(n = 38), both HBV and HCV (n = 2), and unknown etiology (n = 26). Paraffin-embedded sections of the tumors and surrounding nontumor liver tissues were examined by hematoxylin-eosin (H&E) staining and immunohistochemistry.

2.6. Immunohistochemical analyses

After deparaffinization and inhibition of endogenous peroxidase activities, the pathologic specimens of surgically resected tumors were stained with rabbit anti-EZH2 (1:100, Zymed), mouse anti-BMI1 (1:100, Upstate Biotechnology) or mouse anti–Ki-67 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Before incubation with the primary antibodies, antigen retrieval was performed with ethylenediaminetetraacetic acid buffer (pH 8.0) in a pressure cooker at 100°C for 1 minute. The proteins were detected using ImmPRESS peroxidase micropolymer staining kits (Vector Laboratories) and 3,3′-diaminobenzidine substrate according to the manufacturer's instructions. To investigate the colocalization of EZH2 and BMI1, we simultaneously stained the clinical specimens with indicated antibodies. Subsequently, they were stained with Alexa-555–conjugated goat anti-rabbit IgG (Molecular Probes) and Alexa-488–conjugated goat anti-mouse IgG (Molecular Probes), respectively.

2.7. Statistical analyses

Statistical differences between 2 groups in MTS assays were analyzed using the Student t test. The correlations between the expression of polycomb group proteins and clinicopathologic features were assessed using the χ^2 test and Student t test to analyze qualitative and quantitative data, respectively. The cumulative survival and recurrent rates were analyzed by the Kaplan-Meier method, and the statistical significance between the 2 groups positive and

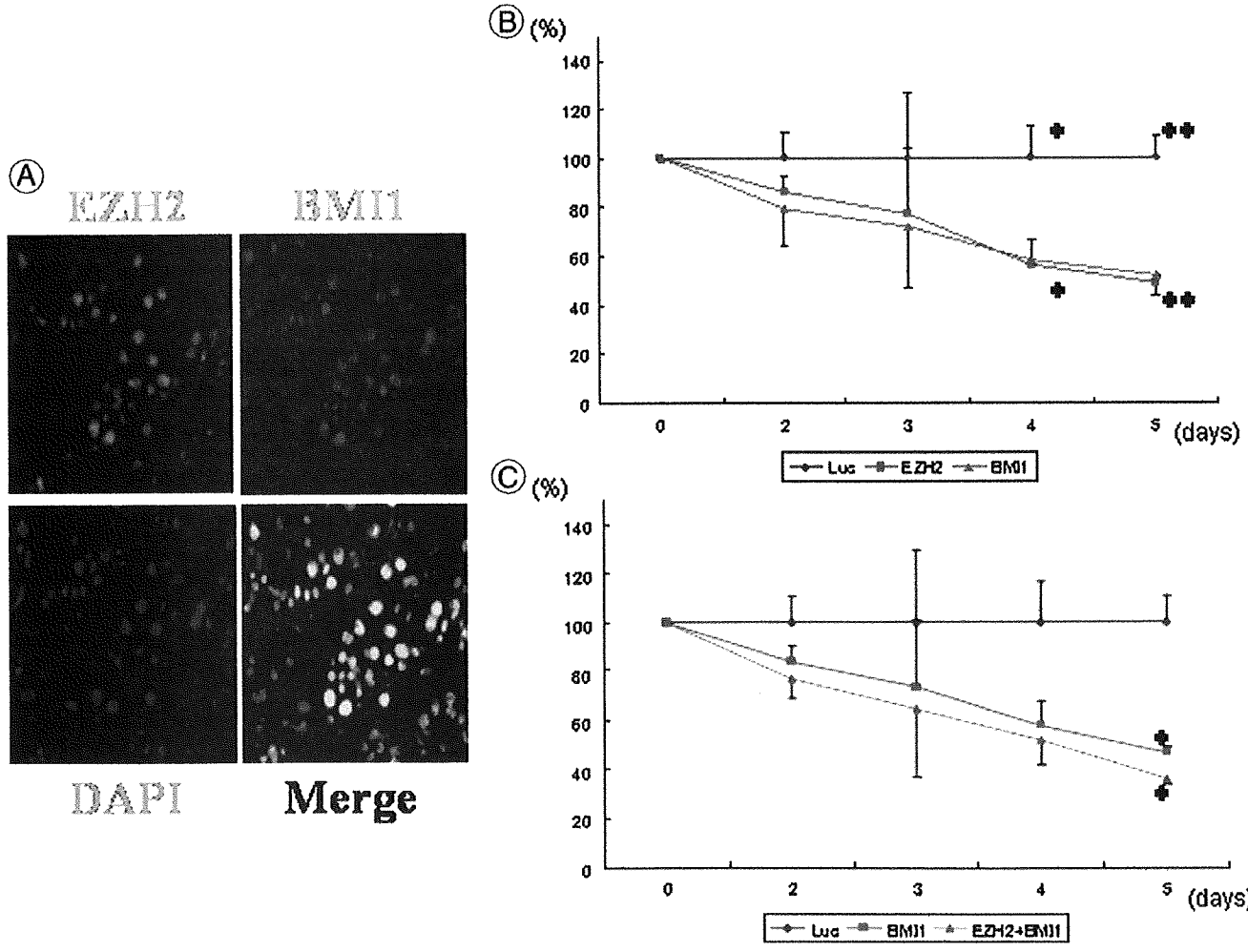

Fig. 1 Loss-of-function assays of EZH2 and BMI1 in HepG2 cells. (A) Immunocytochemical analyses of EZH2 and BMI1. Immunofluorescent labeling of EZH2 (green), BMI1 (red), and nuclear DAPI staining (blue) is merged. (B) Cell growth inhibition of EZH2 or BMI1 knockdown HepG2 cells compared with luciferase knockdown HepG2 cells as a control. Mean ± SD values are shown for each group, $*P < .05$, $**P < .005$. (C) Augmented growth inhibition of both EZH2 and BMI1 knockdown HepG2 cells. Mean ± SD values are shown for each group, $*P < .005$.

