50% inhibition, cell growth was dose-dependently affected by the cytotoxicity of these minerals. Selenium (in the form of Na_2SeO_4), a typical antioxidant, slightly enhanced HCV RNA replication (Fig. 2K). We also confirmed these results using authentic HCV RNA-replicating cells (Fig. 1B and 2A through K).

These results suggest that the ordinary nutrients tested here have different profiles in terms of their effects on HCV RNA replication. The results are summarized in Table 1. Most of the nutrients were found to have no effect on HCV RNA replication. Eight nutrients enhanced HCV RNA replication, and the antioxidant nutrients VA, VC, VE, and Se were included in this group. Among the 46 nutrients tested with the OR6 assay system, we found that BC, VD2, and LA exerted anti-HCV effects without cytotoxicity. To the best of our knowledge, this is the first study to demonstrate the anti-HCV effects of BC and VD2. Therefore, we focused on the anti-HCV effects of BC, VD2, and LA in the following study.

The effects of BC, VD2, LA, and VE on polyclonal genome-length and subgenomic HCV RNA replication. OR6 cells are among the cloned cell lines selected by G418. Therefore, we examined polyclonal cells harboring genome-length HCV RNA (ORN/C-5B/KE/poly) to exclude the possibility that the anti-HCV effects of BC, VD2, and LA were an OR6 clone-specific phenomenon. Furthermore, polyclonal cells harboring subgenomic HCV RNA (ORN/3-5B/KE/poly) were also used to examine the effects of the anti-HCV nutrients on RNA replication in the absence of the structural HCV proteins. The

results revealed that all of these three nutrients exhibited a dose-dependent suppression of HCV RNA replication in both cell systems, although the three nutrients had stronger anti-HCV effects in the polyclonal genome-length HCV RNA-replicating cells than they did in the subgenomic HCV RNA-replicating cells (Fig. 4). These results indicated that the anti-HCV activities of these nutrients were not due to cell clonality, and the sensitivities of the reagents were found to differ between subgenomic and genome-length HCV RNA-replicating cells. One possible explanation of this difference is that the different genome sizes of subgenomic (9-kb) and genome-length (12-kb) HCV RNA might affect the replication efficiencies and lead to the difference in the sensitivities of antiviral reagents. These differences were significant, especially in BC- and LA-treated cells. A subgenomic replicon system may underestimate the effects of anti-HCV reagents and therefore might fail to identify potentially effective anti-HCV reagents. Therefore, our genome-length HCV RNA replication system (OR6) is advantageous for evaluating anti-HCV candidates.

We also tested VE's effect on subgenomic and genome-length HCV RNA-replicating cells. VE enhanced the replication of genome-length HCV RNA. However, interestingly, VE did not affect subgenomic HCV RNA replication. These results suggest that the subgenomic HCV RNA replication system may not be able to evaluate the reagent-enhancing HCV RNA replication.

Anti-HCV activities of three nutrients were not due to inhibition of cell growth. Since it has been reported that HCV RNA replication is dependent on cell growth (34), we examined whether the anti-HCV activities of three nutrients were due to their respective cytotoxicities. OR6 cells were treated with each nutrient (BC, 5 and 10 μM; VD2, 2.5 and 5 μM; LA, 25 and 50 μM) for 72 h. These results suggest that the anti-HCV effects of BC, VD2, and LA are not due to their cytotoxicities.

Time course assay of inhibitory effects of three nutrients on HCV RNA replication. A kinetics analysis of the anti-HCV effects of reagents provides information about inhibitory mechanisms and optimized drug administration. Therefore, we conducted a time course assay (24, 72, and 120 h after treatment) of the anti-HCV effects of three nutrients, BC, VD2, and LA, using our OR6 assay system. The results revealed that BC and VD2 exhibited stronger inhibition of HCV RNA replication than did LA at 24 h after treatment. However, the anti-HCV activities of BC and VD2 only slightly increased at 72 or 120 h after treatment (Fig. 5A). On the other hand, LA inhibited HCV RNA replication in dose- and time-dependent manners. It is noteworthy that about 90% inhibition of RL activity was observed at 120 h after LA (50 μM) treatment of OR6 cells (Fig. 5A).

We examined whether these reductions in relative RL activity induced by all three nutrients at 120 h were due to the suppression of cell growth. Compared to the number of untreated cells, at 120 h after treatment with each nutrient (BC, 5 and 10 μM; VD2, 2.5 and 5 μM; LA, 25 and 50 μM), no significant reduction in the number of treated cells was observed (Fig. 5B). These results indicate that the anti-HCV effects of these three nutrients were not due to their respective cytotoxicities.

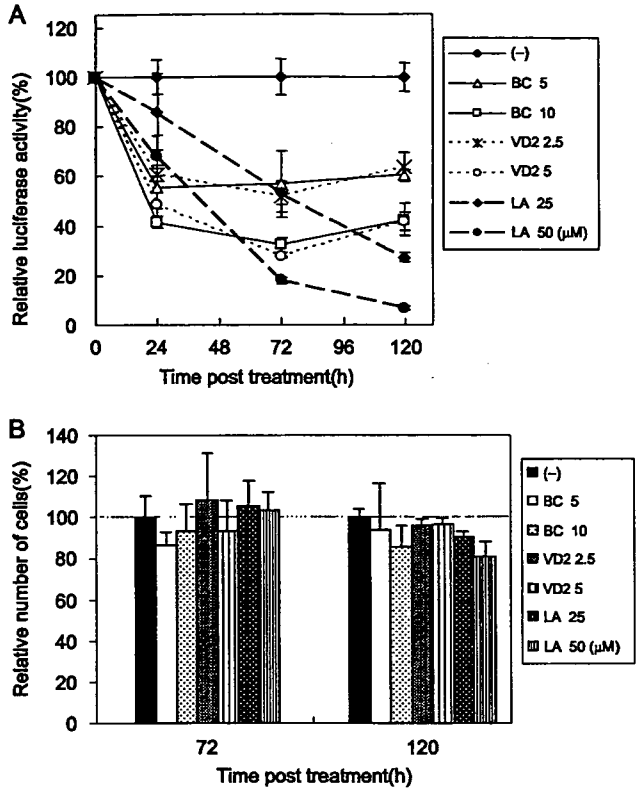


FIG. 5. Time course assay of the anti-HCV activities of three nutrients. (A) Time course of the inhibitory effects of three nutrients on HCV RNA replication. OR6 cells were treated with control medium [(−)], BC (5 and 10 μM), VD2 (2.5 and 5 μM), or LA (25 and 50 μM), and the RL assay was performed at 24, 72, and 120 h postapplication. Relative RL activity was calculated as described in the legend to Fig. 2. (B) Time course of cell viability after the application of three nutrients. OR6 cells were cultured in the control medium and in the presence of BC (5 and 10 μM), VD2 (2.5 and 5 μM), or LA (25 and 50 μM), and at 72 and 120 h postapplication, the number of viable cells was counted after trypan blue dye treatment. Shown here are the percent relative cell numbers calculated as described in the legend to Fig. 3.

HCV RNA replication was additively inhibited by each combination of three nutrients and was synergistically inhibited by all three. As described above, we found that BC, VD2, and LA possessed anti-HCV activities. However, these nutrients appeared to be insufficient for eliminating HCV by mono-treatment. Therefore, we examined the anti-HCV effects of two or three nutrients in combination.

To evaluate the effects of each combination treatment, OR6 cells were cotreated with two nutrients at the listed concentrations for 72 h (BC, approximately 0 to 5 μM; VD2, approximately 0 to 3 μM; LA, approximately 0 to 20 μM). Isoboles of 50% inhibition of HCV RNA replication were obtained for each data point. An analysis of the 50% isoboles of each combination treatment graphed nearly a straight line in each case. These results indicate that the inhibitory effects of all combinations on HCV RNA replication were additive (Fig. 6A).

Treatment with all three nutrients at various concentrations resulted in stronger suppression of HCV RNA replication in OR6 cells than we had predicted as an additive effect (Fig. 6B).

