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Interleukin-29 Suppresses Hepatitis A and C Viral Internal Ribosomal Entry Site-Mediated Translation

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Abstract

Our aim was to investigate the effects of interferons (IFNs)-λ (interleukin-29 [IL-29], IL-28A, and IL-28B) on hepatitis C virus (HCV) and hepatitis A virus (HAV) internal ribosomal entry site (IRES)-mediated translation. The effects of these IFNs on HCV/HAV translation from HAV/HCV IRES were investigated using bicistronic reporter constructs. We transfected HCV/HAV IRES constructs into these IFN-expressing cell lines. IL-29 showed stronger inhibition of their IRES-mediated translation. Combining IL-29 with IFN-α or amantadine resulted in stronger inhibition of HAV IRES activity. Our findings demonstrated a novel antiviral effect of IFNs-λ against HAV and HCV through the suppression of IRES-mediated translation.

Introduction

Hepatitis A virus (HAV), a nonenveloped RNA virus of the Picornaviridae family, is the major causative agent of acute viral hepatitis, and occasionally leads to acute liver failure including fulminant hepatitis (17,31,32). The HAV genome is approximately 7600 nt in length and consists of a 5′ nontranslated region (5′ NTR), a single open reading frame that encodes both structural (VP4, VP2, VP3, and VP1) and nonstructural proteins (2A, 2B, 2C, 3A, 3B, 3C, and 3D), and a 3′ NTR with a polyadenylation signal (polyA) tail. The HAV genome also has an internal ribosomal entry site (IRES) that can promote 5′-end-independent initiation of RNA translation (2,11,12,15,19,20,45).

Hepatitis C virus (HCV), an enveloped RNA virus of the Flaviviridae family, causes a spectrum of diseases ranging from an asymptomatic carrier state to end-stage liver disease, including cirrhosis and hepatocellular carcinoma (HCC) (1,14,16). HCV has a 5′ NTR, a long open reading frame, and a 3′ NTR. The HCV genome is approximately 9600 nt in size and encodes a polyprotein precursor of about 3000 amino acids, which is cleaved by both viral and host proteases into structural (core, E1, E2, and p7) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. HCV also has IRES containing a 5′ NTR and part of the core coding region which forms a secondary structure, and supports translation initiation of an HCV genome in a cap-independent manner (18,24).

IFNs-λ are the most recently defined members of the class III cytokine family, consisting of IL-28A (IFN-λ2), IL-28B (IFN-λ3), and IL-29 (IFN-λ1), and a component of their receptor, IL28-Rα. IL-28 and IL-29 represent an evolutionary link between type I IFNs and the IL-10 family (39). This receptor-ligand system might contribute to antiviral or other defenses by a mechanism similar to, but independent of, type I IFN (25). Additional study is necessary to determine whether IFN-λ can synergize with IFN-α in viral infections, or whether it plays an independent primary role in the antiviral system (41).

There are several reports that IFN-λ exerts antiviral activity against HBV (36), HCV (36), West Nile virus (WNV) (29), influenza A virus, influenza B virus, respiratory syncytial virus, human metapneumovirus, and severe acute respiratory syndrome (SARS) coronavirus (34,41). Human hepatocytes express the IFN-λ receptor complex and IFNs-λ induce signal transducer and activator of transcription 1 (STAT1) phosphorylation (5). Activation of STAT1 is an important factor for the eradication of HCV after antiviral treatments (33). In addition, cellular proteins known as IRES trans-acting factors (ITAFs) are also required for efficient IRES-mediated translation (23,26). The subset of ITAFs that regulates translation initiation appears to be specific for each IRES element (23,26). IFNs including IFNs-λ affect host factors (21,27).

We reported that HAV IRES is an attractive target of anti-HAV drugs because IRES is located in the 5′ NTR, the most

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conserved region among different HAV strains (19,20,45), and is well conserved among clinical isolates (15,17). It has also been reported that HCV IRES is an attractive target of anti-HCV drugs because IRES is located in the 5′ NTR, the most conserved region among different HCV strains (18,24). We focused on the IRES as an antiviral target of these viruses because IRES activity seems to be correlated with translation of viral protein, which is important for viral replication, although there are contrary opinions. Recently, several groups reported that IL-28B SNP predicts hepatitis C treatment-induced viral clearance and natural clearance (10,16,36,42). In the present study, we examined the inhibitory effects of IFN-λ against HAV and HCV IRES-mediated translation.

Materials and Methods

Cells and virus

Huh7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO_2. The plasmids pcDNA3.1-IL28A, pcDNA3.1-IL28B, and pcDNA3.1-IL29 [kindly provided by Prof. T. Betakova, Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic (41)] were plasmids expressing IL-28A, IL-28B, and IL-29, respectively. Huh7 cells were transfected with the expression plasmids pcDNA3.1-IL28A, pcDNA3.1-IL28B, pcDNA3.1-IL29, or pcDNA3.1 in Effectene transfection reagent (Qiagen, Hilden, Germany). After 48 h, G418 (Promega, Madison, WI) was added at 1000 μg/mL for selection of Huh7-IL28A, Huh7-IL28B, Huh7-IL29, or Huh7-pcDNA3.1. After 3 wk, to avoid monoclonal selection, all cells were collected for further analysis.

Cell culture-grown HCV JFH1 (genotype 2a) was used in Huh7-derived cell lines (14,43). Tissue culture-adapted HAV strain KRM003 (genotype IIIB) was used in African green monkey kidney GL37 cells (22,37). A HAV DNA-based subgenomic replicon [kindly provided by Prof. V. Gauss-Muller, Institute of Virology, University of Luebeck, Luebeck, Germany (9)] was also used in HuhT7 cells that stably express T7-RNA polymerase in cytoplasm (9,38).

Reagents

The chemicals used were human recombinant IFN-α (Sigma-Aldrich, St. Louis, MO), human recombinant IL29 (IFN-λ1; Acris Antibodies GmbH, Herford, Germany), and amantadine hydrochloride (Sigma-Aldrich).

Bicistronic reporter plasmids

The bicistronic plasmid pSV40-HAV IRES-luc, encoding *Renilla reniformis* luciferase (Rluc) and firefly luciferase (Fluc), was separated by HAV IRES derived from pHM175 (kindly provided by Prof. S.U. Emerson, U.S. National Institutes of Health) under the control of SV40 promoter, with a polyadenylation signal (polyA; Fig. 1A) (19). The bicistronic plasmid pSV40-HCV IRES-luc (kindly provided by Prof. M. Kruger, Medizinische Hochschule Hannover, Hannover, Germany), carries the Rluc gene, the HCV IRES, including the full-length HCV core, and the Fluc gene under control of the SV40 promoter, with a polyadenylation signal (polyA; Fig. 1B) (18,24).

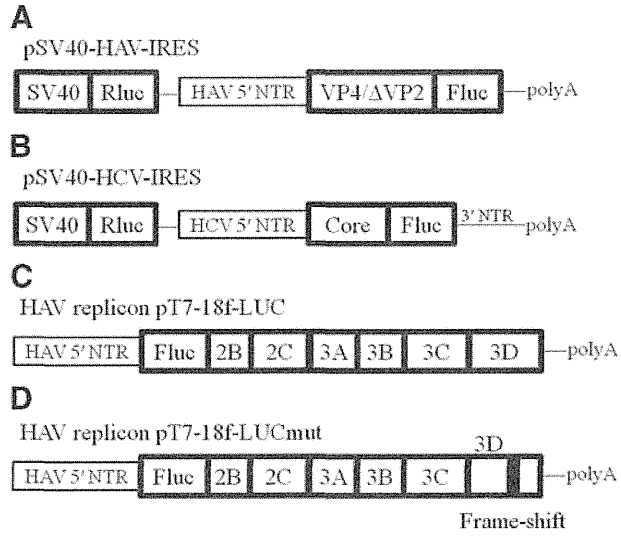


FIG. 1. Structure of HAV/HCV reporter constructs used in this study. (**A**) pSV40-HAV IRES-luc encodes the *Renilla reniformis* luciferase (Rluc), the internal ribosomal entry site (IRES) of HAV HM175, and firefly luciferase (Fluc) under the control of SV40 promoter, with a polyadenylation signal (polyA) (19). (**B**) pSV40-HCV IRES-luc encodes Rluc, the HCV IRES, including the full-length HCV core, and the Fluc gene under control of SV40 promoter, with a polyadenylation signal (polyA) (18,24). (**C**) Replication-competent HAV replicon pT7-18f-LUC contains an open-reading frame of Fluc flanked by the first four amino acids of HAV polyprotein, and by 12 C-terminal amino acids of VP1. This segment is followed by P2 and P3 domains of HAV strain HM175 18f (9). (**D**) Replication-incompetent HAV replicon pT7-18f-LUCmut contains a frameshift mutation in the polymerase 3D (9) (5′ NTR, 5′ nontranslated region; 3′ NTR, 3′ nontranslated region).

Transfection and in vitro *reporter assays*

Approximately 1.0×10^5 cells per well were placed in a six-well plate (Iwaki Glass, Tokyo, Japan) 24 h prior to transfection. The cells were transfected with 0.4 μg of pSV40-HAV (HCV) IRES-luc using Effectene Transfection Reagent (Qiagen) following the manufacturer's protocol. Forty-eight or 72 h after transfection, the cells were harvested using reporter lysis buffer (Toyo Ink, Tokyo, Japan), and luciferase activity was determined by luminometer (Luminescencer-JNR II AB-2300; ATTO, Tokyo, Japan) (44). To control for variations in transcription, IRES activity was assessed by measuring the ratio of Rluc and Fluc activities. The relative ratio of Fluc activity to Rluc activity (Fluc:Rluc) was defined as 100% in the untreated condition. We accept more than 10^2 of Fluc/Rluc as being positive for IRES activity. All samples were run in triplicate.