negative for polycomb group immunostaining was analyzed using the Wilcoxon test. Cox proportional regression analysis was performed to estimate rate ratios for the effect of EZH2 and BMI1 staining in tumors for recurrence. Potential risk factors assessed for recurrence included the following variables: BMI1 in tumors (presence versus absence), EZH2 in tumors (presence versus absence), sex (male versus female), age (>65 versus ≤65 years), size of tumors (>45 versus ≤45 mm), differentiation (poorly versus well, moderately), TNM stage [16] (I + II versus III + IVa), protein induced by vitamin K absence II (PIVKA II) level (>100 versus ≤100 mAU/mL), α-fetoprotein (AFP) level (>40 versus ≤40 mg/mL), etiology (HBV and HCV versus HBV alone, HCV alone, non-HBV, and non-HCV), chronic hepatitis (versus liver cirrhosis), aspartate aminotransferase (AST) level (>50 versus ≤50 IU/L), alanine aminotransferase (ALT) level (>50 versus ≤50 IU/L), total bilirubin level (>1.0 versus ≤1.0 mg/dL), albumin level (>3.8 versus ≤3.8 g/dL), platelet count (>15 × 10^9/L versus ≤15 × 10^9/L), prothrombin time (>80% versus ≤80%), the presence of portal invasion (versus no portal invasion), and tumor number (>2 versus ≤2). Variables statistically significant by univariate Cox proportional regression analysis were further studied by multivariate analysis. The differences were considered significant at $P < .05$.

3. Results

3.1. Stable short hairpin RNA-mediated knockdown of EZH2 and BMI1 mediated growth inhibition in HepG2 cells

To examine the basal expression of EZH2 and BMI1 in hepatocellular carcinoma cells, we conducted dual immunocytochemical analyses in HepG2 cells. EZH2 and BMI1 were widely detected in the nucleus and were coexpressed in more than 70% of HepG2 cells (Fig. 1A). To gain insight into the role of the polycomb gene products in HepG2 cells, we performed loss-of-function assays using lentivirus-mediated knockdown techniques. MTS assays showed that cell growth activity was consistently repressed, after EZH2 and BMI1 knockdown, for the 5 days of the observation period (Fig. 1B). Cell growth activity at days 2, 3, 4, and 5 was 86% ± 6.2%, 77% ± 27%, 56% ± 10%, and 49% ± 2.4%, respectively, after EZH2 knockdown and 79% ± 15%, 72% ± 25%, 58% ± 2.5%, and 52% ± 8.3%, respectively, after BMI1 knockdown compared with the luciferase knockdown cells, with there being a statistically significant difference at day 4 ($P < .05$) and 5 ($P < .005$) in the case of EZH2 or BMI1 knockdown. Intriguingly, simultaneous knockdown of EZH2 and BMI1 mediated an even greater degree of growth inhibition of HepG2 cells (Fig. 1C). Cell growth activity at days 2, 3, 4, and 5 was 77% ± 7.9%, 65% ± 27%, 52% ± 10%, and 36% ± 1.8%, respectively, after simultaneous knockdown compared with luciferase knockdown cells, with there being a statistically significant difference at day 5 ($P < .005$) between knockdown of both EZH2 and BMI1 and that of BMI1 alone.

Table 1 EZH2 and BMI1 expression in hepatocellular carcinoma and equivalent nontumor tissues

	Tumor	Nontumor
EZH2	57/86 (66.3%)*	5/86 (5.8%)*
BMI1	52/86 (60.5%)**	4/86 (4.7%)**

*, **$P < .05$.

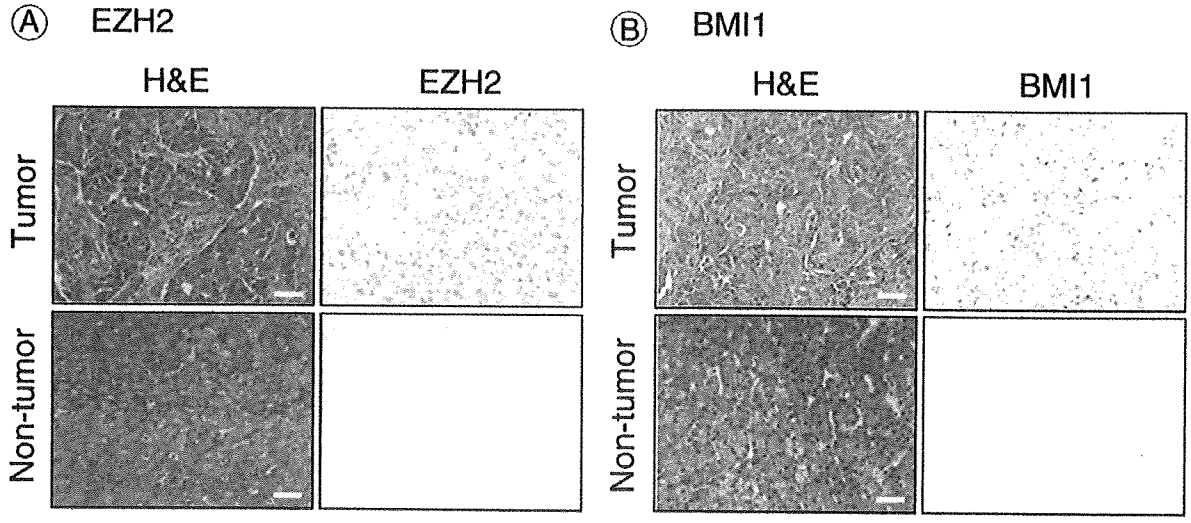

Fig. 2 Representative histopathologic analyses of EZH2 and BMI1. (A) H&E staining and immunohistochemical analysis of EZH2 in hepatocellular carcinoma tissue and the corresponding nontumor tissue. (B) H&E staining and immunohistochemical analysis of BMI1 in hepatocellular carcinoma tissue and the corresponding nontumor tissue (scale bar = 50 μm).