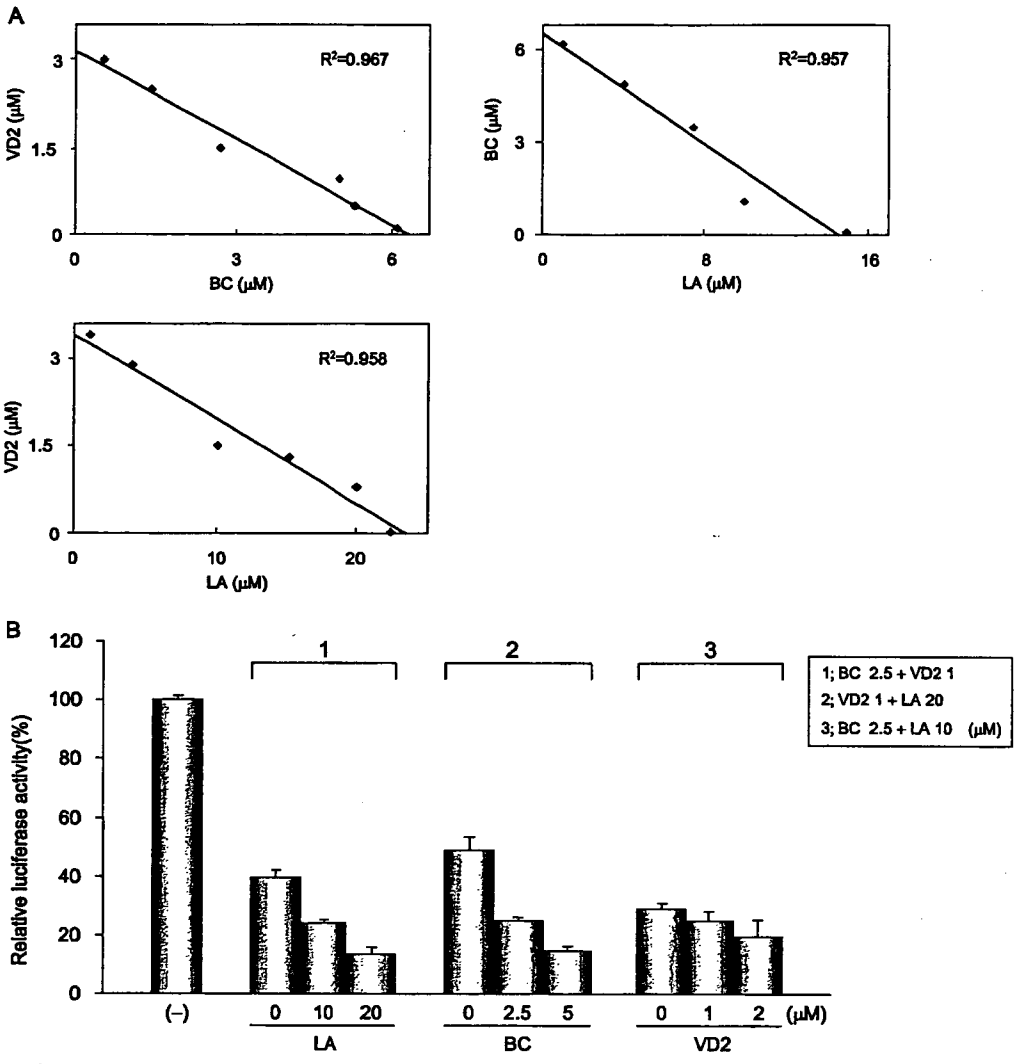


FIG. 6. Effects of treatment with each combination or all of the three nutrients on HCV RNA replication. (A) Isobole plots of 50% inhibition of HCV RNA replication. OR6 cells were treated with each combination of three nutrients, BC (0, 0.5, 1, 2, 3, 4, and 5 μM), VD2 (0, 0.1, 0.5, 1, 1.5, 2, and 3 μM), and LA (0, 1, 5, 10, 15, and 20 μM), for 72 h, and RL assay was performed as described in the legend to Fig. 2 to obtain 50% isoboles. The R^2 value indicates the coefficient of determination. (B) The effect of treatment with all three nutrients on HCV RNA replication was synergistic. OR6 cells were treated with LA (0, 10, and 20 μM) in addition to 2.5 μM BC plus 1 μM VD2, BC (0, 2.5, and 5 μM) in addition to 1 μM VD2 plus 20 μM LA, or VD2 (0, 1, and 2 μM) in addition to 2.5 μM BC plus 10 μM LA. After 72 h of treatment, the RL assay was performed, and then relative RL activity was calculated as described in the legend to Fig. 2.

For instance, in the sample cotreated with 2.5 μM BC ($\approx EC_{20}$) in addition to 1 μM VD2 ($\approx EC_{30}$) plus 20 μM LA ($\approx EC_{50}$) (Fig. 2A through C), the actual effect on HCV RNA replication was 90% inhibition, which was 20% greater than we had originally estimated (i.e., approximately 70%; $1 - 0.8 \times 0.7 \times 0.5 = 0.72$) (Fig. 6B). In addition, no suppression of cell growth was observed during these cotreatments (data not shown). These results suggest that treatment with a mixture of these three nutrients may exert synergistic inhibitory effects on HCV RNA replication.

Treatment with each of three nutrients in combination with IFN or FLV additively inhibited HCV RNA replication, and CsA synergistically inhibited HCV RNA replication. Recently, CsA was proposed as a novel candidate to be paired with IFN in similar studies using a cell culture system (41). We have also reported findings obtained with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, statins, exerted diverse anti-HCV effects, and FLV was found to exert the strongest inhibitory effect on HCV RNA among the statins tested (14).

Therefore, we examined the anti-HCV effects of each of three nutrients in combination with IFN, FLV, or CsA by using OR6 cells. OR6 cells were treated for 72 h with IFN-α (0, 0.2, 0.5, and 1 IU/ml) in combination with each of the nutrients at various concentrations (BC, approximately 0 to 5 μM; VD2, approximately 0 to 4 μM; LA, approximately 0 to 20 μM) (Fig. 7A). FLV (approximately 0 to 2 μM) or CsA (approximately 0 to 1 μg/ml) was also used for treatment in combination with BC, VD2, or LA at the concentration mentioned above (Fig. 7B and C). Isoboles of 50% inhibition of HCV RNA replication were generated from each sample. An analysis of 50%

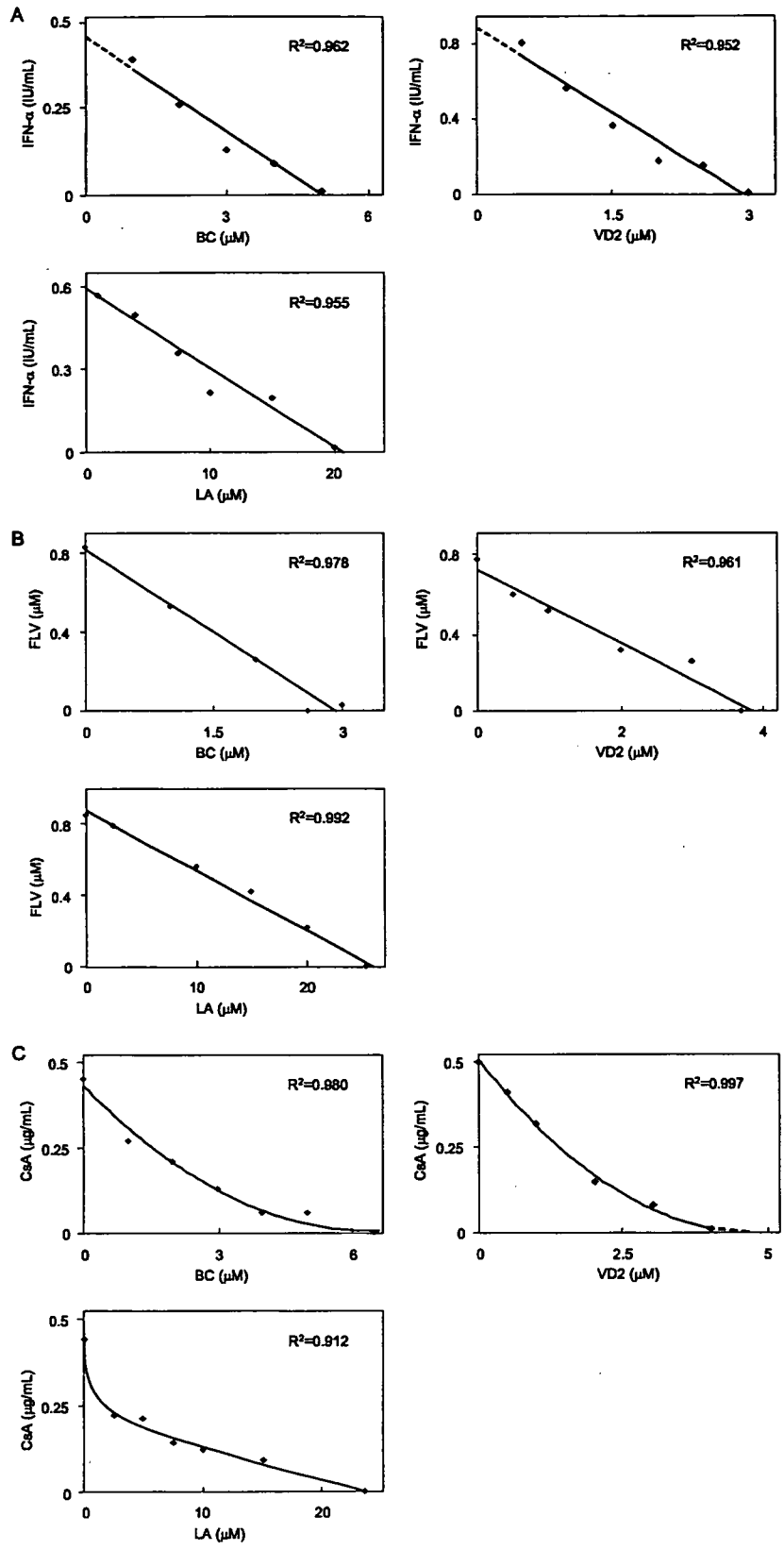
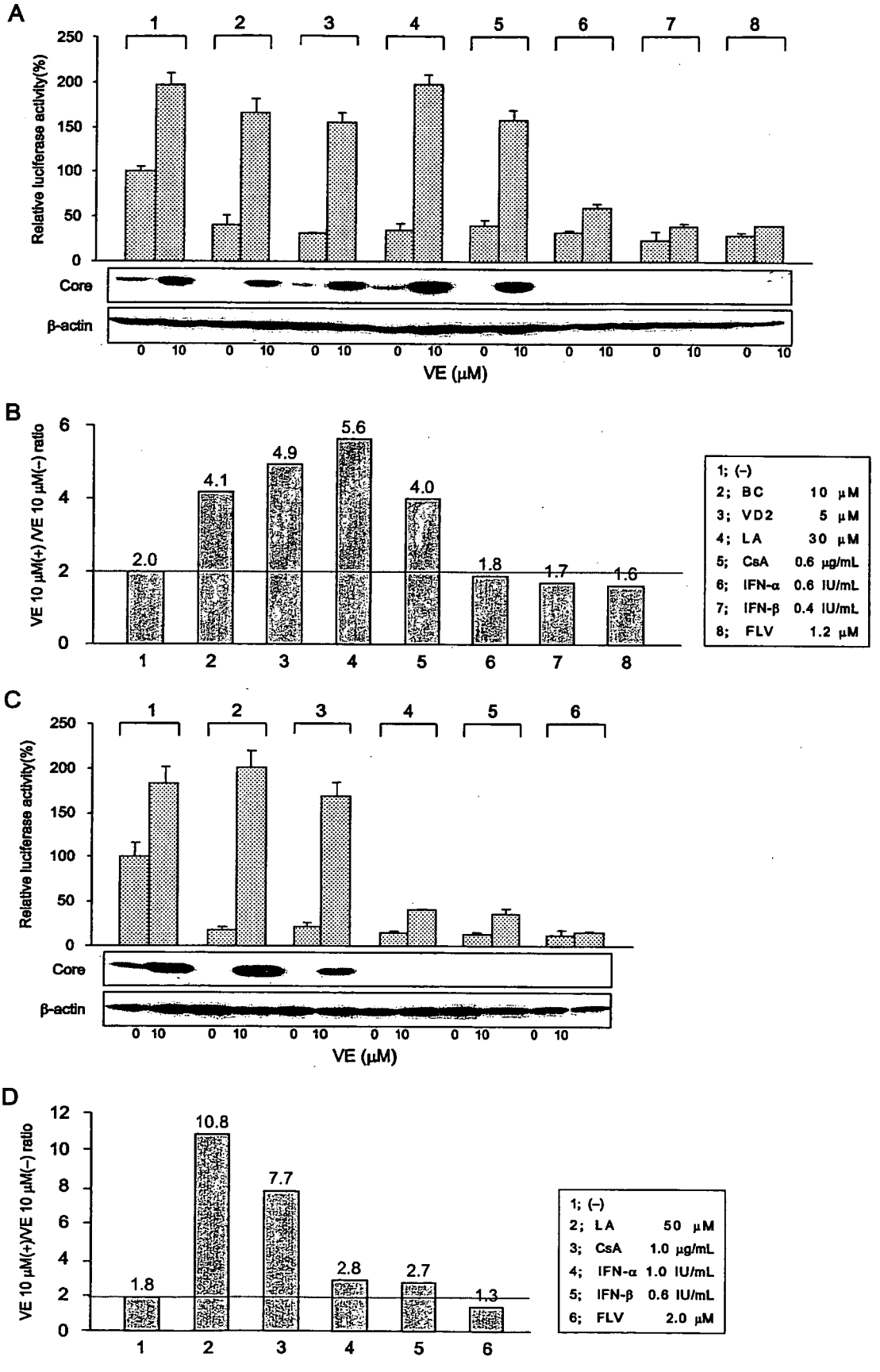