RNA extraction, cDNA synthesis, and RT-PCR

The cells were seeded into 6-well plates, and total cellular RNA was extracted 48 h later with the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA samples were then stored at −80°C until use. RNA quality was examined using the A_{280}:A_{260} ratio (Pharmacia Biotech, Bedford, MA). cDNA synthesis was performed with a random hexamer using Prime Script reverse transcriptase

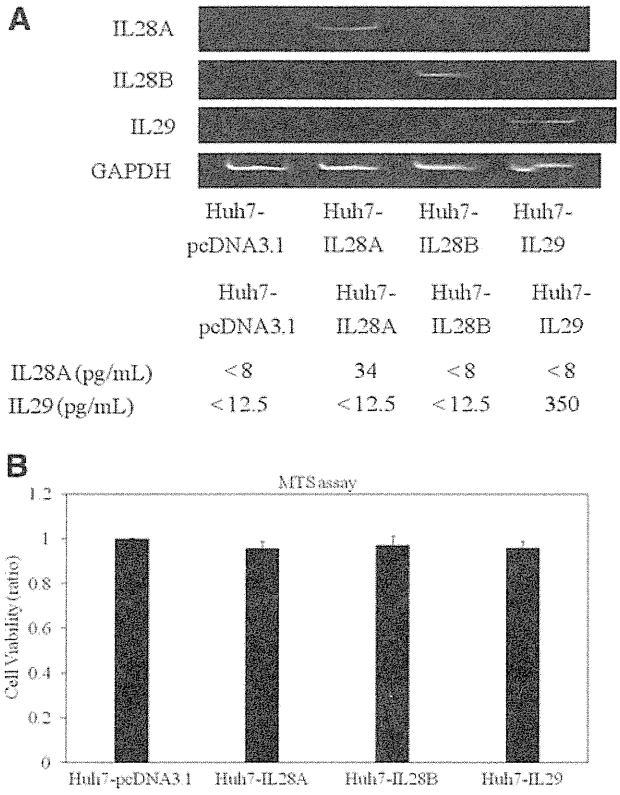


FIG. 2. Overexpression of IL-28A, IL-28B, or IL-29 in human hepatoma cell line Huh7. (**A**) Expression of IFNs-λ mRNA in Huh7-pcDNA3.1, Huh7-IL28A, Huh7-IL28B, and Huh7-IL29 cells (upper panel). RT-PCR was performed using each specific primer. ELISA results for IL-28A and IL-29 are shown in the lower panel. The sensitivities for human IL-28A and IL-29 by these ELISA kits were 8 pg/mL and 12.5 pg/mL, respectively. (**B**) IL-28A, IL-28B, or IL-29 expression does not inhibit cell growth and viability. Huh7-derived cells were plated at a density of 0.5×10^6, and MTS assay was performed at 24 h. The value of Huh7-pcDNA3.1 was set at 1. Data are expressed as mean ± SD of triplicate determinations from one experiment representative of four independent experiments.

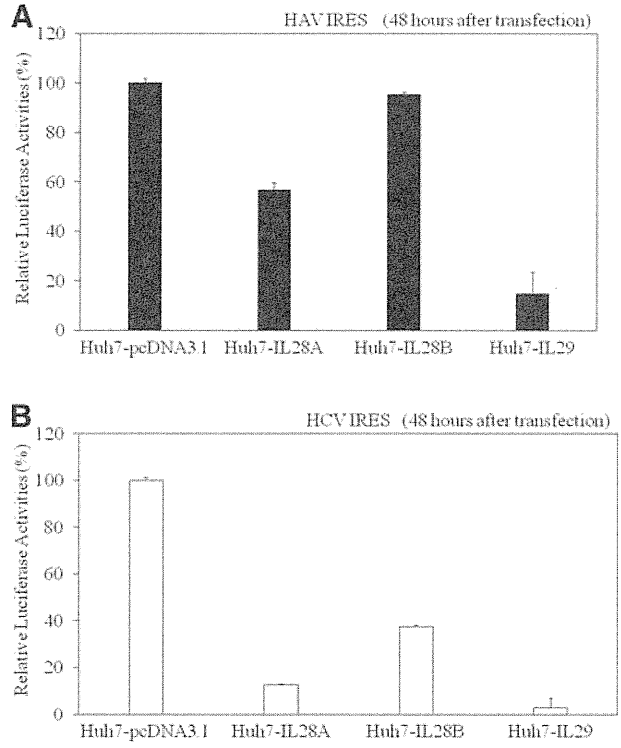


FIG. 3. Interferon-λ inhibits hepatitis A virus (HAV) (**A**) and HCV (**B**) internal ribosomal entry site (IRES)-mediated translation in human hepatoma cells. Huh7-IL28A, Huh7-IL28B, Huh7-IL29, and Huh7-pcDNA3.1, were transfected with pSV40-HAV IRES reporter vector (19) (**A**), and pSV40-HCV IRES reporter vector (18,24) (**B**), and 48 h later, luciferase activity was measured and IRES activity was determined. Relative luciferase activity (Fluc/Rluc) in Huh7-pcDNA3.1 was set at 100%. Data are expressed as mean ± SD of triplicate determinations from one experiment representative of three independent experiments.

(Takara Bio Inc., Otsu, Shiga, Japan). For detection of ectopic expression of IL-28A, IL-28B, and IL-29, RT-PCR was performed with a Thermal Cycler (TP3000; Takara Bio Inc.) using PrimeSTAR HS DNA polymerase (Takara Bio Inc.) with primers as previously described (41), together with primers for GAPDH (44).

Enzyme-linked immunosorbent assay (ELISA) for IL-28A and IL-29

Cell culture fluid was analyzed for human IL-28A and for IL-29 by ELISAs (R&D Systems, Minneapolis, MN and eBioscience, San Diego, CA, respectively), following the manufacturers' protocols. Briefly, cell culture fluid samples were incubated in plates at 37°C overnight, followed by incubation with biotinylated monoclonal antibodies. Avidin-conjugated peroxidase was added to the plates, and enzyme activity was detected with a Bio-Rad iMark microplate reader (Bio-Rad, Hercules, CA) (44). The sensitivities of human IL-28A and IL-29 by these ELISA kits were 8 pg/mL and 12.5 pg/mL, respectively.

MTS assay

To evaluate cell growth and cell viability, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed with the CellTiter 96 Aqueous One-Solution cell proliferation assay (Promega).

Statistical analysis

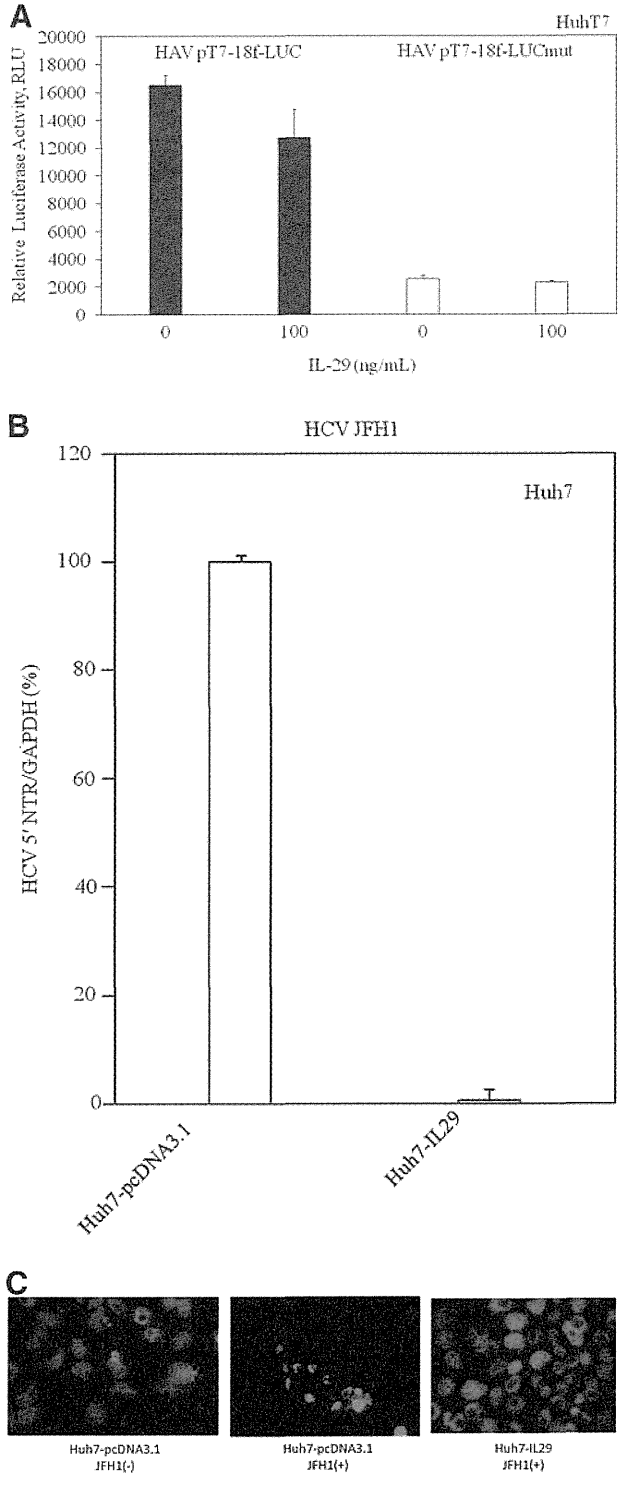
Data were expressed as mean ± SD. Statistical analysis was done by Student's *t*-test. A value of $p < 0.05$ was considered significant.

Results

Overexpression of IL-28A, IL-28B, or IL-29, in Huh7 cells

We used three protein plasmid vectors under control of the CMV promoter: pcDNA3.1-IL28A, pcDNA3.1-IL28B, and pcDNA3.1-IL29 (41). We established three IFN-λ-overexpressing Huh7 cells, designated as Huh7-IL28A, Huh7-IL28B, and Huh7-IL29. We also used pcDNA3.1 for the establishment of a control cell, Huh7-pcDNA3.1. For the generation of stable cell lines, Huh7 cells were transfected

with these vectors and treated with G418. Antibiotic-resistant colonies were expanded for further analysis. To test the ability of these cells to express IL-28A and IL-29, we detected these mRNAs by RT-PCR and measured these cytokines by ELISA (Fig. 2A, upper and lower panels). IL-28A or IL-29 mRNAs were detected only in Huh7-IL28A or Huh7-IL29 cells, respectively. IL-28A or IL-29, respectively, could be measured in each cell culture fluid of Huh7-IL28A or Huh7-IL29. We confirmed the expression of IL-28B mRNA in the cellular RNA of Huh7-IL28B since we could not use ELISA for IL-28B at this time (Fig. 2A, upper panel). We next examined whether overexpression of IFN-λ had any effect on cell proliferation. Equal numbers of control Huh7-pcDNA3.1 and IFN-λ-overexpressing Huh7 cells (Huh7-IL28A, Huh7-IL28B, and Huh7-IL29) were plated, and cell viability was counted at 24 h by MTS assay (Fig. 2B). There were no differences in cell viabilities among these cell lines.



HAV IRES activity in Huh7-IL28A, Huh7-IL28B, and Huh7-IL29

Next, we examined the effects of these IFNs-λ on HAV IRES-mediated translations using a luciferase reporter assay. Huh7-IL28A, Huh7-IL28B, Huh7-IL29, and Huh7-pcDNA3.1 were transfected with pSV40-HAV IRES reporter vector encoding SV40 promoter driven-Rluc and Fluc, separated by HAV IRES (19), and 48 h later, luciferase activity was measured and IRES activity was determined (Fig. 3A). HAV IRES activity was inhibited in Huh7-IL28A (56.7%; n=3, $p<0.0001$), Huh7-IL28B (95.3%, n=3, $p=0.0021$), and Huh7-IL29 (14.9%, n=3, $p<0.0001$), compared to that in control Huh7-pcDNA3.1 (n=3, 100%). IL-28A and IL-28B demonstrated inhibitory effects on HAV IRES activity, but they seemed less efficient than IL-29 (Fig. 3A).