Table 2 The expression pattern of EZH2 and BMI1 in tumor and nontumor tissues

	Tumor				Nontumor			
	BMI1+	BMI1−	Total	*P*	BMI1+	BMI1−	Total	*P*
EZH2+	48	9	57	<.01	0	5	5	>.99
EZH2−	3	26	29		4	77	81	
Total	51	35	86		4	82	86	

3.2. Preferential expression of EZH2 and BMI1 in hepatocellular carcinoma samples

We evaluated the expression levels of polycomb group proteins EZH2 and BMI1 in hepatocellular carcinoma tissues and the corresponding nontumor tissues obtained from 86 patients by histopathologic analyses. Representative immunohistochemical staining of EZH2 and BMI1 is shown in Fig. 2A and B. The expression of EZH2 and BMI1 was diffusely detected in the nuclei of cancer cells, and no patients showed focal expression of these proteins in tumor tissues.

Fifty-seven (66.3%) of 86 hepatocellular carcinoma samples were positive for EZH2, whereas only 5 (5.8%) of 86 nontumor samples showed EZH2 expression ($P < .01$) (Table 1). On the other hand, BMI1 expression was detected in 52 (60.5%) of 86 hepatocellular carcinoma samples, whereas its expression was detected in only 4 (4.7%) of 86 nontumor samples ($P < .01$) (Table 1). Of importance, 48 (55.8%) of 86 hepatocellular carcinoma samples expressed both EZH2 and BMI1 (Table 2). Consequently, 26 (30.2%) of 86 samples did not express either protein. These results showed a statistically significant difference with respect to EZH2-positive ratio between BMI1-positive and BMI1-negative tumor tissues ($P < .01$). In contrast, 77 (89.5%) of 86 nontumor samples were negative for both EZH2 and BMI1 (Table 2). These results indicate that the expression of EZH2 and BMI1 is preferentially up-regulated in hepatocellular carcinoma samples, and their expression patterns could segregate hepatocellular carcinoma samples into subgroups.

3.3. Coexpression of EZH2 and BMI1 in hepatocellular carcinoma tissues

To determine whether EZH2 and BMI1 are coexpressed in the same hepatocellular carcinoma cells, we performed dual immunofluorescence staining on 10 randomly selected hepatocellular carcinoma tissues that were positive for both EZH2 and BMI1 by conventional immunohistochemical analysis. As shown in Fig. 3, EZH2 and BMI1 were detected in the nucleus and were coexpressed in more than 70% of hepatocellular carcinoma cells in all samples analyzed. These results strongly indicate cooperative contributions of EZH2 and BMI1 to the development and progression of hepatocellular carcinoma.

3.4. Correlation between the expression of EZH2 or BMI1 and that of Ki-67

We performed immunohistochemical analysis of Ki-67 in tumor and nontumor tissues and compared the results with those of EZH2 and BMI1 (Table 3). EZH2 was positive in 47 (80%) of 59 Ki-67–positive tumors and in 10 (37%) of 27 Ki-67–negative tumors ($P = .0002$). BMI1 was also positive in 44 (75%) of 59 Ki-67–positive tumors and in 8 (30%) of 27 Ki-67–negative tumors ($P = .0001$). In nontumor tissues, the staining of Ki-67 was found only in 11 cases, and we found no correlation between these 2 proteins and Ki-67 (Table 3).

3.5. Relationship between EZH2 or BMI1 expression and clinicopathologic features

We next evaluated the relationship between increased expression of polycomb group proteins and clinicopathologic parameters of the 86 hepatocellular carcinoma patients (Table 4). Significant correlation was observed between increased EZH2 expression in hepatocellular carcinoma tissues and hypoalbuminemia ($P = .01$) or advanced TNM stage ($P = .04$). In contrast, increased BMI1 expression in the

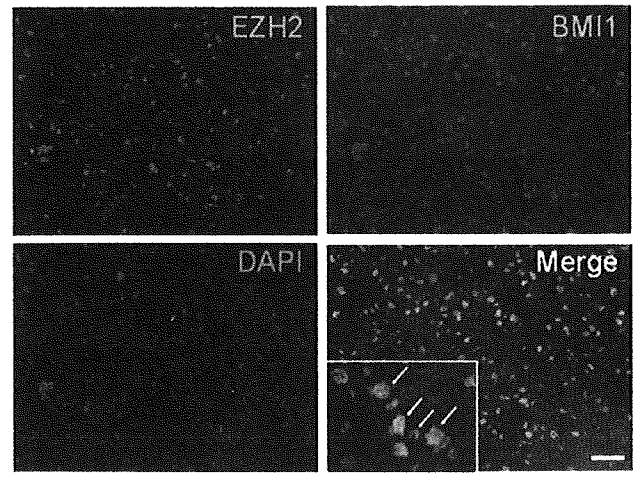

Fig. 3 Representative dual immunostaining of EZH2 (green) and BMI1 (red) in hepatocellular carcinoma tissue and the corresponding nontumor tissue. The nuclei were stained with DAPI (blue). Arrows indicate a typical nucleus positive for EZH2, BMI1, and DAPI (scale bar = 50 μm).

Table 3 Correlation between the expression of EZH2 or BMI1 and that of Ki-67

	Tumor				Nontumor			
	Ki-67+	Ki-67−	Total	*P*	Ki-67+	Ki-67−	Total	*P*
EZH2+	47	10	57	.0002	1	4	5	>.50
EZH2−	12	17	29		10	71	81	
Total	59	27	86		11	75	86	
BMI1+	44	8	52	.0001	1	3	4	.43
BMI1−	15	19	34		10	72	82	
Total	59	27	86		11	75	86	

hepatocellular carcinoma samples had no significant correlation with clinicopathologic factors.

3.6. Prognostic value of EZH2 or BMI1 expression in hepatocellular carcinoma patients

To elucidate the role of polycomb group proteins in hepatocellular carcinoma progression, we performed prognostic analyses of 61 hepatocellular carcinoma patients in whom curative operation was conducted and whose postoperative course could be followed. The cumulative recurrent rates and survival rates were analyzed by the Kaplan-Meier method (Fig. 4A-D). During the follow-up period (27.2 ± 19.6 months), 39 patients developed a recurrence of hepatocellular carcinoma and 19 patients died from relapsed hepatocellular carcinoma. Our results demonstrated higher cumulative recurrence rates in EZH2-positive patients than in EZH2-negative patients (P = .029). In contrast, the cumulative survival rates showed no significant differences between EZH2-positive and EZH2-negative patients. Of interest, results of the prognostic analysis correlating BMI1 expression were similar to those of the EZH2 expression-based analysis. The cumulative recurrence rates were higher in BMI1-positive patients than in BMI1-negative patients (P = .039), although the difference in survival rates between the 2 groups was not significant. Given that EZH2 and BMI1 are frequently coexpressed in the same samples, these prognostic findings appear quite reasonable.