FIG. 7. Additive inhibitory effects of each of three nutrients in combination with IFN-α or FLV on HCV RNA replication, and synergistic effects observed with Cs. (A to C) Isobole plots of 50% inhibition of HCV RNA replication. OR6 cells were treated with BC (0, 1, 2, 3, 4, and 5 μM), VD2 (0, 0.5, 1, 2, 3, and 4 μM), and LA (0, 2.5, 5, 10, 15, and 20 μM) in combination with IFN-α (0, 0.2, 0.5, and 1 IU/ml) (A), FLV (0, 0.5, 1, and 2 μM) (B), or CsA (0, 0.2, 0.5, and 1 μg/ml) (C) for 72 h, and the RL assay was performed as described in the legend to Fig. 2 to obtain 50% isoboles. The R^2 value indicates the coefficient of determination.


A
Relative luciferase activity(%)
Core
β-actin
VE (μM)
B
VE 10 μM(+)/VE 10 μM(−) ratio
1; (−)
2; BC 10 μM
3; VD2 5 μM
4; LA 30 μM
5; CsA 0.6 μg/mL
6; IFN-α 0.6 IU/mL
7; IFN-β 0.4 IU/mL
8; FLV 1.2 μM
C
Relative luciferase activity(%)
Core
β-actin
VE (μM)
D
VE 10 μM(+)/VE 10 μM(−) ratio
1; (−)
2; LA 50 μM
3; CsA 1.0 μg/mL
4; IFN-α 1.0 IU/mL
5; IFN-β 0.6 IU/mL
6; FLV 2.0 μM

isoboles in combinations using each nutrient and IFN-α or FLV graphed nearly straight lines in each case, indicating that the suppressive effects of these cotreatments on HCV RNA replication were additive (Fig. 7A and B). Similar additive effects were obtained in combination with IFN-β (data not shown). It was noteworthy that all cotreatments with each nutrient and CsA resulted in curved, concave plots of 50% isoboles, thus suggesting that these combinations with CsA exerted synergistic inhibitory effects on HCV RNA replication (Fig. 7C). These results indicate that these three nutrients, administered as a supportive nutritional therapy, could potentially improve the SVR rate associated with IFN therapy alone.

The anti-HCV activities of BC, VD2, and LA, as well as that of CsA but not those of IFN and FLV, were completely canceled by VE. Among the 46 nutrients tested, BC and VD2 exhibited inhibitory effects on HCV RNA replication up to 70%, and LA exhibited inhibitory effects up to 90%, without exhibiting any cytotoxicity (Fig. 5A). In contrast, most of the liposoluble vitamins enhanced HCV RNA replication in OR6 cells. We used VE in the following studies because VE is one of the most common vitamins in the daily diet and it exerts a strong enhancing effect on HCV RNA replication. To clarify the mechanism of these opposing effects, we investigated whether the anti-HCV effects of BC, VD2, and LA were canceled by the addition of VE. We also tested representative anti-HCV compounds (i.e., CsA, IFN-α, IFN-β, and FLV) in combination with VE. We first examined the influence of 10 μM VE on the nutrients and compounds at the 70% inhibitory concentration level (Fig. 8A and B). The inhibitory effects of IFN-α, IFN-β, and FLV were hardly influenced by cotreatment with VE, whereas the anti-HCV effects of BC, VD2, LA, and CsA were canceled to a significant level by VE in the OR6 cells (Fig. 8A, upper panel). These results were also confirmed using authentic HCV RNA-replicating cells (Fig. 8A, lower panel). To normalize these results, we divided the luciferase value observed in the presence of VE by that in the absence of VE, and we considered this value to represent the effects of VE. When this value was larger than the value obtained in the absence of anti-HCV reagent (2.0; column 1 in Fig. 8B), we interpreted it as indicative of a reagent whose anti-HCV effects were canceled by VE. According to this criterion, BC (4.1), VD2 (4.9), LA (5.6), and CsA (4.0) were evaluated to be reagents for which the anti-HCV effects were canceled by VE (columns 2, 3, 4, and 5 in Fig. 8B). The anti-HCV effects of IFN-α, IFN-β, and FLV were not affected by VE (columns 6, 7, and 8 in Fig. 8B). We next examined the influence of 10 μM VE on the anti-HCV nutrients and compounds at the 90% inhibitory concentration level (Fig. 8C and D). BC and VD2 were not assessed in this experiment, because the maximum inhibitory effect was 70% in the case of these nutrients (Fig. 5A). Similar results were obtained in this experiment. LA (10.8) and CsA (7.7) were evaluated to be reagents for which the anti-HCV effects were canceled by VE (compare columns 2 and 3 to column 1 in Fig. 8D), although IFN-α (2.8) and IFN-β (2.7) were slightly affected by VE at this concentration (Fig. 8D, compare columns 4 and 5 to column 1). Judging by these results, it appears that BC, VD2, LA, and CsA may share some mechanism by which VE negated their anti-HCV activities.

DISCUSSION

The differential effects of BC and VA, as well as those of VD2 and VD3, which belong to the same categories as VA and VD, respectively, are of interest. We observed that whereas BC and VD2 inhibited HCV RNA replication, VA enhanced it, and VD3 exhibited basically no effect. The mechanism governing how these vitamins from the same category exert different effects on HCV RNA remains to be elucidated. However, liposoluble vitamins have been reported to exhibit various physiological activities with each nuclear receptor, consequently acting as hormone-like substances (19, 20, 27, 35). Differences in the gene products induced by each of these vitamins may lead to differences in the effects on HCV RNA replication. Another explanation might be considered in the light of findings suggesting that VA is an antioxidant, and yet recently, BC has been reported to induce oxidative stress (32, 43). This diversity of activities among vitamins in the same category, VA, might result in a variety of influences on HCV RNA replication. Further studies are still needed to account for why these different consequences are generated.