HCV IRES activities in Huh7-IL28A, Huh7-IL28B, and Huh7-IL29

It is known that HCV also has IRES structures and plays an important role in the translation of HCV proteins (18,24). In order to compare the effects of IFNs-λ on HCV IRES-mediated translation with those on HCV, we next tested their effects on HCV IRES-mediated translation using a luciferase reporter assay. Huh7-IL28A, Huh7-IL28B, Huh7-IL29, and Huh7-pcDNA3.1 were transfected with pSV40-HCV IRES reporter vector encoding SV40 promoter driven-Rluc and Fluc, separated by HCV IRES (24), and 48 h later, luciferase activity was measured and IRES activity was determined (Fig. 3B). HCV IRES activity was inhibited in Huh7-IL28A (12.5%, n=3, $p<0.0001$), Huh7-IL28B (37.5%, n=3, $p<0.0001$), and Huh7-IL29 (2.7%, n=3, $p<0.0001$), compared to that in control Huh7-pcDNA3.1 (n=3100%). Similarly to HAV IRES, IL-28A and IL-28B demonstrated inhibitory effects on HCV

FIG. 4. IL29 suppresses hepatitis A virus (HAV) (**A**) and HCV (**B** and **C**) replication. (**A**) HuhT7 cells were transfected with replication-competent HAV replicon pT7-18f-LUC or replication-incompetent HAV replicon pT7-18f-LUCmut (9). At 60 h post-transfection, the cells were treated with 0 or 100 ng/mL IL-29. At 72 h post-transfection, reporter assays were performed to evaluate HAV subgenomic replication. (**B**) Huh7-IL29 and Huh7-pcDNA3.1 were infected with HCV JFH1 (genotype 2a) (14,43), and 72 h later, HCV RNA was measured by real-time RT-PCR and HCV 5'NTR/GAPDH ratios were measured by ddCt methods. (**C**) After 72 h of infection, HCV was detected by immunofluoresence using antibody to the core protein. Shown are representative photomicrographs of JFH1 virus production in Huh7-IL29 and Huh7-pcDNA3.1 cells. Data are expressed as mean ± SD of triplicate determinations from one experiment representative of three independent experiments.

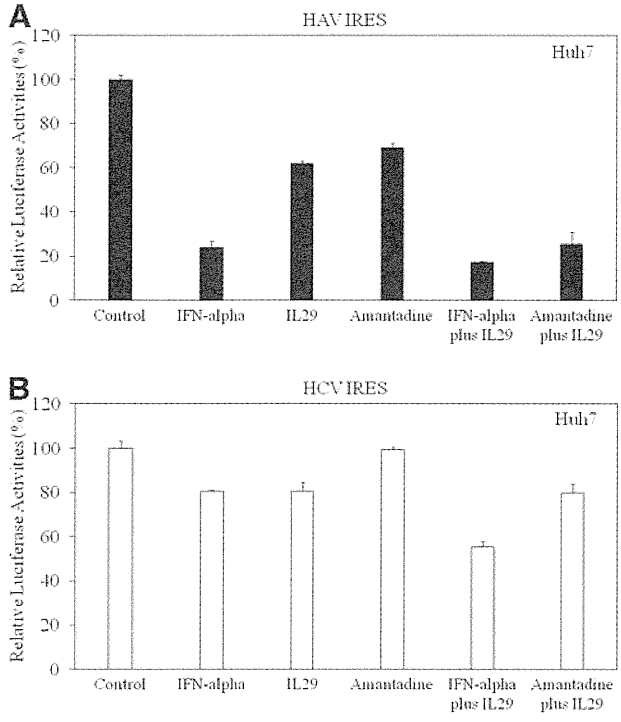


FIG. 5. Effects of IL-29 with or without interferon (IFN)-α or amantadine on hepatitis A virus (HAV) (**A**) and HCV (**B**) internal ribosomal entry site (IRES)-mediated translation in Huh7 cells. Huh7 cells were transfected with pSV40-HAV IRES reporter vector (19) (**A**), or pSV40-HCV IRES reporter vector (18,24) (**B**), and 48 h later, the cells were treated with 50 ng/mL IL-29 with or without 50 μg/mL amantadine or 100 IU/mL IFN-α, and 24 h later luciferase activity was measured and IRES activity was determined. Relative luciferase activity (Fluc/Rluc) without treatment was set at 100%. Data are expressed as mean ± SD of triplicate determinations from one experiment representative of three independent experiments.

IRES activity, but they seemed less efficient than IL-29 (Fig. 3B).

IL-29 inhibits both HAV and HCV replication

Next we investigated the effect of IL-29 on HAV subgenomic replication in HuhT7 cells (9). IL-29 at 100 ng/mL led to 22.8% (n = 3, $p = 0.038$) inhibition of HAV replication, but we observed no reduction of HAV mut replicon replication (Fig. 4A). We also examined whether IL-29 inhibits HAV strain KRM003 propagation in GL37 cells, but at 50 ng/mL of IL-29 we could not observe any effect on the inhibition of HAV propagation. Two-hundred and fifty and 500 ng/mL of IL-29 showed a tendency to inhibit HAV propagation without cell damage. However, it was difficult to obtain a stable reaction. Further study will be needed.

We also examined whether IL-29 inhibits HCV replication in Huh7-derived cell lines. Huh7-IL29 and Huh7-pcDNA3.1 were infected with HCV JFH1 (genotype 2a) (14,43), and 72 h later, HCV RNA was detected less in Huh7-IL29 (0.6%; n = 3, $p < 0.0001$) than in Huh7-pcDNA3.1 (100%; n = 3, $p < 0.0001$; Fig. 4B). HCV core protein expression was also less observed in Huh-IL29 than in Huh7-pcDNA3.1 (Fig. 4C).

Exogenous IL-29 with or without IFN-α or amantadine inhibits HAV IRES activity in Huh7

As Huh7-IL29 cells had the strongest inhibitory effect on HAV IRES-mediated translation (Fig. 3A), we investigated whether exogenous IL-29 had similar effects on HAV IRES-mediated translation using a luciferase reporter assay (Fig. 5A). Huh7 cells were transfected with pSV40-HAV IRES reporter vector (19), and 48 h later, cells were treated with IL-29 with or without amantadine or IFN-α, and 24 h after this, luciferase activity was measured and IRES activity was determined (Fig. 5A).

We previously reported that amantadine with or without IFN-α inhibits HAV IRES-mediated translation in human hepatoma cells (19,45). HAV IRES activity was significantly inhibited, to 24.1% (n = 3, $p < 0.0001$), 62.1% (n = 3, $p < 0.0001$), and 69.1% (n = 3, $p < 0.0001$), by 100 IU/mL IFN-α, 50 ng/mL IL-29, and 50 μg/mL amantadine, respectively (Fig. 5A). The combination of IL-29 with IFN-α or amantadine led to 82.7% (n = 3, $p < 0.0001$), or 74.6% (n = 3, $p < 0.0001$) inhibition of HAV IRES activity, respectively, with these combinations demonstrating stronger effects than IL-29 alone (Fig. 5A).

In order to compare the effects of exogenous IL-29 on HAV IRES-mediated translation with those on HCV, we next tested the effects of IL-29 with or without amantadine or IFN-α on HCV IRES-mediated translation using a luciferase reporter assay. Huh7 cells were transfected with pSV40-HCV IRES reporter vector (24), and 48 h later, the cells were treated with IL-29 with or without amantadine or IFN-α, and 24 h after this, luciferase activity was measured and IRES activity was determined (Fig. 5B). HCV IRES activity demonstrated significant inhibition, to 80.6% (n = 3, $p = 0.00061$) and 80.6% (n = 3, $p = 0.00027$) by IFN-α and IL-29, respectively, but showed no inhibition by amantadine only (99.3%, n = 3; Fig. 5B). The combination of IL-29 with IFN-α led to a 44.5% (n = 3, $p < 0.0001$) inhibition of HCV IRES activity, with this combination demonstrating stronger effects than IL-29 alone. However, the combination of IL-29 with amantadine resulted in only 20% inhibition (n = 3, $p = 0.00027$) of HCV IRES activity, similarly to the effect of IL-29 alone (Fig. 5B).

Discussion

We demonstrated that IL-29 inhibited HAV as well as HCV IRES activity in human hepatoma cell line Huh7, and that Huh7-IL29 had stronger effects than Huh7-IL28A, Huh7-IL28B, and Huh7-pcDNA3.1. The combination of IL-29 with IFN-α or amantadine seemed to have stronger inhibitory effects on HAV IRES activity than IL-29 alone.

IFNs-λ modulate innate and adaptive immune responses to environmental pathogens and protect the host against diseases such as cancer. Expression of IFNs-λ is tightly regulated by viral infection, including hepatitis viral infection (6,7). IFNs-λ utilize a receptor complex different from IFN-α, but both types of IFN induce STAT1 and STAT2, as well as STAT3 activation (33,48). Binding of IFN to the IFN receptor leads to the activation of receptor-associated Janus tyrosine protein kinase (Jak1). IFN stimulation results in tyrosine phosphorylation, dimerization, and nuclear import of STATs (3). STATs and the IFN-stimulated gene factor 3 (ISGF3) transcription factor complex move into the nucleus and bind to IFN-stimulated response elements (ISRE) in the promoters of the

IFN-stimulated genes (ISGs). ISGs inhibit viral replication and activate numerous downstream cellular responses.

In contrast to IFN-α, IFNs-λ bind to a heterodimeric receptor consisting of IL-28Rα subunit and IL10Rβ receptor subunit, that is also shared by IL-10, IL-22, and IL-26. Because the IFN-λ receptor is different from that of IFN-α, their uses as alternative therapies for viral hepatitis need to be examined (6). Although the biological activities of IFNs-λ could overlap with IFN-α, the expression of IL-28Rα receptor is limited in contrast to the ubiquitously expressed IL10Rβ, and IFNs-λ might have fewer adverse events than type I IFN (28). We also found that the combination of IL-29 with IFN-α or amantadine demonstrated stronger inhibitory effects on HAV IRES activity. The combination of IL-29 with amantadine may also be useful in some HAV patients.

We did not observe any differences in HAV 5′ NTR or HCV 5′ NTR RNA detection by RT-PCR among Huh7-pcDNA3.1, Huh7-IL28A, Huh7-IL28B, and Huh7-IL29 at 72 h after transfection of pSV40-HAV IRES-luc or pSV40-HCV IRES-luc (data not shown), although we could not completely exclude the destruction of IRES mRNAs, because IFNs-λ as well as IFN-α activate double-stranded protein kinase PKR and 2′,5′-oligo A (2–5A) synthetases (21). Several noncanonical translation initiation factors such as La protein and polypyrimidine tract binding protein (PTB) have been implicated in translation from HAV and HCV IRESes (4,13,40,46). The effects of IL-29 on these proteins should be examined in future studies.