Table 4 Correlation between polycomb group protein expression and clinicopathologic background in 86 hepatocellular carcinoma samples

Variables	EZH2 expression			BMI1 expression		
	Negative (n = 29)	Positive (n = 57)	*P*	Negative (n = 34)	Positive (n = 52)	*P*
Sex (male/female)	27/2	46/11	.13	31/3	42/10	.46
Age (y)	64.7 ± 9.8	62.6 ± 12.5	.45	63.6 ± 11.6	63.1 ± 11.8	.86
Liver function						
AST (IU/L)	66.8 ± 79.4	94.8 ± 126.0	.30	67.4 ± 75.0	97.2 ± 131.2	.25
ALT (IU/L)	71.4 ± 64.8	90.4 ± 122.3	.46	71.9 ± 64.3	92.0 ± 126.8	.42
Total bilirubin (mg/dL)	1.00 ± 0.38	1.12 ± 0.64	.39	0.96 ± 0.36	1.15 ± 0.66	.15
Albumin (g/dL)	4.02 ± 0.48	3.70 ± 0.53	.01[a]	3.92 ± 0.50	3.74 ± 0.55	.14
Prothrombin time (%)	84.4 ± 12.9	84.7 ± 19.3	.95	83.6 ± 12.5	85.1 ± 20.0	.76
Platelet (×10^4/mL)	15.60 ± 6.62	15.89 ± 6.66	.85	15.94 ± 6.88	15.70 ± 6.5	.88
PIVKA II (mAU/mL)	4027.0 ± 9934.8	5040.6 ± 12 873.8	.76	5654.6 ± 13 844.7	4250.1 ± 11 063.9	.65
AFP (mg/mL)	108.0 ± 181.6	9570.6 ± 37 690.7	.21	3710.9 ± 19 937.8	8283.1 ± 36 691.8	.53
Tumor factors						
Tumor size (mm)	47.4 ± 33.5	51.4 ± 33.6	.63	51.3 ± 34.5	49.4 ± 33.0	.81
No.	1.19 ± 0.51	1.40 ± 0.61	.18	1.26 ± 0.59	1.38 ± 0.58	.40
VP (+) (%)	3/19 (15.8%)	17/50 (34%)	.23	7/24 (29.2%)	13/45 (28.9%)	>.99
Well/moderate/poorly[b]	4/16/4	6/36/9	.84	5/20/4	5/32/9	.64
TNM (I/II/III/IVa)	5/15/4/0	5/25/20/8	.04[a]	4/17/8/0	6/23/16/8	.14
Etiology						
AIH/B/C/B and C/BC (−)	0/5/12/0/12	1/14/26/2/14	.63	1/6/14/0/13	0/13/24/2/13	.42
CH/LC	16/11	34/21	.81	21/11	29/21	.64

Abbreviations: VP, invasion to portal vein; AIH, autoimmune hepatitis; CH, chronic hepatitis; LC, liver cirrhosis; B, hepatitis B; C, hepatitis C.
NOTES: Reference range—AST, 10 to 35 IU/L; ALT, 5 to 40 IU/L; total bilirubin, 0.4 to 1.2 mg/dL; albumin, 3.9 to 5.2 g/dL; prothrombin time, 70% to 140%; platelet, 15 to 35 × 10^4/mL; PIVKA II, <40 mAU/mL; AFP, <20 ng/mL.

[a] Statistically significant.

[b] Histologic differentiation of the tumor.

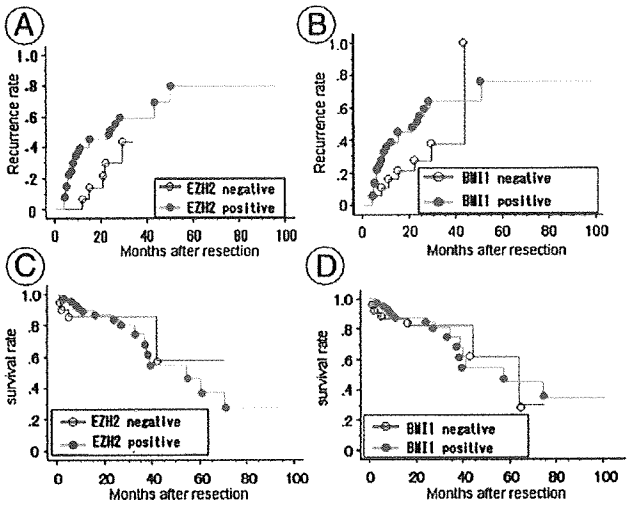

Fig. 4 Kaplan-Meier curves for the prognostic analyses. A and B, Cumulative recurrence rate with regard to EZH2 expression (A) and BMI1 expression (B). C and D, Cumulative survival rate with regard to EZH2 expression (C) and BMI1 expression (D).

The potential risk factors affecting tumor recurrence were analyzed by univariate Cox proportional hazards regression, and variables of P value less than 0.1 (the presence of BMI1, risk ratio, 2.3, $P = .053$; the presence of EZH2, risk ratio, 2.5, $P = .060$; age ≤65 years, risk ratio, 2.4, $P = .018$; AFP >40 mg/mL, risk ratio, 2.5, $P = .013$; albumin ≤3.8 mg/dL, risk ratio, 2.3, $P = .020$; the presence of portal vein invasion, risk ratio, 2.1, $P = .050$; TNM stage I + II, risk ratio, 0.40, $P = .015$) were further studied by multivariate analysis. However, we found that no variables examined in this multivariate analysis showed a statistically significant correlation with recurrence (the presence of BMI1, risk ratio, 1.4, $P = .51$; the presence of EZH2, risk ratio, 1.6, $P = .47$; age ≤65 years, risk ratio, 2.4, risk ratio, 2.0, $P = .07$; AFP >40 mg/mL, risk ratio, 2.0, $P = .12$; albumin ≤3.8 mg/dL, risk ratio, 2.2, $P = .11$; the presence of portal vein invasion, risk ratio, 0.64, $P = .43$; TNM stage I + II, risk ratio, 0.52, $P = .10$).