Previous studies have demonstrated that PUFAs such as AA, EPA, and DHA inhibit HCV RNA replication in cell culture systems (17, 21). However, saturated and mono-unsaturated fatty acids have been shown to enhance HCV RNA replication (17). In the prior studies, the cells tolerated the presence of PUFAs at concentrations of up to 50 μM. In contrast, in our study, 50 μM PUFAs were toxic, with the exception of LA. Furthermore, saturated and mono-unsaturated fatty acids hardly exhibited any effects on HCV RNA replication in our OR6 cell culture system. These discrepancies might be due to differences in both the clonalities of the cells and the HCV strains used in each experiment.

FIG. 8. VE canceled the anti-HCV activities of BC, VD2, LA, and CsA. (A and B) Effects of VE on the nutrients and compounds at the 70% inhibitory concentration. Both OR6 cells and OR6c cells, into which authentic HCV RNA was introduced, were treated with control medium [(−)], 10 μM BC, 5 μM VD2, 30 μM LA, 0.6 μg/ml of CsA, 0.6 IU/ml of IFN-α, 0.4 IU/ml of IFN-β, or 1.2 μM FLV in either the absence or presence of 10 μM VE for 72 h. After treatment, an RL assay of harvested OR6 cell samples was performed, and then the relative RL activity was calculated as described in the legend to Fig. 2. Subsequently, the production of HCV core antigen in OR6c cells was analyzed by immunoblotting using antibody specific to HCV core antigen. β-Actin was used as a control for the amount of protein loaded per lane (A). Then, the ratio of RL activity in the presence of 10 μM VE (+) to the RL activity in the absence of VE (−) was calculated. The horizontal line indicates the promotive effect of 10 μM VE alone on HCV RNA replication as a baseline (B). (C and D) Effects of VE on the nutrients and compounds at the 90% inhibitory concentration. Both OR6 cells and OR6c cells were treated with control medium, 50 μM LA, 1 μg/ml of CsA, 1 IU/ml of IFN-α, 0.6 IU/ml of IFN-β, and 2 μM FLV in either the absence (−) or presence (+) of 10 μM VE for 72 h. After treatment, the RL assay and Western blot analysis were performed (C), and then the ratio of RL activity in the presence of 10 μM VE to the RL activity in the absence of VE was calculated in the same manner as that described above (D).

Here, we demonstrated that three nutrients, BC, VD2, and LA, exhibited anti-HCV effects in polyclonal genome-length and subgenomic HCV RNA (strain O of genotype 1b)-replicating cells. These results indicated that the inhibitory activities of at least three anti-HCV nutrients are not limited to a specific cell clone (OR6).

Moreover, IFN or FLV exhibited additive anti-HCV effects when the cells were cotreated with each of the three anti-HCV nutrients. However, CsA showed synergistic anti-HCV effects in combination with each of these three nutrients. Interestingly, these results coincided with the experiment using VE, as VE canceled the anti-HCV effects of CsA but not those of IFN or FLV. It was recently demonstrated that the anti-HCV effects of CsA are related to the inhibition of cyclophilin (31, 42). CsA is also known as an oxidant that can cause renal or vascular dysfunction, and interestingly, antioxidants, including VE, attenuate these CsA-induced side effects (16, 22). Furthermore, we confirmed that another antioxidant, Se, also weakened the anti-HCV effects of BC, VD2, and LA (data not shown). Therefore, BC, VD2, and LA may possess an anti-HCV mechanism similar to that of CsA, and oxidative stress may be involved in these anti-HCV effects to some extent. Among the nutrients tested, VA, VC, VE, and Se enhanced HCV RNA replication, and these nutrients functioned as antioxidants. In contrast, four PUFAs inhibited HCV RNA replication, and they served as oxidants (29, 44). These results are further evidence of the involvement of oxidative stress in HCV RNA replication.

CH C patients may take excessive doses of VE during the course of interferon therapy, because as an antioxidant, VE has been expected to prevent injury to hepatocytes caused by oxidative stress. However, our results suggest that the potentially negative effects of VE on therapy for CH C patients should be carefully considered. To date, the significance of the role played by ordinary nutrients in viral infections has not been well characterized and has even been underestimated. We believe that our findings will shed light on the field of viral infection from the perspective of the nutritional sciences.

It is difficult to determine the blood concentrations of the nutrients tested in this study because the administration conditions may affect the concentrations in the blood. Rühl et al. (35) reported that the concentrations of BC in human serum are between 0.34 to 0.54 μM and that the average concentration in the human liver is 4.4 μM. Hagenlocher et al. (12) reported that the concentration of LA in human serum is 0.8 to 11.9 μg/100 μl. Armas et al. (3) reported that the concentration of VD2 in human serum at 24 h after a 50,000-IU administration is about 50 nM. The concentration of the nutrient in this study is higher than that in those reports. Therefore, monotreatment of the nutrient may not eliminate HCV. However, these nutrients may boost the effect of IFN treatment in combination like ribavirin does.

It is worth trying to examine the effects of BC, VD2, and LA on the recently developed JFH1 infectious virus production system in a future study. Here, it remains unclear whether these three nutrients affect the production of the virus. Furthermore, the comparison of the effects of these three nutrients between HCV genotypes 1 and 2 will provide useful information for the HCV therapy, as HCV genotypes 1 and 2 respond differentially to IFN treatment.

The precise mechanism underlying the anti-HCV activities of the nutrients has not been clarified in this study. The nutrients may inhibit viral RNAs and proteins, including the internal ribosome entry site, NS3-4A serine protease, and NS5B polymerase. Further in vitro study will be needed to clarify the targets of the nutrients responsible for their anti-HCV activities. Another possibility is that the nutrients inhibit the cellular proteins required for HCV RNA replication. We are now planning a study to clarify the mechanism underlying the nutrients' anti-HCV activities.

In conclusion, we found that three nutrients, BC, VD2, and LA, inhibited HCV RNA replication in a cell culture system and that Se, tryptophan, and various vitamins (A, C, E, and K) enhanced HCV RNA replication. The anti-HCV effects of BC, VD2, and LA were reversed by VE. These results are expected to provide useful information for the improvement of the SVR rates of patients receiving the currently standard IFN therapy. In addition, these findings may contribute to the development of a nutritional supplement specific to the treatment of people with CH C.

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Original Article

Fucosylated fraction of alpha-fetoprotein, L3, as a useful prognostic factor in patients with hepatocellular carcinoma with special reference to low concentrations of serum alpha-fetoprotein

Makoto Kobayashi, Takashi Kuroiwa, Takeshi Suda, Yasushi Tamura, Hirokazu Kawai, Masato Igarashi, Yasuo Fukuhara and Yutaka Aoyagi

Division of Gastroenterology and Hepatology, Department of Cellular Function, Course for Molecular and Cellular Medicine, Graduate School of Medical and Dental Sciences, Niigata University, Niigata, Japan

Background: The aim of the present study was to establish L3 fraction before initial treatment as a useful prognostic factor in a prospective fashion in hepatocellular carcinoma (HCC) where the alpha-fetoprotein (AFP) was very low.

Methods: From 1990 to 2004, 298 HCC patients in whom L3 could be measured were examined in the present study. Enrolled patients with HCC underwent operation, transcatheter arterial chemoembolization and percutaneous ablation therapy. The current patient status was confirmed as of the end of March 2005. L3 was determined by crossed immunoaffinoelectrophoresis when AFP was ≥30 ng/mL. It was carried out by liquid-phase binding assay system on cases where AFP < 30 ng/mL. The tentative discriminating line of L3 was set at 15%.

Results: The HCC group included four subgroups: 110 patients with AFP concentrations ≤100 ng/mL, 70 with AFP ≤ 50 ng/mL, 38 with AFP ≤ 30 ng/mL and 29 with AFP ≤ 25 ng/mL. The mean survival rate in the HCC group, whose L3 was >15% (high L3), was significantly lower than that in the HCC group whose L3 was ≤15% (low L3). There were also statistically significant differences in survival rates between high and low L3 in the four HCC subgroups. The statistically significant differences were more distinct in the subgroups with low AFP concentrations.

Conclusions: The present study indicates that the L3 fraction before treatment serves as a useful prognostic indicator when the serum concentrations of AFP were very low.