We previously demonstrated that siRNAs targeted against HAV IRES, amantadine, and IFN-α, inhibited HAV IRES-mediated translation and HAV replication (15,19,33,45). In the present study, we planned to examine the effects of IFN-λ on HAV IRES-mediated translation. IFNs are proteins induced by lymphocytes and other cells including hepatocytes in response to viruses such as HAV. Our study also supports the notion that IFNs-λ might inhibit HAV IRES-mediated translation as one of the host defense mechanisms against HAV infection.

It has been reported that genetic variations in IL-28B SNPs predict hepatitis C treatment-induced viral clearance and natural clearance (10,16,33,42). Tanaka *et al.* (42) reported that IL-28B minor SNP was associated with a null virological response in the treatment of Japanese patients infected with HCV genotype 1. Yu *et al.* (47) also reported that the IL-28B rs8099917 TT genotype is significantly independently predictive of RVR, which is the single best predictor of SVR, in Asian HCV genotype 2 patients. Pegylated IL-29 induces antiviral gene expression and represses hepatitis B and C replication *in vitro* (6), and HCV replication *in vivo* (35). Among IFNs-λ, it was reported that IL-28A inhibits HCV IRES-mediated translation and suppresses HCV replication (49). Kato *et al.* (21) reported that IFN-α, as well as IFN-β, specifically suppress the translation from HCV IRES. We also demonstrated that siRNAs targeted against HCV IRES were potent inhibitors of HCV IRES-mediated translation and HCV replication (18). In the present study, we demonstrated that IL-29, as well as IFN-α, inhibited HCV IRES-mediated translation, although amantadine did not inhibit HCV IRES-mediated translation in our experimental condition.

In conclusion, we demonstrated that IL-29 suppressed HAV as well as HCV IRES-mediated translation. Viral IRES activity may influence the level of replication (8,15,18), although it was reported that the preponderance of host factors might determine the clinical presentation (30). To inhibit HAV or HCV IRES-mediated translation, the combination IL-29 with IFN-α or amantadine has a stronger inhibitory effect. IFNs-λ might also play an important role in host defense mechanisms and in HAV pathogenesis.

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Author Disclosure Statement

No competing financial interests exist.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Algorithm to determine the outcome of patients with acute liver failure: a data-mining analysis using decision trees

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Abstract
Background We established algorithms to predict the prognosis of acute liver failure (ALF) patients through a data-mining analysis, in order to improve the indication criteria for liver transplantation.
Methods The subjects were 1,022 ALF patients seen between 1998 and 2007 and enrolled in a nationwide survey. Patients older than 65 years, and those who had undergone liver transplantation and received blood products before the onset of hepatic encephalopathy were excluded. Two data sets were used: patients seen between 1998 and 2003 (n=698), whose data were used for the formation of the algorithm, and those seen between 2004 and 2007 (n=324), whose data were used for the validation of the algorithm. Data on a total of 73 items, at the onset of encephalopathy and 5 days later, were collected from 371 of the 698 patients seen between 1998 and 2003, and their outcome was analyzed to establish decision trees. The obtained algorithm was validated using the data of 160 of the 324 patients seen between 2004 and 2007.
Results The outcome of the patients at the onset of encephalopathy was predicted through 5 items, and the patients were classified into 6 categories with mortality rates between 23% and89%. When the prognosis of the patients in the categories with mortality rates greater than 50% was predicted as "death", the accuracy, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the algorithm were 79, 78, 81, 83, and 75%, respectively. Similar high values were obtained when the algorithm was employed in the patients for validation. The outcome of the patients 5 days after the onset of encephalopathy was predicted through 7 items, and a similar high accuracy was found for both sets of patients.
Conclusions Novel algorithms for predicting the outcome of ALF patients may be useful to determine the indication for liver transplantation.

Keywords Hepatic encephalopathy · Liver transplantation · Fulminant hepatitis · Late-onset hepatic failure

Abbreviations

ALF	Acute liver failure
LOHF	Late-onset hepatic failure
HBV	Hepatitis B virus
SOM	Self-organizing map
DIC	Disseminated intravascular coagulation
PPV	Positive predictive value
NPV	Negative predictive value
HAV	Hepatitis A virus
HEV	Hepatitis E virus
ALS	Artificial liver support
CHDF	Continuous hemodiafiltration
HDF	Hemodiafiltration

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Introduction

Acute liver failure (ALF) is a clinical syndrome characterized by hepatic encephalopathy and a bleeding tendency due to the severe impairment of liver function caused by massive or submassive liver necrosis. In Japan, patients showing 40% or less of the standardized prothrombin time value or INRs of 1.5 or more caused by severe liver damage within 8 weeks of onset of the symptoms are diagnosed as having ALF, where the liver function prior to the current onset of liver damage was estimated to be normal [1]. ALF is classified into the categories of "acute liver failure without hepatic coma" and "acute liver failure with hepatic coma," depending on the severity of the hepatic encephalopathy; the latter is further classified into 2 types, the "acute type" and the "subacute type", in which grade II or more severe hepatic coma develops within 10 days and between 11 and 56 days, respectively, after the onset of disease symptoms. Also, patients with less than 40% of the standardized prothrombin time value or INRs of 1.5 or more and grade II or more severe hepatic coma occurring between 8 and 24 weeks of the onset of disease symptoms are diagnosed as having late-onset hepatic failure (LOHF), as a disease related to ALF. ALF in Japan has been typically regarded as fulminant hepatitis, for which the diagnostic criteria were established by the Inuyama Symposium held in 1981 [2]. Among patients with ALF, those showing histological findings of hepatitis (characterized by inflammatory lymphocyte infiltration), as well as 40% or less of the standardized prothrombin timeand grade II or more severe hepatic encephalopathy, are diagnosed as having "fulminant hepatitis", which is classified as acute and subacute types in the same manner as ALF [2, 3]. Thus, fulminant hepatitis is almost synonymous with ALF in the United States and Europe as well as in Japan, except that patients without histological evidence of hepatitis are excluded from both disease conditions in Japan. Thus, ALF caused by viral infections, autoimmune hepatitis, and drug allergy-induced liver injury is included in the diagnosis of fulminant hepatitis, while ALF caused by drug/chemical intoxication (such as acetaminophen intoxication) microcirculatory disturbances, Wilson's disease, acute fatty liver of pregnancy, and Reye's syndrome is excluded from that. A history of chronic liver disease preceding the onset of acute liver injury also precludes the diagnosis of fulminant hepatitis and LOHF, while inactive hepatitis B virus (HBV) carriers showing normal serum alanine aminotransferase (ALT) values before acute exacerbation of hepatitis are included in both these disease conditions.

According to a nationwide survey conducted by the Intractable Liver Diseases Study Group of Japan constituted under the aegis of the Ministry of Health, Welfare and Labour [4], artificial liver support with plasma exchange and/or hemodiafiltration was performed in almost all patients with fulminant hepatitis and LOHF between 1998 and 2003. Also, about 70 and 60% of the patients, respectively, received intravenous glucocorticoid treatment and anticoagulant therapy with an antithrombin III concentrate. Moreover, patients with HBV infection have received antiviral therapy with lamivudine or entecavir since 1998. Despite the use of these therapeutic modalities, however, the outcome of the patients receiving these treatments had not improved; the survival rates of patients with the acute and subacute types of fulminant hepatitis not treated with liver transplantation were 54 and 24%, respectively, , and in those with LOHF not treated with liver transplantation the survival rate was 12% [4]. In contrast, the outcome of the patients receiving liver transplantation was excellent, with the survival rate being 78% among those with fulminant hepatitis and 75% among those with LOHF, suggesting that liver transplantation is the optimal therapeutic strategy for the rescue of patients with ALF, irrespective of the disease types in Japan.

The indications for liver transplantation in patients with ALF are currently determined according to the guideline published by the Acute Liver Failure Study Group of Japan in 1996 [5, 6]. The predictive accuracy, however, decreased when the guideline was adopted for patients seen between 1998 and 2003; the accuracy values in the patients not receiving liver transplantation were 67 and 78% among those with the acute and subacute types of fulminant hepatitis, respectively, and the specificity of the guideline was extremely low especially in patients with the subacute type of fulminant hepatitis [6]. Thus, the guideline to determine the indication for liver transplantation in ALF patients in Japan needs to be updated.

Recently, we performed a cluster analysis of the patients with fulminant hepatitis and LOHF seen between 1998 and 2007 to evaluate the validity of the classification of ALF in Japan [7]. We adopted the self-organizing map (SOM), one of the data-mining methods introduced by Kohonen as an artificial neural network [8], which has been shown to be suitable for analyses of complex multidimensional relationships in various medical science fields [9–15]. Consequently, we found that ALF patients could be classified into three clusters independent of the interval between the onset of disease symptoms and the development of hepatic encephalopathy, and the outcome of the patients differed markedly among the clusters [7]. These observations prompted us to postulate that data-mining methods may be useful to revise the above-mentioned guideline.

We report on algorithms to predict the outcome of ALF patients under intensive medical care without liver transplantation; these algorithms were established based on the data-mining analysis using decision trees. The algorithms were constructed using the data from ALF patients without

liver transplantation, because there may have been many patients among those receiving liver transplantation who could have been rescued by intensive medical care.

Patients and methods

Patients

The subjects of this study were 1,022 patients with ALF who were enrolled in the nationwide survey of fulminant hepatitis and LOHF conducted by the Intractable Hepato-Biliary Disease Study Group of Japan between 1999 and 2008 (formerly the Intractable Liver Diseases Study Group of Japan, before 2003). All of the patients showed grade II or more severe hepatic encephalopathy and prothrombin times of less than 40% of the standardized value and were admitted to 610 hospitals specializing in hepatology in Japan between 1998 and 2007. Patients without histological evidence of hepatitis, such as those with hepatitis due to drug-toxicity, circulatory disturbance, and metabolic diseases, were excluded from the analysis. The interval between the onset of the hepatitis symptoms and the development of encephalopathy was 10 days or less in 472 patients (group-A; acute type of fulminant hepatitis), between 11 and 56 days in 468 patients (group-B; subacute type of fulminant hepatitis), and more than 56 days in 82 patients (group-C; LOHF). The patients were classified into two data sets; 698 patients (316, 318, and 64 in group-A, group-B, and group-C, respectively) seen between 1998 and 2003, and 324 patients (156, 150, and 18, respectively, in each group) seen between 2004 and 2007. The former data set was used for the formation of the algorithms to predict the outcome of the patients and the latter data set was used for the validation of the established algorithms. The clinical features of all patients were obtained until either of the following time-points: they died in hospital, or received liver transplantation, or were discharged following improvement of liver function; the outcomes of the patients were expressed as "dead", "transplanted", and "rescued", respectively. Missing data were managed through available-case analysis, in which all relevant data were used.