We found no effect on survival and recurrence in tumors that simultaneously expressed both EZH2 and BMI1 as compared with those that expressed only one of these proteins ($P = .61$ for recurrence and $P = .58$ for survival, as determined by Kaplan-Meier analysis).

4. Discussion

Hepatocellular carcinoma is one of the most common malignancies, and chronic viral infection by hepatitis B or C virus is well recognized as a major risk factor [17]. Therapeutic advancements such as nucleotide analogues and interferon has successfully improved hepatitis viremia and reduced the incidence of hepatocellular carcinoma, but the mortality rate of hepatocellular carcinoma remains high in spite of recent advances in cancer therapy [18]. In hepatocarcinogenesis, it appears that repeated injury and regeneration of damaged liver cells induce genetic and epigenetic dysregulation, which ultimately contribute to cancer development [19,20]. Recent studies highlight the pivotal role of polycomb group proteins in cancer [1]. However, our knowledge on their implications in hepatocarcinogenesis remains limited [21].

At first, we examined the growth activity of HepG2 cells in loss-of-function assays. The MTS assays showed that EZH2 or BMI1 knockdown mediated significant cell growth inhibition. This finding appears to be consistent with previous observations suggesting a role for EZH2 or BMI1 in a variety of immortalized and transformed cells [6,7,22]. Interestingly, knockdown of both EZH2 and BMI1 augmented cell growth inhibition, which indicated that the function of EZH2 might partly differ from that of BMI1 in molecular mechanisms underlying proliferation of hepatocellular carcinoma cells.

On the other hand, immunohistochemical analyses showed that increased expression of either EZH2 or BMI1 protein was observed in 60 (69.8%) of 86 hepatocellular carcinoma samples. Meanwhile, only 5.8% and 4.7% of surrounding nontumor tissues expressed EZH2 and BMI1, respectively. These results unveil preferential up-regulation of polycomb group protein expression during hepatocarcinogenesis and might implicate a special role for polycomb group proteins in the development and progression of hepatocellular carcinoma. Given that EZH2 and BMI1 were frequently coexpressed in the same samples, there might be functional crosstalk between EZH2 and BMI1 in the pathogenesis of hepatocellular carcinoma. Conversely, 26 (30.2%) of 86 samples exhibited expression of neither protein, which might be reflective of inherent heterogeneity with respect to origin of hepatocellular carcinoma and/or underlying molecular mechanisms.

Analyses of EZH2 mRNA expression in hepatocellular carcinoma samples based on real-time reverse transcriptase polymerase chain reaction have previously documented no significant correlation between EZH2 expression and disease-free survival [21]. Recently, another group reported that overexpression of EZH2 and BMI1 is associated with aggressive biologic behavior including vascular invasion and lymph node metastasis [23,24]. To examine whether protein expression of EZH2 and BMI1 are good biomarkers for recurrence and survival in our samples, we conducted prognostic analyses. The present analyses demonstrate that increased expression of both EZH2 and BMI1 proteins significantly correlated with recurrence after hepatectomy ($P = .029$ and $P = .039$, respectively), although there was no significant correlation between the expression of these polycomb group proteins and survival. Analyses of clinicopathologic parameters showed lower levels of serum albumin and advanced stage of TNM in EZH2-positive patients compared with EZH2-negative patients, which might indicate advanced liver dysfunction and tumor stage in EZH2-positive patients. In contrast, the significant

correlation between high EZH2 expression and portal vein invasion of the tumor, which was previously reported by mRNA expression-based analyses [21], was not detected in this study ($P = .23$).

In conclusion, our studies showed that polycomb proteins, in particular, EZH2 and BMI1, can have a strong effect on proliferation of hepatocellular carcinoma cells and that simultaneous knockdown of EZH2 and BMI1 has more marked effect on cell growth inhibition than knockdown of either protein alone. Immunohistochemical analyses further demonstrated a clear association between EZH2 and BMI1 expression and the development and progression of hepatocellular carcinoma, as well as recurrence after curative surgery. Thus, EZH2 and BMI1 could be target molecules in the development of new treatment strategies against hepatocellular carcinoma.

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Blood flow parameters in the short gastric vein and splenic vein on Doppler ultrasound reflect gastric variceal bleeding

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ABSTRACT

Purpose: Hemodynamic features associated with the bleeding from gastric fundal varices (FV) have not been fully examined. The purpose of this study was to elucidate hemodynamics in the short gastric vein (SGV) which is a major inflow route for FV and flow direction of the splenic vein (SV) in relation to bleeding FV.

Materials and Methods: The subject of this retrospective study was 54 cirrhotic patients who had medium- or large-sized FV (20 bleeders, 34 non-bleeders) on endoscopy with SGV on both angiogram and sonogram. Diameter, flow velocity, flow volume of SGV and flow direction in the SV were evaluated by Doppler ultrasound.

Results: Diameter, flow velocity and flow volume of SGV were significantly greater in bleeders (9.6 ± 3.1 mm, 11.4 ± 5.2 cm/s, 499 ± 250.1 ml/min) than non-bleeders (6.5 ± 2.2 mm, $p = 0.0141$; 7.9 ± 3.3 cm/s, $p = 0.022$; 205 ± 129.1 ml/min, $p = 0.0031$). SV showed forward flow in 37 (68.5%), to and fro in 3 (5.6%) and reversed flow in 14 patients (25.9%). The frequency of FV bleeding was significantly higher in case with reversed or "to and fro" SV flow (11/17) than forward SV flow (9/37, $p = 0.0043$). The cumulative bleeding rate at 3 and 5 years was significantly higher in patients without forward SV flow (38.8% at 3 years, 59.2% at 5 years) than in patients with forward SV flow (18.7% at 3 years, 32.2% at 5 years, $p = 0.0199$).

Conclusion: Advanced SGV blood flow and reversed SV flow direction may be a hemodynamic features closely related to the FV bleeding.

1. Introduction

Gastric fundal varices (FV) are well known as a considerable hemodynamic feature in patients with portal hypertension [1–3]. Although the incidence is less than that of esophageal varices (EV), they provide a large hemorrhage volume, resulting in a serious condition in case of bleeding [4–6]. Management of bleeding FV is actually important issue for the clinicians.