Key words: alpha-fetoprotein, hepatocellular carcinoma, L3, prognostic factor, survival rate

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most prevalent human cancers,[1] and measurements of serum concentrations of alpha-fetoprotein (AFP)[2,3] have been widely used for the early diagnosis of hepatocellular carcinoma (HCC). Recently, the determination of the L3 fraction of *Lens culinaris* agglutinin (LCA)-reactive species of AFP was introduced to discriminate HCC from non-neoplastic liver diseases when slight increments of serum AFP concentration were observed.[4–10] The molecular basis of the LCA-reactive species of AFP was the fucosylation of the biantennary sugar chain.[11,12]

Our previous results together with others by multivariate analysis showed that L3 was considered to be an independent prognostic factor in patients with HCC.[13,14] However, a measurement of the L3 fraction was not always reliable by crossed immunoaffinoelectrophoresis when the serum AFP concentrations were low. Accordingly, the limited usefulness of L3 fraction at low concentrations of AFP was observed. In the present study, we aim to establish the measurements of L3 fraction before initial treatment as a useful

Correspondence: Professor Yutaka Aoyagi, Division of Gastroenterology and Hepatology, Department of Cellular Function, Course for Molecular and Cellular Medicine, Graduate School of Medical and Dental Sciences, Niigata University, 757 Asahimachi Dori-1-Bancho, Chuo-ku, Niigata 951-8122, Japan. Email: aoy@med.niigata-u.ac.jp
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Table 1 Clinical features of 298 patients with hepatocellular carcinoma

Clinical features		No. (%) patients
Male		200
Female		98
Age mean age (years ± SD)		62 ± 10
Complication of cirrhosis		211 (71%)
Complication of chronic hepatitis		74 (25%)
Positive for HBsAg		78 (26%)
Positive for anti-HCV		170 (57%)
Positive for both HBsAg and anti-HCV		5 (2%)
Stage	UICC	Liver Cancer Study Group of Japan
I	32 cases	42 cases
II	87	81
III	101	92
IVA	60	65
IVB	18	18
Child–Pugh classification		
A		216 cases
B		66
C		16
Alpha-fetoprotein (ng/mL)†		13 742 ± 69 209 (12–770 000)
L3		28 ± 28 (0–100%)
Des-γ-carboxy prothrombin (mAU/mL)‡		52 838 ± 616 670 (26–2 120 000)
Treatment		
Operation		61
Radiofrequency ablation or microwave coagulation therapy		40
Percutaneous ethanol injection		47
Transcatheter arterial embolization and/or chemoembolization with/without percutaneous ethanol injection		150

†Value in parentheses is the range of AFP for patients in the present study with HCC.
‡Value in parentheses is the range of DCP for patients in the present study with HCC.
HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

prognostic factor in HCC patients whose serum concentrations were very low by liquid-phase binding assay system (LiBASys).[15]

METHODS

INFORMED CONSENT WAS obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by our institution's human research committee.

Patients and study design

Two hundred and ninety-eight patients with HCC, in whom an L3 of serum AFP could be determined before initial treatment in a prospective fashion, were examined in the present study. Of the 298 enrolled patients with HCC, 61 underwent operation, 150 were treated by transcatheter arterial emobolization and/or chemoembolization, 47 were treated by percutaneous ethanol injection, and 40 were treated by radiofrequency ablation and/or percutaneous microwave coagulation therapy from 1990 to 2004 (Table 1). The current patients statuses were confirmed as of the end of March 2005. Two hundred and forty-seven of 298 (82.8%) patients with HCC were diagnosed histologically by biopsy and autopsy. The remainder showed typical results indicating the presence of HCC by use of several imaging modalities such as computed tomography,[16] magnetic resonance imaging,[17] ultrasonography and selective celiac angiography together with the measurement of AFP, L3 and/or des-γ-carboxy prothrombin (DCP). Patients in early stages of HCC (Stage I) or patients with no typical results indicating the presence of HCC using the imaging modalities underwent histological examination.

L3 determination by crossed immunoaffinoelectrophoresis or LiBASys

L3 (fucosylated fraction) of AFP was determined by crossed immuno-affinoelectrophoresis in the presence of *Lens culinaris* agglutinin when the serum concentrations of AFP were ≥30 ng/mL.[4,18] In our previous study, the minimum concentration of AFP for an application of crossed immuno-affinoelectrophoresis was 30 ng/mL.[6] L3 was determined by LiBASys in cases where AFP was <30 ng/mL.[15] The tentative discriminating line of L3 was set at 15%.

Tumor stage

Tumor stage was ranked according to the TNM classification of malignant tumors by the International Union Against Cancer (UICC),[19] and the Liver Study Group of Japan.

Statistical analysis

Survival rates were determined by the method of Kaplan–Meier, and the differences in the survival rates between the HCC groups were compared using the generalized Wilcoxon's and the log-rank tests. The univariate and multivariate analyses in the prognostic factors for the patient's background was carried out with the Cox's proportional hazards model. The differences in the distributions of tumor stage, Child–Pugh classification and treatment between two HCC groups were determined by chi-squared analysis. Statistical significance was defined as a *P*-value of less than 0.05.

RESULTS

Clinical features of patients with HCC

TABLE 1 SHOWS the clinical features of 298 patients with HCC. The 298 HCC patients comprised 32 patients with stage I, 87 with stage II, 101 with stage III, 60 with stage IVA and 18 with stage IVB by the International Union Against Cancer; and comprised 42 patients with stage I, 81 with stage II, 92 with stage III, 65 with stage IVA and 18 with stage IVB by the Liver Cancer Study Group of Japan. The comparisons between high and low L3 groups in the baseline characteristics of 110 patients with AFP ≤ 100 ng/mL are shown in Table 2. A significant deviation of the distribution of tumor stage by the Liver Cancer Study Group of Japan was observed between the two HCC groups of L3 > 15% and L3 ≤ 15% with chi-squared analysis ($P = 0.0051$), but was not obtained by the UICC ($P = 0.165$). Significant deviations were not obtained between the two HCC groups in the distributions of Child–Pugh classification ($P = 0.099$) and treatments ($P = 0.895$). There were also no remarkable differences in clinical features between the two HCC groups except for positive rates of viral markers.

Range and distribution of AFP L3 in the high L3 group

The distribution of AFP L3 in the high L3 HCC group with AFP concentration ≤100 ng/mL is shown in Figure 1. Seventeen out of 36 cases (47%) were distributed in the L3 ranges from 15 to 25%.

L3 levels after treatments in the high L3 group

The decrement of L3 levels was observed in 71% of patients with high L3 after effective treatment. Table 3 shows the results of univariate analysis in the prognostic factors of patients with HCC evaluated by the Cox's proportional hazards model. L3 was a statistically significant prognostic factor when AFP concentrations of patients with HCC were limited to low ranges, AFP concentrations ≤100, 50, 30 and 25 ng/mL, respectively. However, the AFP concentration was not a statistically significant prognostic factor in low ranges of AFP concentrations.

Multivariate analysis in prognostic factors of HCC patients with AFP ≤ 100 ng/mL

Table 4 shows the results of multivariate analysis in the prognostic factors evaluated by Cox's proportional hazards model when AFP concentrations of patients with HCC were ≤100 ng/mL. The L3, DCP and tumor stage were statistically significant prognostic factors, but the AFP concentration was not a statistically significant prognostic factor. Essentially the same statistical result was obtained when the tumor stage by the Liver Cancer Study Group of Japan was applied rather than the tumor stage by the UICC.

Comparisons of the survival rates according to L3

As the mean percentage of L3 in 143 patients with hepatic cirrhosis was 4.0 ± 5.7% in our previous report,[6] L3 at 15% which corresponds to a mean + 2SD in cirrhosis was used as a tentative discriminating line. The

Table 2 Comparison of clinical features between high and low L3 HCC groups in 110 patients with AFP ≤100 mg/mL

Clinical features	L3 ≥ 15 No (%) of patients		L3 ≤ 15	
Male	26		47	
Female	10		27	
Age mean age (years ± SD)	65 ± 9		64 ± 8	
Complication of cirrhosis	29 (81%)		59 (80%)	
Complication of chronic hepatitis	7 (19%)		15 (20%)	
Positive for HBsAg	7 (19%)		7 (10%)	
Positive for anti-HCV	24 (67%)		62 (86%)	
Positive for both HBsAg and anti-HCV	1 (3%)		1 (1%)	
Stage	UICC	LCSGJ	UICC	LCSGJ
I	2 cases	2	16	20
II	10	8	22	26
III	18	13	27	19
IVA	4	11	8	8
IVB	2	2	1	1
Child–Pugh classification				
A	25 cases		62	
B	8		11	
C	3		1	
Alpha-fetoprotein (ng/mL)†	56 ± 25 (7–96)		39 ± 20 (11–89)	
Des-γ-carboxy prothrombin (mAU/mL)‡	1479 ± 4545 (22–20 750)		34 549 ± 252 747 (20–2 120 000)	
Treatment				
Operation	8		16	
Radio frequency ablation or microwave coagulation therapy	6		10	
Percutaneous ethanol injection	7		19	
Transcatheter arterial embolization and/or chemoembolization with/without percutaneous ethanol injection	15		29	

†Value in parentheses is the range of AFP for patients in the present study with HCC.
‡Value in parentheses is the range of DCP for patients in the present study with HCC.
HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LCSGJ, Liver Cancer Study Group of Japan.

mean survival rate in the HCC group, whose L3 (before treatment) was over 15% (high), was significantly lower than that in the other HCC group, whose L3 was ≤15% (low) by the generalized Wilcoxon test and the log-rank test ($P < 0.0001$) (Fig. 2).

In the next analysis, overall survival rates were compared on the basis of L3 in four HCC subgroups of AFP ranges. A statistically significant difference of survival rate was observed between HCC subgroups with high and low L3 when the serum AFP concentrations of patients with HCC subgroup were ≤100 ng/mL (Fig. 3a). A significant difference was also observed between HCC groups with high and low L3 when the serum AFP concentrations of patients in the HCC group were ≤50 ng/mL (Fig. 3b). Essentially identical results were seen in the other HCC groups with high and low L3 in the very low AFP ranges of ≤30 ng/mL (Fig. 4a) and 25 ng/mL (Fig. 4b).