The etiology of ALF was determined based on the definition proposed by the Intractable Liver Diseases Study Group of Japan constituted under the aegis of the Ministry of Health, Welfare and Labour [1, 4]. Criteria for complications were defined as follows: *Infection*; (1) manifestation of organic symptoms and/or imaging findings, (2) body temperature of 38°C or more, (3) white blood cell counts of 10,000 cells/mm^3 or more, (4) positive for causative bacteria in organs suspicious of infection and/or increase of white blood cell counts in body fluid. Patients were diagnosed as having infection when two or more of these criteria were present. *Brain edema*; (1) typical findings on computed tomography (CT) images, or (2) intracranial pressure of 25 mmHg or more. *Gastrointestinal bleeding*; (1) hematemesis and/or drainage of blood from a catheter in the upper gastrointestinal tract, (2) tarry stool or melena, (3) endoscopic findings of bleeding. Patients were diagnosed as having gastrointestinal bleeding when one or more of these criteria were present. *Renal failure*; (1) urine volume output of 400 mL or less per day, or (2) serum creatinine levels of 2.0 mg/dL or higher. *Disseminated intravascular coagulation (DIC)*; patients were diagnosed as having DIC when the score on the scoring system for DIC revised by the Japanese Association for Acute Medicine (JAAM) [16] was four or more. *Heart failure*; (1) chest X-ray showing an enlarged cardiac silhouette, (2) chest X-ray showing pulmonary congestion, (3) an ejection fraction of 40% or less. Patients were diagnosed as having heart failure when two or more of these criteria were present. Atrophy of the liver was assessed by each practitioner subjectively based on imaging through ultrasound and/or CT scan examinations.

The demographic and clinical features, the therapies undertaken, and the consequent outcomes of the patients are shown in the various sections of Table 1. Of the total study population seen between 1998 and 2007, 40.2% had underlying diseases such as metabolic syndrome, and most of such patients were on daily medications. The etiology of hepatitis was viral infection in 69.3, 31.2, and 17.1% of the patients in group-A, group-B, and group-C, respectively. In most cases, the causative virus was hepatitis B virus (HBV); transient infection was predominant in the patients in group-A, whereas inactive carriers showing acute exacerbation of hepatitis predominated in group-B. The etiology was indeterminate in 41.5 and 47.6% of the patients in group-B and group-C, respectively. Autoimmune hepatitis and drug-induced liver injury were found in 12.0 and 13.0%, respectively, of the patients in group-B, and in 17.1 and 15.9%, respectively, of those in group-C. The survival rates of the 811 patients who were treated conservatively without liver transplantation were 53.4, 24.5, and 12.1%, respectively, in group-A, group-B, and group-C patients. The remaining 211 patients (20.6%) underwent liver transplantation, and the survival rates were 56.4, 39.7, and 25.6%, respectively, in the patients in group-A, group-B, and group-C.

The demographic and clinical features in the patients seen between 1998 and 2003 and those seen between 2004 and 2007 were similar, except for the following items (Table 1a): the ages of the patients seen between 2004 and 2007 were significantly higher than the ages in those seen between 1998 and 2003 irrespective of the groups to which they belonged . On the other hand, the percentage of HBV

Table 1 Demographic and clinical features of acute liver failure patients in Japan seen between 1998 and 2003 and those seen between 2004 and 2007

(a) Demographic features and the etiology of acute liver failure

1998–2003	Total (n = 698)	Group-A[a] (n = 316)	Group-B (n = 318)	Group-C (n = 64)
Male:female (:unknown)[b]	346:351 (:1)	167:148 (:1)	152:166	27:37
Age (years)[c]	47.0 ± 16.8[†]	45.1 ± 16.6[†]	47.8 ± 17.1[†]	51.9 ± 15.0[†]
HBV carrier[d]	14.1 (93/658)*	12.7 (37/291)*	17.4 (53/305)	4.8 (3/62)
Underlying diseases[d, e]	38.5 (265/689)	32.7 (102/312)	41.5 (130/313)	51.6 (33/64)
History of medication[d]	42.0 (282/672)*	36.6 (112/306)*	45.7 (139/304)*	50.0 (31/62)
Etiology[d]				
Viral infection	48.0 (335)	71.2 (225)	31.8 (101)	14.1 (9)
HAV	6.4 (45)[#]	12.0 (38)	1.9 (6)	1.6 (1)
HBV	38.8 (271)	56.6 (179)	27.0 (86)	9.4 (6)
Transient infection	23.2 (162)	41.8 (132)	8.8 (28)	3.1 (2)
Carrier	13.5 (94)	12.0 (38)	16.7 (53)	4.7 (3)
Undetermined	2.1 (15)[#]	2.8 (9)[#]	1.6 (5)	1.6 (1)
HCV	1.4 (10)	1.6 (5)	1.3 (4)	1.6 (1)
HEV	0.4 (3)	0 (0)[#]	0.9 (3)	0 (0)
Other virus	0.9 (6)	0.9 (3)	0.6 (2)	1.6 (1)
Autoimmune hepatitis	6.9 (48)	1.6 (5)	10.7 (34)	14.1 (9)
Drug allergy-induced	9.3 (65)[#]	6.0 (19)[#]	11.3 (36)	15.6 (10)
Indeterminate	32.8 (229)	18.7 (59)	42.8 (136)	53.1 (34)
Insufficient examinations[f]	3.0 (21)[#]	2.5 (8)	3.5 (11)	3.1 (2)
2004–2007	Total (n = 324)	Group-A[a] (n = 156)	Group-B (n = 150)	Group-C (n = 18)
Male:female [b]	152:172	82:74	64:86	6:12
Age (years)[c]	51.1 ± 16.1	48.6 ± 15.5	52.7 ± 16.5	60.3 ± 11.5
HBV carrier[d]	11.7 (33/282)	9.5 (12/126)	13.7 (19/139)	11.8 (2/17)
Underlying diseases[d, e]	44.0 (139/316)	39.7 (60/151)	47.6 (70/147)	50.0 (9/18)
History of medication[d]	60.3 (184/305)	51.7 (75/145)	66.9 (95/142)	77.8 (14/18)
Etiology[d]				
Viral infection	46.9 (152)	65.4 (102)	30.0 (45)	33.3 (6)
HAV	3.1 (10)	6.4 (10)	0.0 (0)	0.0 (0)
HBV	41.0 (133)	56.4 (88)	26.7 (40)	27.8 (5)
Transient infection	21.9 (71)	38.5 (60)	6.7 (10)	5.6 (1)
Carrier	12.3 (40)	6.4 (10)	18.0 (27)	16.7 (3)
Undetermined	6.8 (22)	11.5 (18)	2.0 (3)	5.6 (1)
HCV	0.9 (3)	0.6 (1)	1.3 (2)	0.0 (0)
HEV	1.2 (4)	1.3 (2)	1.3 (2)	0.0 (0)
Other virus	0.6 (2)	0.6 (1)	0.7 (1)	0.0 (0)
Autoimmune hepatitis	9.9 (32)	3.2 (5)	14.7 (22)	27.8 (5)
Drug allergy-induced	14.5 (47)	12.2 (19)	16.7 (25)	16.7 (3)
Indeterminate	27.8 (90)	17.3 (27)	38.7 (58)	27.8 (5)
Insufficient examinations[f]	0.9 (3)	1.9 (3)	0.0 (0)	0.0 (0)

(b) Complications of acute liver failure[g]

1998–2003	Total (n = 698)	Group-A[a] (n = 316)	Group-B (n = 318)	Group-C (n = 64)
Infection	39.1 (247/632)	35.0 (100/286)	40.8 (117/287)	50.8 (30/59)
Brain edema	31.0 (173/558)*	35.3 (91/258)*	29.0 (73/252)*	18.8 (9/48)
Gastrointestinal bleeding	20.1 (134/668)	22.2 (67/302)*	16.7 (51/305)	26.2 (16/61)

Table 1 continued

(b) Complications of acute liver failure[g]

1998–2003	Total (n = 698)	Group-A[a] (n = 316)	Group-B (n = 318)	Group-C (n = 64)
Renal failure	36.5 (249/682)	41.5 (129/311)*	29.9 (92/308)	44.4 (28/63)
DIC	41.5 (271/653)	43.4 (129/297)*	41.3 (124/300)	33.9 (19/56)
Congestive heart failure	10.5 (70/664)*	11.2 (34/303)	9.6 (29/301)*	11.7 (7/60)
2004–2007	Total (n = 324)	Group-A[a] (n = 156)	Group-B (n = 150)	Group-C (n = 18)
Infection	35.7 (109/305)	33.8 (49/145)	35.9 (51/142)	50.0 (9/18)
Brain edema	16.7 (47/282)	20.1 (28/139)	11.7 (15/128)	26.7 (4/15)
Gastrointestinal bleeding	15.4 (48/312)	12.5 (19/152)	17.4 (25/144)	25.0 (4/16)
Renal failure	35.4 (113/319)	35.7 (55/154)	35.4 (52/147)	33.3 (6/18)
DIC	35.1 (108/308)	30.6 (45/147)	37.1 (53/143)	55.6 (10/18)
Congestive heart failure	7.6 (23/303)	8.7 (13/150)	5.8 (8/137)	12.5 (2/16)

(c) Therapeutic strategies undertaken following the onset of hepatic encephalopathy[g]

1998–2003	Total (n = 698)	Group-A[a] (n = 316)	Group-B (n = 318)	Group-C (n = 64)
Glucocorticoids	67.6 (470/695)	60.5 (190/314)	76.0 (241/317)	75.0 (48/64)
Glucagon/insulin	43.2 (300/694)*	37.6 (118/314)*	47.5 (150/316)*	50.0 (32/64)*
BCAA-rich solution	32.9 (227/689)*	27.6 (86/312)	35.8 (112/313)*	45.3 (29/64)
Plasma exchange	91.1 (634/696)	90.1 (283/314)	93.4 (297/318)	84.4 (54/64)
Hemodiafiltration	74.7 (518/693)	75.2 (236/314)	77.2 (244/316)	60.3 (38/63)
Prostaglandin E1	23.2 (160/691)*	19.4 (61/314)*	25.8 (81/314)*	28.6 (18/63)*
Cyclosporin A	13.9 (96/691)*	11.1 (35/314)	15.9 (50/314)	17.5 (11/63)
Interferon	19.5 (135/691)*	22.0 (69/314)*	19.7 (62/314)*	6.3 (4/63)
Nucleoside analog	23.9 (164/687)*	30.9 (96/311)*	20.4 (64/314)*	6.5 (4/62)*
Anticoagulation therapy	59.6 (413/693)*	57.3 (180/314)*	60.1 (190/316)	68.3 (43/63)*
Liver transplantation	20.3 (142/698)	14.6 (46/316)	26.4 (84/318)*	18.8 (12/64)
2004–2007	Total (n = 324)	Group-A[a] (n = 156)	Group-B (n = 150)	Group-C (n = 18)
Glucocorticoids	71.8 (232/323)	66.7 (104/156)	75.8 (113/149)	83.3 (15/18)
Glucagon/insulin	15.5 (50/323)	16.7 (26/156)	14.1 (21/149)	16.7 (3/18)
BCAA-rich solution	23.7 (76/321)	18.2 (28/154)	26.2 (39/149)	50.0 (9/18)
Plasma exchange	90.7 (293/323)	92.3 (144/156)	91.3 (136/149)	72.2 (13/18)
Hemodiafiltration	69.9 (225/322)	69.7 (108/155)	73.8 (110/149)	38.9 (7/18)
Prostaglandin E1	7.4 (24/323)	7.7 (12/156)	7.4 (11/149)	5.6 (1/18)
Cyclosporin A	9.0 (29/323)	6.4 (10/156)	11.4 (17/149)	11.1 (2/18)
Interferon	13.3 (43/323)	14.7 (23/156)	12.1 (18/149)	11.1 (2/18)
Nucleoside analog	39.1 (126/322)	51.6 (80/155)	27.5 (41/149)	27.8 (5/18)
Anticoagulation therapy	45.5 (147/323)	39.1 (61/156)	54.4 (81/149)	27.8 (5/18)
Liver transplantation	21.3 (69/324)	12.8 (20/156)	30.0 (45/150)	22.2 (4/18)