Hepatic functional reserve, size of varices, and red spots on varices by endoscopic examination were reported as significant factors for FV bleeding [5,7]. However, as these parameters are not quantitatively well defined, they are not always satisfactory in clinical practice. Although portal venous pressure (PVP) also accounts for the FV bleeding, measurement of PVP still requires an invasive procedure and patients without high PVP sometimes present developed FV or FV bleeding [8,9]. Pathophysiological difference in the FV between bleeders and non-bleeders has not been fully resolved.

Portal hypertension frequently results in the development of collateral vessels and a change in blood flow direction. Left gastric vein (LGV) supplies blood flow for EV, and its hemodynamics reflect the grade and bleeding of EV [10–12]. Similarly, gastric veins such as LGV, short gastric vein (SGV) and posterior gastric vein (PGV) are known as inflow routes for FV, and hemodynamics of these vessels might be closely related to the pathophysiology of FV [13,14]. Additionally, it is reported that reversed flow of splenic vein (SV) was frequent in patients with advanced FV accompanied by chronic portal systemic encephalopathy [13]. Blood flow in the SV might also reflect the potential development of FV.

Pulsed and color Doppler ultrasound (US) has the advantage of allowing real-time observation of the portal hemodynamics in patients with portal hypertension, repeatedly and non-invasively, compared with other imaging modalities [15–18]. With the use of

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these US techniques, we focused on the hemodynamics of the SGV and flow direction in the SV in FV patients. The purpose of our study was to elucidate the hemodynamic features of the SGV and SV on Doppler sonograms in relation to the bleeding FV.

2. Patients and methods

2.1. Patients

There were 149 consecutive patients who had medium- or large-sized FV on endoscopic examination in our department between April 1994 and December 2003. Among them, this retrospective study enrolled subjects according to the following criteria; (i) subjects received angiographic examination, that is arterial portography for the treatment of HCC or percutaneous transhepatic portography (PTP) for the portal hemodynamic evaluation, 72/149, (ii) subjects who had SGV as inflow route for FV on the angiogram, 62/72, (iii) subjects underwent Doppler US examination for the portal hemodynamic evaluation prior to treatments for FV, 59/62, (iv) subjects who had SGV on Doppler US, 54/59. Therefore, the subjects were 54 patients who consisted of 34 males and 20 females, aged 37–79 years (63.8 ± 8.2). All subjects were diagnosed as cirrhosis on the basis of imaging findings with clinical symptoms and biochemistry findings. The cause of cirrhosis was viral in 40 patients (hepatitis C virus 33, hepatitis B virus 7), alcohol abuse in 7, cryptogenic in 5 and primary biliary cirrhosis in 2. The severity of liver dysfunction as classified by the Child-Pugh scoring system was A in 18, B in 19, and C in 17. Forty-two patients had hepatocellular carcinomas (HCCs), which were controlled by non-surgical treatment. None had thrombosis or tumor thrombosis in the portal vein on both the sonogram and angiogram. The beginning of follow-up period was the time of initial Doppler US examination, and the end of that was bleeding from FV, death, changing hospital or denial to hospital visit, or the time of the latest Doppler US observation. The duration of clinical observation of FV was 18–4380 (1159 ± 1088) days in this study. The informed written consent was obtained from all patients, and the research was carried out in accordance with the Helsinki Declaration. The ethics committee in our hospital deemed this retrospective study as an appropriate design for the publication.

2.2. Endoscopy

Endoscopic findings of FV and EV were classified according to the General Rules for Recording Endoscopic Findings set by the Japan Research Society for Portal Hypertension [19]: F1 (straight), F2 (winding), and F3 (nodule-beaded), corresponding to the grades of small, medium and large, respectively. The grades of FV were F2 in 32, and F3 in 22; 12 of the FV patients were accompanied by EV (F1 in 5 and F2 in 7). Twenty FV patients were bleeders: 12 confirmed by emergency endoscopy and 6 of them received endoscopic sclerotherapy after the Doppler US examination, and 8 by clinical symptoms of hematemesis or melena. The latter patients underwent endoscopic examination within 10 days after appearance of symptoms, and other causes for gastrointestinal bleeding were not found except for FV. The other thirty-four FV patients were non-bleeders with no history of hematemesis or melena. Endoscopic examination was performed by HI, TI and TT.

2.3. US examination

The US system used in our study was SSA-260A, 270A and 390A (Toshiba, Tokyo, Japan) with a 3.75-MHz convex probe. The imaging modes were fundamental grey-scale imaging, pulsed and color Doppler US. The fundamental grey-scale imaging was used for the measurement of maximum diameter of the vessels, and Doppler US

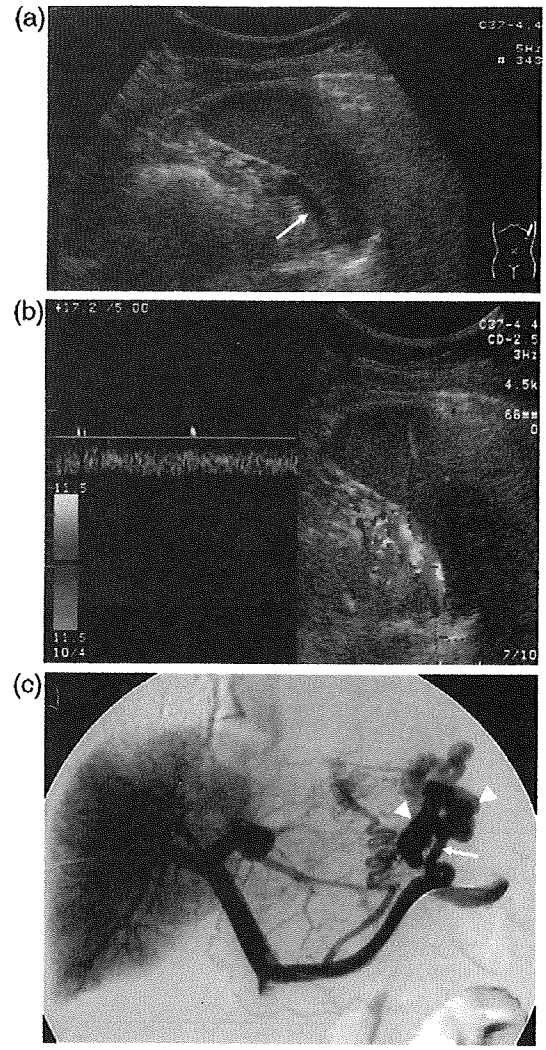

Fig. 1. 59-year-old female, cirrhotic patient with FV. (a) B-mode US by left intercostal scan. The short gastric vein (SGV, arrow) was demonstrated beside the spleen by left intercostal scan. (b) Pulsed and color Doppler US by left intercostal scan. Flow direction of SGV was hepatofugal. (c) Portogram. SGV (arrow) was observed running toward the FV (arrow heads). FV, gastric fundal varices.