Comparisons of the survival rates according to the serum AFP concentration

There was a statistically significant difference between the HCC group with AFP from 12 to 200 ng/mL and the other HCC group with AFP >200 ng/mL by the generalized Wilcoxon test ($P < 0.0001$) and the log-rank test ($P < 0.0035$). However, there was no significant difference in the low AFP ranges. Statistically significant differences were not observed between the HCC group with AFP concentrations from 12 to 50 ng/mL and the other HCC group with AFP from 51 to 100 ng/mL by the generalized Wilcoxon test ($P = 0.647$) and the log-rank test ($P = 0.343$). No significant difference was obtained between the HCC group with AFP from 12 to 25 ng/mL and the other HCC group with AFP from 26 to 50 ng/mL by the generalized Wilcoxon test ($P = 0.159$) and the log-rank test ($P = 0.279$).

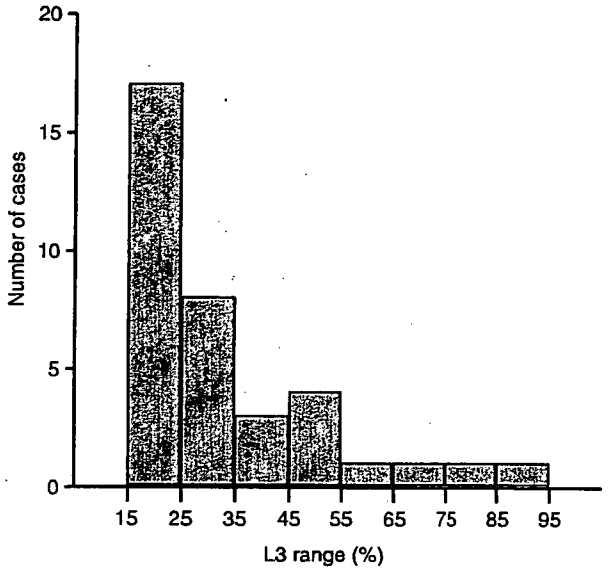

Figure 1 Distribution of alpha-fetoprotein (AFP) L3 in the high L3 hepatocellular carcinoma (HCC) group with AFP concentration ≤100 ng/mL. Seventeen out of 36 cases (47%) were distributed in the L3 ranges from 15 to 25%.

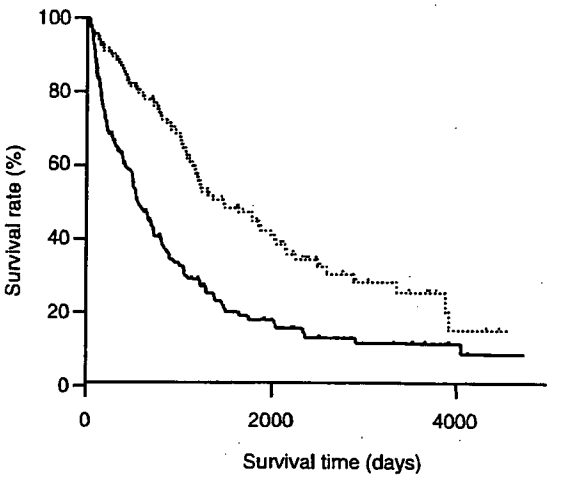

Figure 2 Comparisons of the survival rates between the hepatocellular carcinoma (HCC) groups with high L3 (more than 15%) before treatment and low L3 (≤15%) by the Kaplan–Meier method. The mean survival rate in the HCC group whose L3 was >15% was significantly lower than that in the HCC group whose L3 was ≤15% by the generalized Wilcoxon test and the log-rank test ($P < 0.0001$). ——, L3 > 15% ($n = 137$); ·····, L3 ≤ 15% ($n = 161$).

DISCUSSION

MEASUREMENTS OF SERUM concentrations of AFP have been widely used for the early diagnosis of HCC. However, a slight increment of serum AFP concentration ≤50 ng/mL was not regarded as an important indicator for the diagnosis and evaluation of therapeutic effects of patients with HCC. Many hepatologists are apt to regard a non-specific increase in serum AFP concentration with chronic liver inflammation.[20–22]

Table 3 Univariate analysis by Cox's proportional hazards model

Variables	Coefficient	Standard error	Chi-square	Relative risk	*P*-value
Total patients (298 cases)					
L3	0.0112	0.0024	21.335	1.0112	<0.0001*
AFP	0.3119	0.0686	20.692	1.3660	<0.0001
AFP ≤ 100 ng/mL (110 cases)					
L3	0.0236	0.0059	16.104	1.0239	<0.0001
AFP	0.6897	0.5393	1.6352	1.9931	0.2010
AFP ≤ 50 ng/mL (70 cases)					
L3	0.0202	0.0072	7.9780	1.0204	0.0047
AFP	0.7307	0.9648	0.5735	2.0765	0.4489
AFP ≤ 30 ng/mL (38 cases)					
L3	0.0556	0.0156	12.678	1.0571	0.0004
AFP	0.9573	1.7772	0.2902	2.6047	0.5901
AFP ≤ 25 ng/mL (29 cases)					
L3	0.0565	0.0185	9.308	1.0581	0.0023
AFP	−0.7686	2.0646	0.1386	0.4637	0.709

*Univariate analysis was carried out separately using L3 and AFP in the prognostic factors for the patient's background with Cox's proportional hazards model.
AFP, alpha-fetoprotein.

Table 4 Multiivariate analysis in the prognostic factors of HCC patients with AFP ≤ 100 ng/mL

Variables	Coefficient	Standard error	Chi-square	Relative risk	*P*-value
DCP	0.8839	0.3282	7.2542	2.4203	0.0071
Tumor stage (UICC)	0.4016	0.1791	5.02799	1.4942	0.0249
L3	0.7945	0.3675	4.6731	2.2134	0.0306
Platelet count	−0.0560	0.0438	1.6332	0.9455	0.2013
ALT	−0.0028	0.0026	1.1338	0.9972	0.2870
Child-Pugh score	0.3118	0.4432	0.4950	1.3659	0.4817
Indocyanin green disappearance rate	2.6758	4.9245	0.2953	14.5239	0.5869
AFP	0.2548	0.6670	0.1458	1.2900	0.7026
Age	0.0068	0.0209	0.1058	1.0068	0.7450

AFP, alpha-fetoprotein; ALT, alanine aminotransferase; DCP, des-γ-carboxy prothrombin.

The measurement of the LCA-reactive species, L3, was previously performed by lectin-affinity electrophoresis followed by antibody-affinity blotting.[18] However, the manual electrophoresis method requires complicated and time-consuming procedures together with low reliability at very low concentrations of AFP ≤ 30 ng/mL. In the present study, the LiBASys automated analyzer system of measuring AFP species was introduced when the AFP concentrations of patients with HCC were relatively low, <30 ng/mL. This automated system provided good quantitative results for measuring AFP-L3 percentage in the very low concentrations of AFP. We could detect the L3 fraction (0–55%) from 12 to 30 ng/mL with high reproducibility in the present study.

Fucosylated species of AFP, L3, is the product of α1–6 fucosyltransferase in the presence of GDP-fucose. The cDNA-cloning of this enzyme, GDP-L-Fuc:*N*-acetyl-β-D-glucosaminide α1–6 fucosyltransferase, was performed

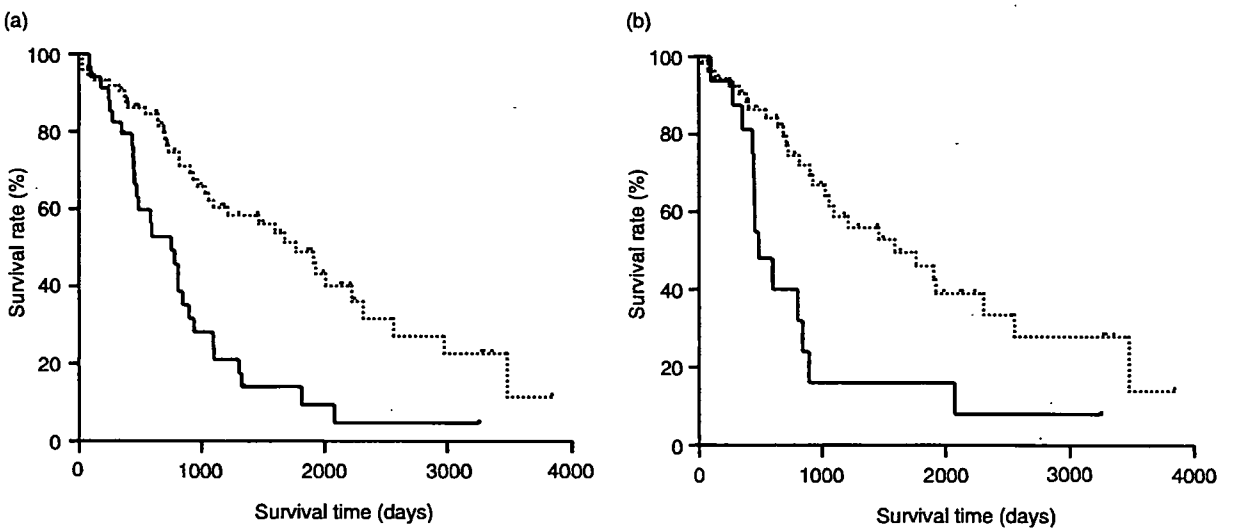

Figure 3 (a) Comparisons of the survival rates between the hepatocellular carcinoma (HCC) groups with high L3 and low L3 before treatment, based on the Kaplan–Meier method, when the serum alpha-fetoprotein (AFP) concentrations of the HCC group were ≤100 ng/mL. There were statistically significant differences between the HCC groups with high and low L3 by the generalized Wilcoxon test ($P = 0.0004$) and the log-rank test ($P < 0.0001$). ——, L3 > 15% ($n = 36$); ·····, L3 ≤ 15% ($n = 74$). (b) Comparisons of the survival rates between the HCC groups with high and low L3 before treatment, based on the Kaplan–Meier method, when the serum AFP concentrations of the HCC group were ≤50 ng/mL. There were statistically significant differences between the HCC groups with high and low L3 before treatment by the generalized Wilcoxon test ($P = 0.0024$) and the log-rank test ($P = 0.0022$). ——, L3 > 15% ($n = 17$); ·····, L3 ≤ 15% ($n = 53$).