(d) The outcome of the patients[g]

1998–2003	Total (n = 698)	Group-A[a] (n = 316)	Group-B (n = 318)	Group-C (n = 64)
Survival rate	45.6 (318/698)	56.3 (178/316)	39.3 (125/318)	23.4 (15/64)
Treated without liver transplantation	37.4 (208/556)	53.7 (145/270)	24.4 (57/234)	11.5 (6/52)
Treated with liver transplantation	77.5 (110/142)	71.7 (33/46)	81.0 (68/84)	75.0 (9/12)

Table 1 continued

2004–2007	Total ($n = 324$)	Group-A[a] ($n = 156$)	Group-B ($n = 150$)	Group-C ($n = 18$)
Survival rate	47.8 (155/324)	56.4 (88/156)	40.7 (61/150)	33.3 (6/18)
Treated without liver transplantation	39.2 (100/255)	52.9 (72/136)	24.8 (26/105)	14.3 (2/14)
Treated with liver Transplantation	79.7 (55/69)	80.0 (16/20)	77.8 (35/45)	100.0 (4/4)

HBV hepatitis B virus, *HAV* hepatitis A virus, *HCV* hepatitis C virus, *HEV* hepatitis E virus, *BCAA* branched-chain amino acid, *DIC* disseminated intravascular coagulation

[a] The interval between the onset of the hepatitis symptoms and the onset of grade II or more severe hepatic encephalopathy was 10 days or less (group-A), between 11 and 56 days (group-B), and more than 56 days (group-C)

[b] Number of patients

[c] Mean ± SD

[d] The values are the percentages of patients (%), and the values in parentheses represent the numbers of patients for the calculation of the percentage

[e] Diseases such as metabolic syndrome, malignancy, and psychiatric disorders

[f] The etiology was unknown because of insufficient examinations

[g] The values are the percentages of patients (%), and the values in parentheses represent the numbers of patients for calculation of the percentage

† $p < 0.05$ versus 2004–2007 by Student's t-test

* $p < 0.05$ versus 2004–2007 by the χ^2 test

# $p < 0.05$ versus 2004–2007 by the χ^2 test and analysis of residuals in cross tabulation

carriers in group-A was greater in patients seen between 1998 and 2003 compared to the percentage in those seen between 2004 and 2007. In contrast, the percentages of patients with previous medication in group-A and group-B were greater in those seen between 2004 and 2007 than in those seen between 1998 and 2003. There were also differences in the incidence of brain edema and congestive heart failure between patients seen between 1998 and 2003 and those seen between 2004 and 2007 (Table 1b). Also, the percentages of patients who received therapies such as glucagon and insulin infusion, administration of branched-chain-rich amino acid, prostaglandin E1, interferon, and nucleoside analogs for HBV, and anticoagulant therapies, were different between the two data sets (Table 1c). However, the survival rates of patients both with and without liver transplantation were equivalent in the two data sets (Table 1d).

The following patients were excluded from both data sets: (1) patients older than 65 years; (2) those who had undergone liver transplantation; and (3) those who had received blood product administration before the onset of hepatic encephalopathy. Patients aged more than 65 years were excluded from the analysis because the Act on Organ Transplantation (Law number: Act No. 104 of 1997) recommends that liver transplantation recipients should be younger than 60 years, and in general, in Japan, liver transplantation has been done in recipients aged 65 years or less. Consequently, the data of 371 patients (male 196, female 175) aged between 2 and 65 years (mean ± SD 44.1 ± 14.2) seen between 1998 and 2003 were used for the formation of the algorithms. The disease types of these patients were group-A, group-B, and group-C in 206, 140, and 25 patients, respectively. Validation of the established algorithms was performed in 160 patients (male 81, female 79), aged between 17 and 65 years (47.5 ± 11.9), seen between 2004 and 2007 (98, 56, and 6 patients in group-A, group-B, and group-C, respectively). The algorithms were also employed for the 211 patients who had received liver transplantation between 1998 and 2007, comprising 80 male and 131 female patients aged between 7 and 70 years (39.6 ± 15.6), with 66, 129, and 16 patients belonging to group-A, group-B, and group-C, respectively.

Formation of the algorithms through decision tree analysis

Two types of algorithms were formed using the different data sets; one for the prediction of the patients' outcome at the onset of hepatic encephalopathy of grade II or more (day 0), and the other for the prediction 5 days later (day 5). Data on a total of 62 items, including: (1) the demographic features of the patients, (2) clinical features and laboratory and imaging data at the onset of hepatic encephalopathy, and (3) the therapies received until the development of hepatic encephalopathy, were collected from 371 patients seen between 1998 and 2003 (Table 2), and used for the formation of the algorithm predicting the patients' outcome on day 0. Data on a total of 73 items, including 62 items for the algorithm predicting the patients' outcome on day 0, and clinical features, laboratory and imaging data, and the therapies received at 5 days after the onset of hepatic encephalopathy, collected from the same patients, were used for the formation of the algorithm predicting the patients' outcome on day 5. Items

Table 2 Items characteristic of acute liver failure patients used in the decision tree analysis to establish the algorithms

(a) Items for construction of the algorithm for the patients at the onset of hepatic encephalopathy (day 0)
The types of hepatitis: acute and subacute types of fulminant hepatitis and LOHF
Outcomes: survived and died among patients treated conservatively without liver transplantation and the patients who underwent transplantation
Gender: male and female
Age (years, continuous variable)
Complications preceding acute liver failure: diseases different from liver diseases such as metabolic syndrome, psychiatric diseases, and malignancies
HBV carrier
Past medical history: operations, blood infusions, alcohol intake, and medications
Family history: liver diseases
Etiology of hepatitis: viral infection [HAV, HBV (transient infection, carrier, undetermined), HCV, HEV, other virus], autoimmune hepatitis, drug-induced, indeterminate, and unknown due to insufficient examinations
Interval between the onset of the hepatitis symptoms and the subsequent events (days, continuous variables): onset of jaundice and grade II or more severe hepatic encephalopathy
Interval between the onset of jaundice and the subsequent events (days, continuous variables): onset of hepatic encephalopathy of grade II or more
Symptoms at the onset of grade II or more severe hepatic encephalopathy: fever, jaundice, ascites, edema, flapping tremor, halitosis, loss of liver dullness, convulsion, tachycardia, and hyperventilation
Laboratory data at the onset of grade II or more severe hepatic encephalopathy (continuous variables): the grading of the encephalopathy, peripheral counts of WBC and platelets, prothrombin time, hepaplastin test, plasma concentrations of antithrombin III and ammonia, serum concentrations of AST, ALT, total albumin, bilirubin, AFP, and HGF, the serum concentration ratios of direct to total bilirubin, molar ratio of BCAA to tyrosine (BTR), and Fischer ratio
Atrophy of the liver at the onset of grade II or more severe hepatic encephalopathy
Complications of acute liver failure at the onset of grade II or more severe hepatic encephalopathy: bacterial and fungal infections, gastrointestinal bleeding, renal failure, cardiac failure, disseminated intravascular coagulation, other complications
Number of complications at the onset of grade II or more severe hepatic encephalopathy (continuous variables)
The therapies received: plasma exchange, hemodiafiltration, glucocorticoids, glucagon and insulin, prostaglandin E1, interferon, lamivudine or entecavir, cyclosporin A, anticoagulants, and fresh-frozen plasma
(b) Items for construction of the algorithm for the patients at 5 days after the onset of hepatic encephalopathy (day 5)
The types of hepatitis: acute and subacute types of fulminant hepatitis and LOHF
Outcomes: survived and died among patients treated conservatively without liver transplantation and the patients who underwent transplantation
Gender: male and female
Age (years, continuous variable)
Complications preceding acute liver failure: diseases different from liver diseases such as metabolic syndrome, psychiatric diseases, and malignancies
HBV carrier
Past medical history: operations, blood infusions, alcohol intake, and medications
Family history: liver diseases
Etiology of hepatitis: viral infection [HAV, HBV (transient infection, carrier, undetermined), HCV, HEV, other virus], autoimmune hepatitis, drug-induced, indeterminate, and unknown due to insufficient examinations
Interval between the onset of the hepatitis symptoms and the subsequent events (days, continuous variables): onset of jaundice and grade II or more severe hepatic encephalopathy
Interval between the onset of jaundice and the subsequent events (days, continuous variables): onset of hepatic encephalopathy of grade II or more
Symptoms at the onset of grade II or more severe hepatic encephalopathy: fever, jaundice, ascites, edema, flapping tremor, halitosis, loss of liver dullness, convulsion, tachycardia, and hyperventilation
Laboratory data at the onset of grade II or more severe hepatic encephalopathy (continuous variables): the grading of the encephalopathy, peripheral counts of WBC and platelets, prothrombin time, hepaplastin test, plasma concentrations of antithrombin III and ammonia, serum concentrations of AST, ALT, total albumin, bilirubin, AFP, and HGF, the serum concentration ratios of direct to total bilirubin, molar ratio of BCAA to tyrosine (BTR), and Fischer ratio
Symptoms and laboratory data 5 days after the onset of encephalopathy (continuous variables): the grading of the encephalopathy, prothrombin time

Table 2 continued

Atrophy of the liver at the onset of grade II or more severe hepatic encephalopathy and 5 days later Complications of acute liver failure at the onset of grade II or more severe hepatic encephalopathy: Bacterial and fungal infections, gastrointestinal bleeding, renal failure, cardiac failure, disseminated intravascular coagulation, other complications
Number of complications at the onset of grade II or more severe hepatic encephalopathy and 5 days later (continuous variables)
Complications of acute liver failure 5 days after the onset of encephalopathy: bacterial and fungal infections, gastrointestinal bleeding, renal failure, cardiac failure, disseminated intravascular coagulation, other complications
Number of complications 5 days after the onset of encephalopathy (continuous variables)
The therapies received: plasma exchange, hemodiafiltration, glucocorticoids, glucagon and insulin, prostaglandin E1, interferon, lamivudine or entecavir, cyclosporin A, anticoagulants, fresh-frozen plasma, and liver transplantation

LOHF late-onset hepatic failure, *HAV* hepatitis A virus, *HBV* hepatitis B virus, *HCV* hepatitis C virus, *HEV* hepatitis E virus *WBC* white blood cell count, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *AFP* alpha-fetoprotein, *HGF* hepatocyte growth factor, *BCAA* branched-chain amino acids

such as age, body weight, and biochemical data were analyzed as continuous variables, while those such as gender, outcomes, and complications were analyzed as nominal variables.