was used for the demonstration of the flow direction, and measurement of mean flow velocity (cm/s) and mean flow volume (ml/min) calculated by multiplying mean flow velocity by cross-section of the vessel by 60 (s) automatically, with sampling width corresponding to the diameter of the vessel. Care was taken to ensure that the angle between the US beam and the vessel was less than 60° in this study. Color Doppler US was done with an optimal level of gain and at 60–65 dB of dynamic range, and these settings were used in all examinations. US examination was performed in a supine position in an intermediate or inspiratory phase of respiration with a fasting state of over 6 h except for the emergency setting in bleeders. The observation for SGV was performed under left intercostal scan or subcostal scan, and SGV was defined as collateral vessels originating from the splenic hilum, running along the splenic surface in a hepatofugal flow direction (Fig. 1a, b) [18,20]. The SV was observed under the transverse scan in the middle part of the upper abdomen.

The US examination was performed by two operators who had more than 5-year career for Doppler US examination at the observation of the initial case, SM for 36 patients and HM for 32 patients. Therefore, 14 patients received US examination by two operators independently, and inter-observer variability of measurement data

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Table 1
Flow direction in the SV in relation to the bleeding from FV.

	Bleeder	Non-bleeder
Forward flow	9	28
Reversed flow[a]	11	6

$p = 0.0043$ (Chi square test); SV, splenic vein; FV, gastric fundal varices.
[a] SV with "to and fro" direction in three cases, bleeder in 2 and non-bleeder in 1.

was evaluated in them, given as a coefficient of variation, calculated by dividing the standard deviation (SD) by mean and multiplying by 100. The number of US examinations of each patient was 1.9 ± 1.9 (1–13 times) during the clinical course.

2.4. Angiography

A portogram was obtained by arterial portography or PTP. The former was performed by MY by means of superior mesenteric arteriography and celiac arteriography using a 5-Fr catheter with rapid injection of iodinated contrast material (30 ml at 5 ml/s), and the latter by HM under US-guided procedure with rapid injection of iodinated contrast material (35 ml at 7 ml/s) into the splenic hilum. Arterial portography was performed in 42 patients who underwent the treatment of HCC, and PTP was done for the evaluation of portal hemodynamics in 12 patients who had neither ascites nor HCC. All of them were done before the Doppler US examination, and the presence or absence of SGV on the portograms were blindly reviewed by SM (Fig. 1c).

2.5. Statistical analysis

All results were expressed as mean ± SD or percentage. Statistical significance of differences in diameter, flow velocity and flow volume of SGV between bleeder and non-bleeder was assessed using Mann–Whitney U-test. Chi square test was used for the comparison of the SV flow direction between bleeders and non-bleeders. The cumulative bleeding rate was calculated by the Kaplan–Meier method and the difference in relation to the SV flow direction was compared by Log-rank test. Statistical analysis was performed using the Dr. SPSS package (version 11.0J for Windows; SPSS Inc., Chicago, Illinois, USA). p values of less than 0.05 were considered to indicate statistical significance.