by Taniguchi and his coworkers from a porcine brain[23] and a human gastric cancer cell line.[24] This enzyme was distributed in many organs in rats, and abundant activities were observed in the brain, spleen and testis. Normal liver showed relatively low activity.[25] However, we showed that α1–6 fucosyltransferase (FUT8) levels in HCC tissues were higher than those in surrounding noncancerous tissues and that α1–6 fucosyltransferase levels of HCC tissues increased in accordance with dedifferentiation of HCC tissues. These results indicate the close relationship between an activation of α1–6 fucosyltransferase with a subsequent increment of L3 fraction of AFP and an increased degree of malignant behavior of HCC tissue. The clinical evidence that an increment of L3 of AFP species resulted in an unfavorable prognosis in the present study is consistent with previous results.[26]

Tada *et al.* recently reported the relationship between positivity of *Lens culinaris* agglutinin-reactive AFP and pathological features of HCC.[27] A total of 111 patients with HCC less than 5 cm in diameter who underwent hepatic resection were studied. Thirty-three (30%) cases were positive for AFP-L3. The prevalence of HCC with infiltrative growth, with capsule infiltration, with septum formation, with portal vein invasion and with hepatic vein invasion was significantly higher in the AFP-L3 positive group. They concluded that AFP-L3-positive HCC had several pathological features of a progressed state of HCC, which accounted for AFP-L3 as an indicator of poor prognosis of HCC.

More recently, Okuda *et al.* reported the clinicopathological features of 14 patients with primary malignant hepatic tumors who were positive for L3 with low AFP concentrations (from 5 to 73 ng/mL) and negative for DCP.[28] Among the 14 patients, three had intrahepatic cholangiocarcinoma, one had cholangiocellular carcinoma, one had combined-type HCC, and one had undifferentiated hepatic sarcoma. The remaining eight patients (57%) had HCC. In the present study, the entire HCC group included 110 HCC patients with AFP concentrations < 101 ng/mL. Twenty out of 110 patients showed seronegativity of DCP. There was no typical patient showing cholangiocellular carcinoma by biopsy and several imaging modalities, although histological examinations were not completely performed in the 20 patients.

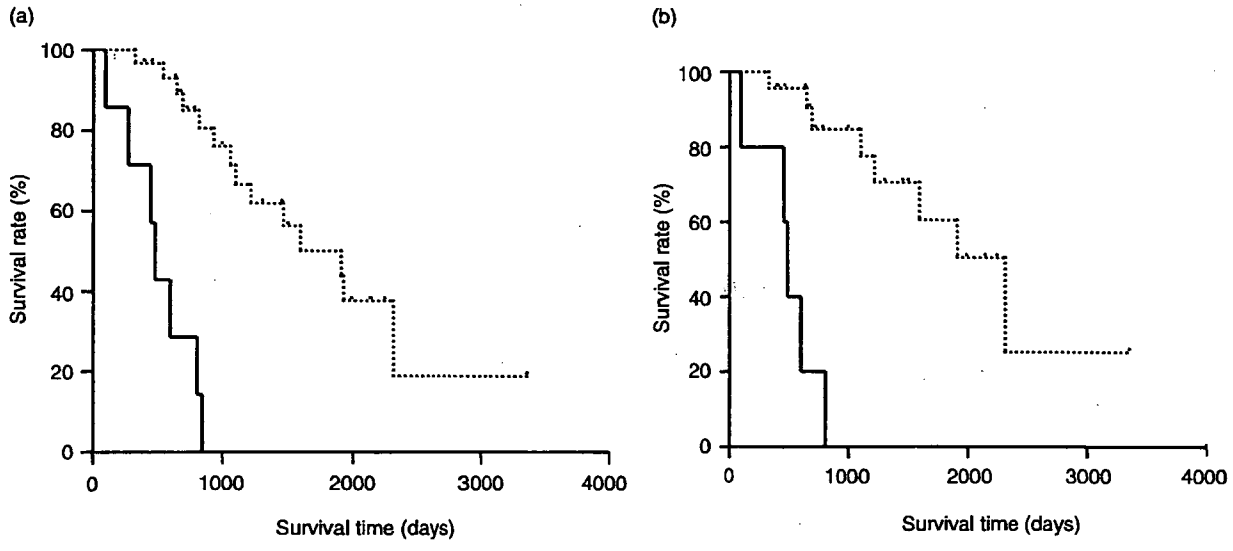

Figure 4 (a) Comparisons of the survival rates between the hepatocellular carcinoma (HCC) groups with high and low L3 before treatment, based on the Kaplan–Meier method, when the serum alpha-fetoprotein (AFP) concentrations of the HCC group were ≤30 ng/mL. There were statistically significant differences between the HCC groups with high and low L3 by the generalized Wilcoxon test ($P < 0.0001$) and the log-rank test ($P < 0.0001$). ——, L3 > 15% ($n = 7$); ·····, L3 ≤ 15% ($n = 31$). (b) Comparisons of the survival rates between the HCC groups with high and low L3 before treatment, based on the Kaplan–Meier method, when the serum AFP concentrations of the HCC group were ≤25 ng/mL. There were statistically significant differences between the HCC groups with high and low L3 by the generalized Wilcoxon test ($P < 0.0001$) and the log-rank test ($P < 0.0001$). ——, L3 > 15% ($n = 5$); ·····, L3 ≤ 15% ($n = 24$).

The treatment of HCC patients with cirrhosis faces a dilemma as to whether or not to ablate the cancer tissue thoroughly including the surrounding non-cancerous liver tissue. Regarding the poor hepatic reserve in HCC patients with cirrhosis, minimal damage to non-cancerous liver tissue by the treatment leads to a long survival. However, an incomplete treatment results in an unfavorable prognosis by subsequent HCC recurrence. Accordingly, if the scale to estimate the biological malignant potential could be available before the initial therapy, there would be several advantages in not only the treatment but also in the follow up of patients with HCC. The present results showed a positive reaction of L3 as an indicator of unfavorable prognosis in HCC with very low concentrations of serum AFP. In conclusion, we must make every effort to prevent an increase of L3 in the treatment of patients with HCC in cases with very low serum concentrations of AFP. When included in anatomical staging together with DCP, the measurement of AFP-L3 improves prognostic estimates and appraisal of therapeutic outcomes.

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Haploinsufficiency of *Bcl11b* for suppression of lymphomagenesis and thymocyte development

Kenya Kamimura [a], Hiroyuki Ohi [a], Tomoyuki Kubota [a], Kiyoshi Okazuka [a], Yoshihiro Yoshikai [a], Yu-ichi Wakabayashi [a], Yutaka Aoyagi [a], Yukio Mishima [a,b], Ryo Kominami [a,b,*]

[a] *Department of Molecular Genetics, Niigata University Graduate School of Medical and Dental Sciences, Asahimachi 1-757, Niigata 951-8510, Japan*
[b] *Center for Transdisciplinary Research, Niigata University, Asahimachi 1-757, Niigata 951-8510, Japan*

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Abstract

Recurrent chromosomal rearrangements at *BCL11B* are found in human hematopoietic malignancies mostly of T-cell origin. However, it is unclear how this disruption contributes to oncogenesis, because the majority of leukemias express BCL11B from an undisrupted allele. Here, we show that $Bcl11b^{+/-}p53^{+/-}$ mice exhibited greater susceptibility to lymphomas than $Bcl11b^{+/+}p53^{+/-}$ mice but most lymphomas retained and expressed the wild-type *Bcl11b* allele. This strongly suggests that *Bcl11b* is haploinsufficient for suppression of thymic lymphoma development in mice of the $p53^{+/-}$ background, a situation in which functional loss of only one allele confers a selective advantage for tumor growth. The haploinsufficiency is further supported by that $Bcl11b^{+/-}$ mouse embryos were impaired in thymocyte development and survival. These results indicate relevance of *BCL11B* aberration to human leukemogenesis.

Keywords: Lymphoma; γ-ray; Haploinsufficiency; P53; Bcl11b

Genetic studies of familial cancers have identified tumor suppressor genes that affect several cellular pathways, such as those controlling proliferation, apoptosis, differentiation, and genomic integrity. Also, the recessive nature of the tumor suppressors is defined, requiring "two-hit" inactivation of both alleles for the suppression [1,2]. Inheritance of a single mutant allele accelerates tumor susceptibility, because only one additional mutation is required for complete loss of gene function. However, a subset of tumor suppressor genes is dominant or haploinsufficient for tumor suppression, a situation in which functional loss of only one allele confers a selective advantage for tumor growth [2–4].