The decision tree analysis was performed using Intelligent Miner software (IBM, Armonk, New York, USA), which can automatically search a data set to find the optimal classification variables leading to the building of a decision tree algorithm [15]. Briefly, all items derived from the patients were evaluated to determine which variables and cutoff points might produce the most significant division into two subgroups showing mortality divergent from each other. Then the same analytic procedures were applied to all newly defined subgroups. These procedures were repeated and were terminated when either no additional significant variables were detected or when the sample size decreased to less than 20.

Evaluation of the established algorithms

The usefulness of the established algorithms was assessed through the following evaluations: (1) comparison of the mortality rates in patients belonging to each category to observe differences between patients used for the formation and those used for the validation of the algorithms; (2) the predictive accuracies, sensitivity, specificity, and positive and negative predictive values (PPV and NPV) among patients for both the formation and the validation of the algorithms, calculated based on the postulation that the outcome of the patients in the categories with mortality rates greater than 50% was predicted as "death"; and (3) the distribution of the patients in each category, when the data of the patients receiving liver transplantation were applied for the algorithms.

In each evaluation, data on the totals of 62 and 73 items, respectively, were selected for the algorithm at the onset of hepatic encephalopathy and that at 5 days after the development of encephalopathy, in a similar manner to the selection of data for the formation of the algorithms.

Statistical analysis

Statistical testing was performed using SPSS version 15.0J (SPSS, Tokyo, Japan). Results are expressed as means ± SD. Continuous variables were compared using Student's *t*-test. Categorical data were compared using the χ^2 test and analysis of residuals in cross tabulation.

Results

Algorithms to predict the outcome of patients with ALF based on decision tree analysis

Three hundred and seventy-one patients with ALF were classified through 5 items into 6 categories on the decision tree based on the data set obtained at the onset of hepatic encephalopathy (day 0) (Fig. 1). The mortality rate of patients with a serum bilirubin concentration of greater than 17.9 mg/dL was 89% (category-1A: $n = 91$). Two hundred and eighty patients with bilirubin concentrations of less than 17.9 mg/dL were divided into 2 groups according to peripheral blood platelet counts and further divided into 6 category groups according to age, the presence of ascites, and the disease etiology. The mortality rate of patients showing peripheral blood platelet counts of less than $10.2 \times 10^4/mm^3$ with ascites was 80% (category-1B: $n = 49$). In contrast, 61 patients with peripheral blood platelet counts of less than $10.2 \times 10^4/mm^3$ without ascites were divided into 2 groups according to the disease etiology; the mortality rate of patients with disease due to hepatitis A virus (HAV) and hepatitis E virus (HEV) infection and drug-allergy induced hepatitis, HBV carriers showing acute hepatitis exacerbation, and those with indeterminate etiology was 23% (category-1C: $n = 31$), whereas the mortality rate of those with other etiologies was 67% (category-1D: $n = 30$). The remaining 170 patients showing platelet counts of $10.2 \times 10^4/mm^3$ or

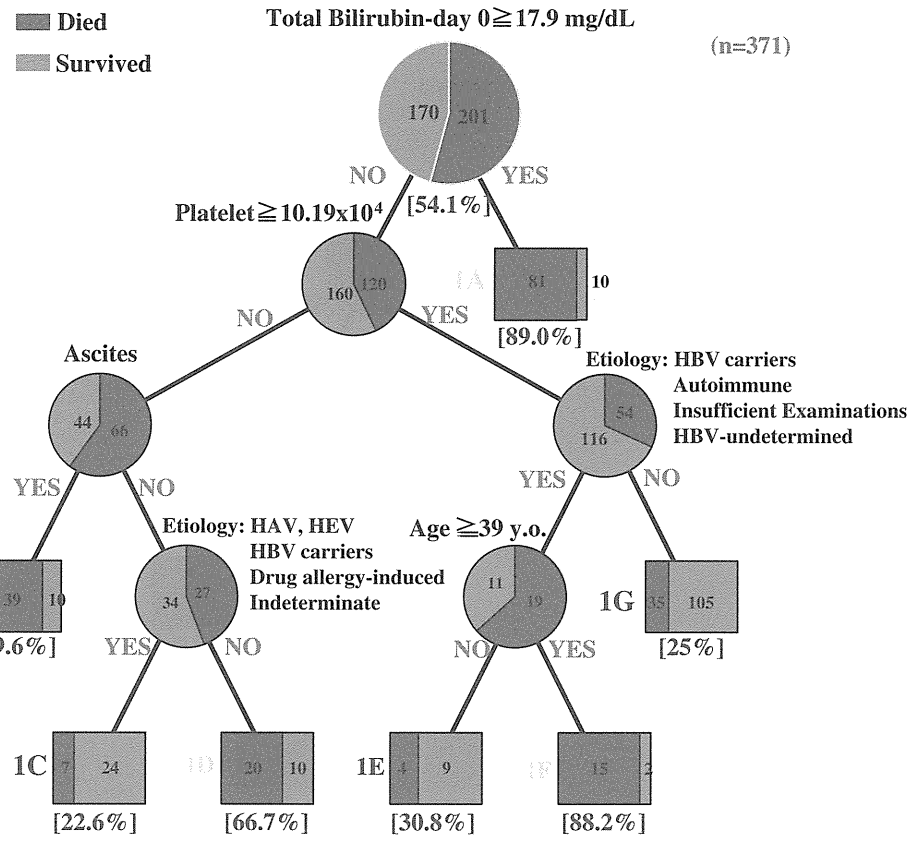

Fig. 1 The decision tree algorithm for outcome prediction at the onset of grade II or more severe hepatic encephalopathy (day 0). *HBV* hepatitis B virus, *HAV* hepatitis A virus, *HEV* hepatitis E virus

more were divided into 2 groups according to the different classification criteria of disease etiology; the mortality rates of HBV carriers showing acute hepatitis exacerbation and patients with autoimmune hepatitis were 31% (category-1E: $n = 13$) if the patient age was less than 39 years and 88% (category-1F: $n = 17$) if the age was 39 years old or more, whereas the mortality rate of those with disease due to other etiologies was 25% (category-1G: $n = 140$).

Based on the data set obtained 5 days after the onset of hepatic encephalopathy (day 5), ALF patients were classified through 7 items into 8 categories (Fig. 2). First, the patients were divided into 2 groups according to prothrombin time at 5 days after the development of encephalopathy. One hundred and ninety-two patients showing a prothrombin time of less than 39.5% of the standardized value were further classified through the presence of brain edema, liver atrophy, and cardiac failure at 5 days after the onset of encephalopathy. The mortality rate of patients with brain edema was 93% (category-2A: $n = 87$), but those without brain edema showed mortality rates of 80% (category-2B: $n = 66$), 16% (category-2C: $n = 31$), and 100% (category-2D: $n = 8$), respectively, when liver atrophy was present, both liver atrophy and cardiac failure were absent, and cardiac failure was present despite the absence of liver atrophy. In contrast, 179 patients showing a prothrombin time of 39.5% or more of the standardized value were classified by the serum bilirubin concentration. The mortality rate of the patients showing serum bilirubin concentrations of 17.45 mg/dL or more was 76% (category-2E: $n = 33$), whereas those with a serum bilirubin concentration of less than 17.45 mg/dL were further classified based on the presence of renal failure both at the onset of hepatic encephalopathy and 5 days later. The mortality rate of the patients without renal failure at 5 days after the onset of the encephalopathy was 11% (category-2F: $n = 109$). In contrast, the mortality rates of those with renal failure at 5 days were 30% (category-2G: $n = 27$) and 90% (category-2H: $n = 10$), respectively, depending on the presence and absence of renal failure at the onset of the encephalopathy.

As shown in Table 3, the predictive accuracies assessed in patients for the establishment of the algorithms were 79% at the onset of hepatic encephalopathy and 84% at 5 days after the onset of encephalopathy, when the estimated prognosis of patients classified in categories-1A, -1B, -1D, and -1F and categories-2A, -2B, -2D, -2E, and -2H was determined as "death". The sensitivity, specificity, PPV, and NPV were 78, 81, 83, and 75%, respectively, at the onset of the encephalopathy, and 83, 85, 87, and 81%, respectively, at 5 days later.

Validation of the established algorithms

One hundred and sixty patients with ALF, seen between 2004 and 2007, were classified into 7 categories through

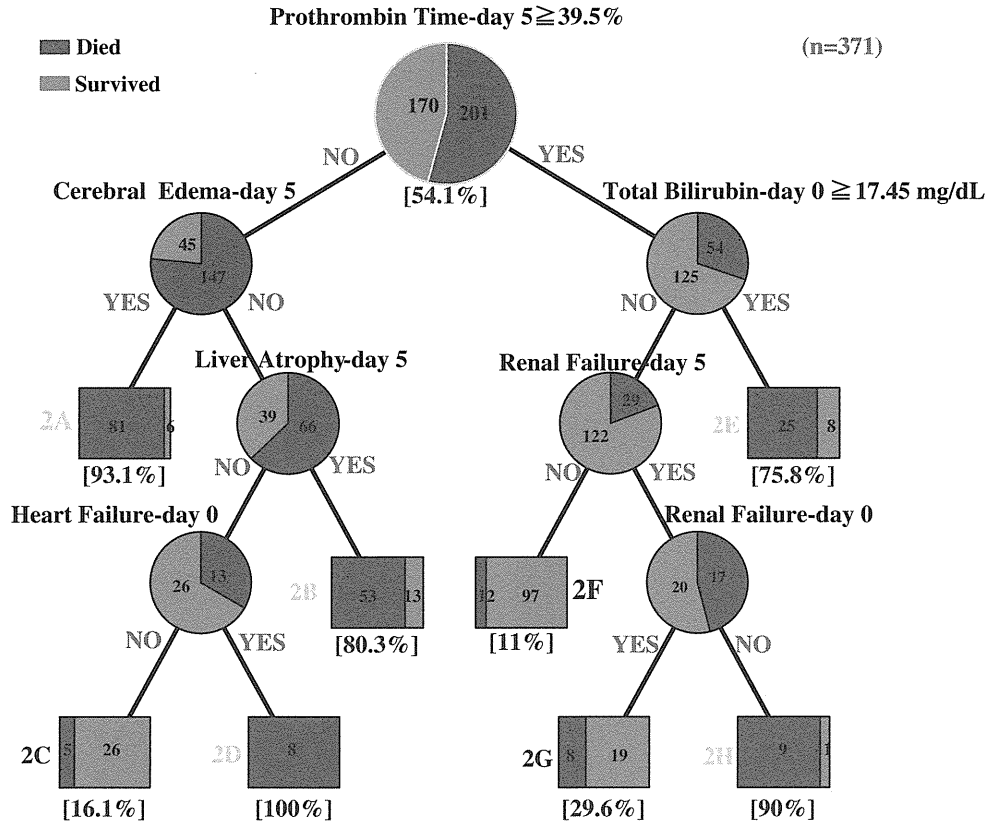

Fig. 2 The decision tree algorithm for outcome prediction at 5 days after the onset of grade II or more severe hepatic encephalopathy (day 5)

Table 3 The accuracy of the decision tree algorithms to predict the prognostic outcome of acute liver failure patients at the onset of hepatic encephalopathy and 5 days later

	At the onset of hepatic encephalopathy	At 5 days after the onset of hepatic encephalopathy
Patients for the formation of the algorithm 1998–2003 ($n = 371$)		
Accuracy	79.0	83.6
Sensitivity	77.6	82.6
Specificity	80.6	84.7
PPV	82.5	86.5
NPV	75.3	80.5
Patients for the validation of the algorithm 2004–2007 ($n = 160$)		
Accuracy	71.2	73.1
Sensitivity	75.0	63.6
Specificity	67.1	82.4
PPV	70.6	77.8
NPV	71.8	70.0

PPV positive predictive value, *NPV* negative predictive value

the analysis using the data set at the onset of hepatic encephalopathy, and 8 categories using the data set at 5 days after the onset of encephalopathy. The number of patients who died and the mortality rates of the patients in each category are shown in Table 4. The distribution of the patients and the mortality rates in each category were almost equivalent to those in the patients used for the formation of the algorithms both at the onset of hepatic encephalopathy and 5 days later, except for category-2C. The mortality rate in patients classified as category-2C was 16.1% in patients used for the formation of the algorithm, while the rate was 91.7% in those used for the validation (Table 4b).

The predictive accuracies assessed in patients for validation of the algorithms were 71 and 73%, respectively, at the onset of hepatic encephalopathy and 5 days later, similar to findings in the patients used for the formation of the algorithms (Table 3). The sensitivity, specificity, PPV, and NPV were 75, 67, 71, and 72%, respectively, at the onset of the encephalopathy, and 64, 82, 78, and 70%, respectively, at 5 days after the onset of encephalopathy.

Application of the algorithms for ALF patients receiving liver transplantation

When the data from the 211 patients who had received liver transplantation were applied for the established algorithms at the onset of hepatic encephalopathy, 141 patients (66.8%) were classified as category-1A, category-1B, category-1D, or category-1F, in which categories the

Table 4 The numbers of deaths and the mortality rates of patients in each category classified through decision tree analysis: comparison among patients used for the formation of the algorithm, those used for the validation of the algorithm, and those who received liver transplantation

Categories classified through decision tree analysis	Mortality rates of patients % (number of patients)		Number of patients
	Patients for algorithm formation 1998–2003 ($n = 371$)	Patients for algorithm validation 2004–2007 ($n = 160$)	Patients receiving liver transplantation 1998–2007 ($n = 211$)
(a) The algorithm for the patients at the onset of hepatic encephalopathy			
1A	89.0 (81/91)	83.9 (26/31)	95
1B	79.6 (39/49)	50.0 (16/32)	34
1C	22.6 (7/31)	37.5 (3/8)	10
1D	66.7 (20/30)	83.3 (10/12)	8
1E	30.8 (4/13)	18.2 (2/11)	7
1F	88.2 (15/17)	80.0 (8/10)	4
1G	25.0 (35/140)	30.2 (16/53)	53
(b) The algorithm for the patients at 5 days after the onset of hepatic encephalopathy			
2A	93.1 (81/87)	86.4 (19/22)	19
2B	80.3 (53/66)	71.4 (15/21)	36
2C	16.1 (5/31)	91.7 (11/12)	16
2D	100.0 (8/8)	– (0/0)	0
2E	75.8 (25/33)	72.7 (8/11)	18
2F	11.0 (12/108)	17.3 (9/52)	20
2G	29.6 (8/27)	25.0 (4/16)	1
2H	90.0 (9/10)	– (0/0)	2

mortality rates were greater than 50% in patients for the formation of the algorithm (Table 4a). In contrast, 53 patients (25.2%) were classified as category-1G, in which the mortality rates were 25.0 and 29.4%, respectively, in patients used for the formation and those used for the validation of the algorithm.

The outcome at 5 days after the onset of hepatic encephalopathy was assessed in 112 (53.1%) of the 211 patients who had received liver transplantation, because the transplantation was done within 5 days after the onset of hepatic encephalopathy in 99 patients (Table 4b). Consequently, 75 (67.0%) of the 112 patients were classified as category-2A, category-2B, category-2D, category-2E, or category-2H for the formation of the algorithm, in which categories the mortality rates were greater than 50%. Sixteen patients (14.3%) were classified as category-2C for validation of the algorithm, in which category the mortality rate was greater than 90%, despite the fact that the mortality in it was only 16.1% in the patients used for formation of the algorithm.

Discussion

In the present study, we established a predictive model to determine the outcome of patients with ALF through decision tree analysis, one of the data-mining methods. Data-mining has been applied to analysis in fields such as business intelligence, marketing, banking and finance, customer relationship management, and engineering, as well as various areas of science, including medicine. In clinical medicine, data-mining techniques are used to construct a predictive model, which supports clinical decisions for researchers as well as practitioners [17]. A decision tree algorithm is one of the most popular data-mining techniques, constructed through recursive data partitioning, where the data are split according to the values of a selected attribute in iteration. Decision trees have already been applied to the field of hepatology; for example, to analyze the characteristic features of hepatocellular carcinoma [18–20], and to evaluate the therapeutic efficacy of pegylated-interferon and ribavirin for patients with chronic hepatitis due to HCV infection [21, 22].

In the present study, algorithms of two types were established; an algorithm for use at the onset of hepatic encephalopathy and one for use 5 days later, because, in Japan, conservative medical care including artificial liver support is generally performed in most patients, including those receiving liver transplantation, following the onset of hepatic encephalopathy. In fact, as shown in Table 1c, plasma exchange and hemodiafiltration were carried out in more than 90 and 70%, respectively, of patients with ALF. Thus, the outcome of the patients could be evaluated 5 days after the onset of hepatic encephalopathy in 53% of patients receiving liver transplantation (Table 4). The data sets obtained from ALF patients seen between 1998 and

2003 were used for the formation of the algorithms and those from the patients seen between 2004 and 2007 for their validation, because the outcomes of the patients seen in the two periods were almost equivalent, although there were some differences between the two periods in the frequencies of the therapeutic procedures undertaken (Table 1c, d).

According to the established decision tree algorithms, the patients with ALF were classified into 7 categories through 6 items at the onset of hepatic encephalopathy and into 8 categories through 7 items at 5 days after the onset of hepatic encephalopathy. Serum bilirubin concentration was selected as the first split item in the former algorithm, and the patients were further classified based on peripheral blood platelet counts, age, presence or absence of ascites, and the etiology of liver injuries. In contrast, the prothrombin time at 5 days after the onset of encephalopathy was the first split item in the latter algorithm, and the patients were then classified based on the serum bilirubin concentration and presence or absence of cerebral edema, liver atrophy, and cardiac and renal failure at the onset of encephalopathy or 5 days later. The interval between the onset of disease symptoms and hepatic encephalopathy has been considered to be one of the most important factors to determine the prognosis of ALF patients [4], and this factor was selected as a parameter in the previous guidelines [5]. The prothrombin time and the ratio of the direct-to-total bilirubin concentration at the onset of hepatic encephalopathy were previously selected as parameters as well [5]. However, these factors were not chosen as items responsible for the prognosis of ALF patients in our novel model established through decision tree analysis. These decisions are in line with findings in our previous report [7], in which ALF patients could be classified into three clusters independent of the interval between the onset of disease symptoms and the onset of hepatic encephalopathy, and the prognosis of the patients differed markedly among the clusters. Moreover, among 7 items in the algorithms at 5 days after the onset of hepatic encephalopathy, the extent of cerebral edema, renal failure, and heart failure may vary depending on the therapeutic devices used, especially regarding methods for artificial liver support (ALS) [23–25]. High-flow continuous hemodiafiltration (CHDF) and on-line hemodiafiltration (HDF) are much more effective than conventional HDF and CHDF [26, 27]. In the present study, most of the patients received conventional CHDF and HDF (data not shown), and such therapeutic devices were not selected as factors affecting the prognoses of the patients.

Certain characteristic features in both our algorithms are deserving of inclusion in the algorithms. First, the categories can be divided into 2 types depending on their mortality rates; the mortality rates in patients used for the formation of the model were greater than 66.7% in 4 categories in both algorithms, while they were less than 33.3% in the remaining 3 and 4 categories, respectively, in the algorithm used at the onset of hepatic encephalopathy and that used 5 days later. Secondly, 341 of the 371 patients used for the establishment of decision trees (91.9%) were classified into 4 major categories, in which the number of patients belonging to each category was greater than 30 in the algorithm at the onset of hepatic encephalopathy. Also, 325 patients (87.6%) were classified into 5 major categories in the algorithm at 5 days after the onset of hepatic encephalopathy. Considering these characteristic features of both algorithms, the novel model constructed through the decision tree analysis seems to be useful for the prediction of the outcome of patients with ALF, because the first characteristic above allowed the analysis to achieve high accuracy rates when the outcomes of the patients were predicted qualitatively as "death" or "survival". In contrast, the second characteristic may enable us to obtain stable results for prediction even after the validation. In fact, as shown in Table 3, the predictive accuracies of both algorithms were high; 79.0 and 83.6%, respectively, in the algorithm at the onset of hepatic encephalopathy and that at 5 days later, when the outcome of patients belonging to the categories with mortality rates greater than 50% was predicted as "death". Moreover, the sensitivity, specificity, PPV, and NPV values were greater than 75% in the algorithm at the onset of hepatic encephalopathy, and greater than 80% in the algorithm at 5 days later. Also, the mortality rates in patients used for the algorithm formation were similar to those in the patients used for the validation in each category, except for category-2C. As a result, the predictive accuracies were also high in patients used for the validation algorithm; 71.2 and 73.1%, respectively, in the algorithm at the onset of hepatic encephalopathy and that at 5 days later, when the outcome of patients was assessed qualitatively. Thus, it is concluded that the present model, consisting of 2 algorithms, may be useful to predict the outcome of ALF patients both quantitatively and qualitatively. Clinicians can obtain the predictive mortality rates of the patients depending on the categories to which the patients belong, and they can also predict the outcome as "death" or "survival" with satisfactory accuracies.

However, there are several weak points in both algorithms to predict the outcome of the ALF patient. Although the reproducibility of the algorithm at the onset of hepatic encephalopathy was generally good in each category, a 29.6% difference in mortality rates was found between the formation and validation data sets in category-1B. Also, there was a 75.6% difference between the two data sets in category-2C. Moreover, the validation could not be done in categories-2D and -2H, because no patients were classified in these categories in the validation groups, and a similar