Bcl11b/Rit1/Ctip2 gene encodes a member of the zinc finger proteins [5–7] that regulate the development of thymocytes of the αβ T-cell lineage [8,9]. We previously found allelic losses at a high frequency and mutations at *Bcl11b* gene in γ-ray-induced mouse thymic lymphomas. The human *BCL11B* locus was also involved in recurrent chromosomal aberrations in leukemias mostly of T-cell origin, although the majority of the leukemias expressed BCL11B from an undisrupted allele [10,11]. These suggest that *Bcl11b* gene is a tumor suppressor gene in hematopoietic malignancies. However, *Bcl11b*-deficient mice die shortly after birth, exhibiting profound apoptosis of thymocytes [8]. Since apoptosis may be a mechanism to eliminate deleterious cells, the apoptotic phenotype of $Bcl11b^{-/-}$ thymocytes seems to contradict with the possibility of Bcl11b as a tumor suppressor.

In this paper, we examine whether or not $Bcl11b^{+/-}$ genotype provides susceptibility to γ-ray-induced thymic

* Corresponding author. Address: Department of Molecular Genetics, Niigata University Graduate School of Medical and Dental Sciences, Asahimachi 1-757, Niigata 951-8510, Japan. Fax: +81 25 227 0757.
E-mail address: rykomina@med.niigata-u.ac.jp (R. Kominami).

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lymphomas. Also, susceptibility to spontaneous thymic lymphomas is examined in *Bcl11b*$^{+/-}$ mice and *Bcl11b*$^{+/-}$*p53*$^{+/-}$ doubly heterozygous mice. Here, we show that *Bcl11b*$^{+/-}$ genotype provides susceptibility and that *Bcl11b* heterozygous state contributes to lymphomagenesis even retaining the expression, suggesting haploinsufficiency for tumor suppression.

Materials and methods

Mice and lymphoma development. *Bcl11b*$^{+/-}$ mice of BALB/c background were mated with MSM mice and 49 progeny were exposed to a single dose of 3-Gy γ-irradiation at 4 weeks of age, followed up for 300 days. On the other hand, *Bcl11b*$^{+/-}$ mice were mated with *p53*$^{-/-}$ MSM mice and 40 progeny of *Bcl11b*$^{+/-}$*p53*$^{+/-}$ doubly heterozygous or *p53*$^{+/-}$ singly heterozygous mice were followed up for 300 days without irradiation. Development of thymic lymphoma was diagnosed by inspection of labored breathing and palpable induration of thymic tumor. Existence of tumors was confirmed upon autopsy of the mice. The Mantel–Cox test with StatView-J 5.0 software on a Macintosh personal computer was used for χ^2 and P values to test the difference between *Bcl11b*$^{+/-}$ and *Bcl11b*$^{+/+}$ genotypes for the development of thymic lymphomas. Mice used in this study were maintained under specific pathogen-free conditions in the animal colony of the Niigata University. All animal experiments comply to the guidelines by the animal ethics committee for animal experimentation of the University.

DNA analysis. Isolation of genomic DNA from brain and thymic lymphomas was carried out by standard protocols. Genotyping of *Bcl11b* and *p53* were carried out with PCR as described previously [7,12,13]. Allelic loss at *Bcl11b* was analyzed by using *D12 Mit181* marker in the vicinity of *Bcl11b* and by detecting a single-nucleotide polymorphism on *Hin*fI restriction site between BALB/c (KO-allele) and MSM genomes. Primers were 5′-GGCTGAATTTACAGGATGAGG and 5′-CTTGAA CCCCAACTTCTGTG, and products were analyzed by gel electrophoresis after *Hin*fI digestion.

Flowcytometry. Two female mice were mated with one male overnight and were checked for copulation plugs in the following morning. The gestational age of the embryos was calculated 0:00 a.m. on the day of plug detection as day 0 and hour 0. On 16.5 or 18.5 day of gestation, thymus was removed from embryos and subjected to flow cytometric analysis [8]. Cells were analyzed with a FACSAria flow cytometer (Becton–Dickinson) using anti-CD4 (RM4-5) and anti-CD8 (53–6.7) that were obtained from PharMingen. Data were analyzed with the Flow-Jo software (Tree-Star, Inc.).

Immunoblot assay. Western blotting was performed as previously described [8]. Thymic lymphomas were suspended in PBS and mixed with an equal volume of lysis buffer, 0.125 M Tris–HCl (pH 6.8), 10% sucrose, 10% SDS, 10% 2-ME, and 0.04% bromophenol blue. The extract was electrophoresed in 8% SDS–PAGE gels and blotted onto Hybond membranes (Amersham Pharmacia Biotech, Piscataway, NJ). Protein bands were visualized using chemiluminescent detection (ECL plus, Amersham Pharmacia Biotech.).

Results

Bcl11b$^{+/-}$ *genotype provides susceptibility to γ-ray-induced mouse thymic lymphomas*

A total of 49 F_1 hybrids between *Bcl11b*$^{+/-}$ BALB/c and MSM mice were produced and subjected to γ-irradiation. They were followed up for 300 days. Forty-one of the mice developed tumors, 40 of which were thymic lymphomas. Genotyping of *Bcl11b* locus revealed that 27 mice were of *Bcl11b*$^{+/-}$ and 22 were of *Bcl11b*$^{+/+}$. Fig. 1A displays the cumulative tumor incidence of mice of *Bcl11b*$^{+/-}$ and *Bcl11b*$^{+/+}$ genotypes. *Bcl11b*$^{+/-}$ mice developed tumors at a higher incidence and a shorter latency than those of *Bcl11b*$^{+/+}$ ($P = 0.0037$ in Mantel–Cox test), indicating that *Bcl11b* is a tumor suppressor gene. Fig. 1B shows examples of analysis of loss of the wild-type allele using D12Mit181 marker in the vicinity of *Bcl11b*. Of the 26 thymic lymphomas examined, 14 (54 %) of the lymphomas lost the wild-type allele. This is consistent with that *Bcl11b* is a tumor suppressor gene, though the loss frequency was lower than that (85%) of the wild-type *p53* allele in the thymic lymphomas developed in *p53*$^{+/-}$ mice [12].

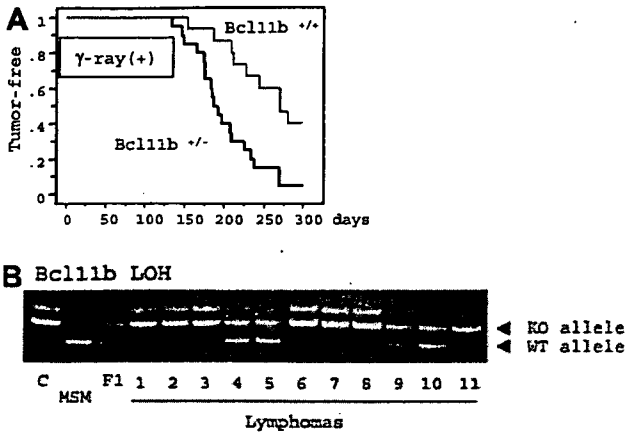

Fig. 1. *Bcl11b*$^{+/-}$ genotype provides susceptibility to thymic lymphomas. (A) Kaplan–Meier analysis of γ-ray induced mouse thymic lymphomas in *Bcl11b*$^{+/-}$ (black line) and *Bcl11b*$^{+/+}$ (gray line) mice. (B) Loss of the wild-type *Bcl11b* allele examined using *D12Mit181* marker in the vicinity of *Bcl11b* locus.

Bcl11b is a haploinsufficient tumor suppressor gene

Fig. 2A displays the cumulative incidences of spontaneously developed tumors in *Bcl11b*$^{+/-}$ mice, *p53*$^{+/-}$ mice and *Bcl11b*$^{+/-}$*p53*$^{+/-}$ mice. Unless *Bcl11b*$^{+/-}$ mice were irradiated, no mice ($n = 60$) developed tumors until 600 days after birth. However, the mice doubly heterozygous for *Bcl11b* and *p53* (*Bcl11b*$^{+/-}$*p53*$^{+/-}$) developed tumors at a higher incidence and a shorter latency than those of *Bcl11b*$^{+/+}$*p53*$^{+/-}$ mice ($P < 0.0001$ in Mantel–Cox test). Fourteen of the 16 *Bcl11b*$^{+/-}$*p53*$^{+/-}$ mice developed thymic lymphomas whereas only two of the 24 *Bcl11b*$^{+/+}$*p53*$^{+/-}$ mice did so. In contrast to the radiogenic lymphomas, 12 of the 14 spontaneous lymphomas of *Bcl11b*$^{+/-}$*p53*$^{+/-}$ mice retained the wild-type *Bcl11b* allele (Fig. 2B). Western blotting revealed that nine out of the 14 lymphomas expressed Bcl11b proteins at the same level to thymocytes, two lower than those, and three (two of them losing the wild-type allele) lacked expression (Fig. 2C). This high rate of retention of Bcl11b expression strongly suggests haploinsufficiency of *Bcl11b* for tumor suppression. This haploinsufficiency may be incomplete,