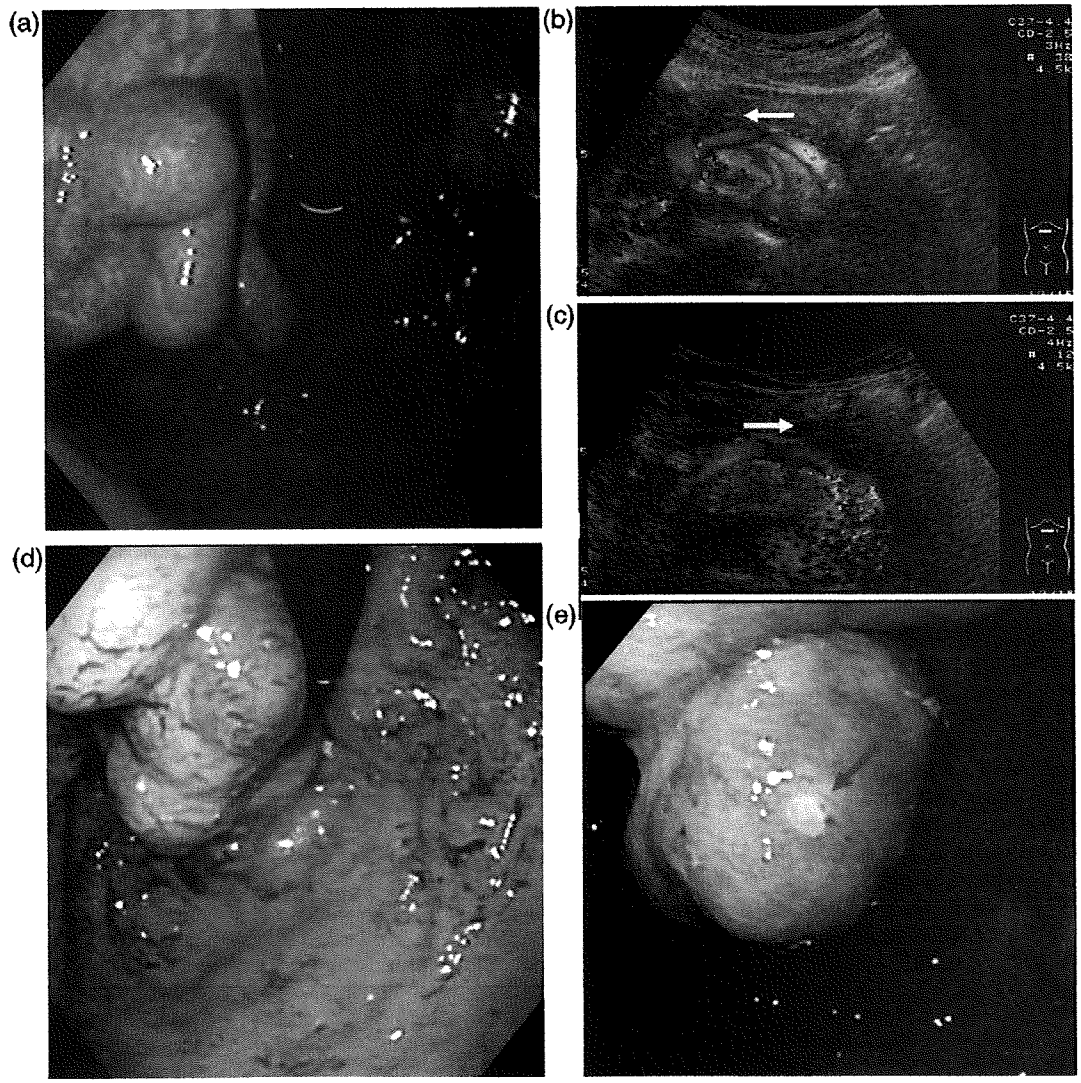

Fig. 2. 64-year-old male, cirrhotic patient with FV. (a) Endoscopic findings on July 30th, 1997. Endoscopy showed medium-sized FV. (b) Doppler US findings by transverse scan on July 30th, 1997. Flow direction of SV was forward (arrow, flow direction). (c) Doppler US findings by transverse scan on February 19th, 2001. Flow direction of SV was reversed (arrow, flow direction). (d) Endoscopic findings on March 15th, 2001. (e) Endoscopic findings on March 15th, 2001. Bleeding from gastric fundal varices (arrow: bleeding point) was noted, after 25 days of the last Doppler US examination. FV, gastric fundal varices; SV, splenic vein.

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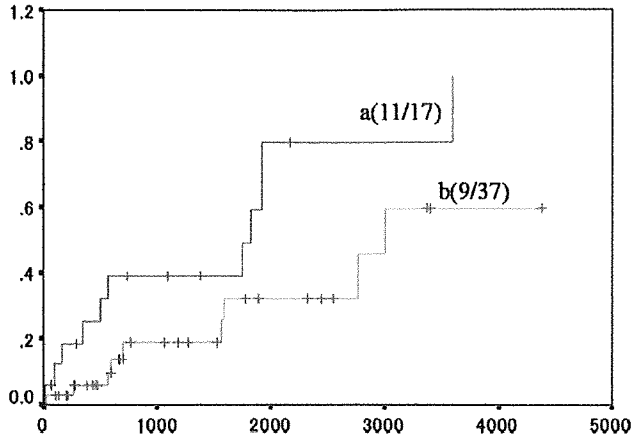

Fig. 3. Comparison of the cumulative bleeding rates of patients with reversed or "to and fro" SV flow and with forward SV flow. The cumulative bleeding rate at 3 and 5 years was significantly higher in patients with reversed or "to and fro" SV flow (38.8% at 3 years, 59.2% at 5 years) than in patients with forward SV flow (18.7% at 3 years, 32.2% at 5 years, $p = 0.0199$). a, patients with reversed or "to and fro" SV flow; b, patients with forward SV flow. SV, splenic vein.

3. Results

3.1. Hemodynamics of SGV in relation to the bleeding FV

Diameter, flow velocity and flow volume of SGV were significantly greater in bleeders (9.6 ± 3.1 mm, 11.4 ± 5.2 cm/s, 499 ± 250.1 ml/min) than non-bleeders (6.5 ± 2.2 mm, $p = 0.0141$; 7.9 ± 3.3 cm/s, $p = 0.022$; 205 ± 129.1 ml/min, $p = 0.0031$) at the latest observation. There were no significant differences of stratification of age and sex, Child-Pugh scoring, and endoscopic variceal size of FV between bleeders and non-bleeders. Inter-observer variability for the measurement results of SGV was 6.1% for diameter, 8.1% for flow velocity and 9.5% for flow volume in 14 patients.

3.2. Flow direction of SV in relation to the bleeding FV

SV had forward flow in 40 (74%), "to and fro" in 1 (1.9%) and reversed flow in 13 patients (24.1%) at the beginning of the observation. As the flow direction changed in three patients, from forward flow to "to and fro" in two patients and from forward flow to reversed flow in one patient, SV showed forward flow in 37 (68.5%), "to and fro" in 3 (5.6%) and reversed flow in 14 patients (25.9%) at the latest observation. None had reversed flow direction in the portal trunk during the clinical course. The frequency of FV bleeding was significantly higher in case with reversed or "to and fro" SV flow (11/17) than in case with forward SV flow (9/37, $p = 0.0043$, Table 1). Diameter, flow velocity and flow volume of SGV were significantly greater in patients with reversed SV flow (10.6 ± 4.4 mm, 13.1 ± 5.1 cm/s, 599 ± 388.9 ml/min) than in patients with forward SV flow (7.2 ± 2.4 mm, $p = 0.0018$; 8.8 ± 4.1 cm/s, $p < 0.0001$; 255.6 ± 171.9 ml/min, $p < 0.0001$). The cumulative bleeding rate at 3 and 5 years was significantly higher in patients with reversed or "to and fro" SV flow (38.8% at 3 years, 59.2% at 5 years) than in patients with forward SV flow (18.7% at 3 years, 32.2% at 5 years, $p = 0.0199$, Figs. 2, 3).

4. Discussion

The portal hemodynamics of patients with FV are quite different from those of patients with EV, the former being characterized by having dominant blood supply from SGV and/or PGV [11–14]. From this aspect, we investigated the blood flow in the SGV in patients with FV using Doppler US, which allowed the sufficient inter-observer variability of the measurement results. As diameter, flow velocity and flow volume of SGV were significantly greater in bleeders than in non-bleeders, it may be suggested that the hemodynamics of SGV on Doppler US were closely related to FV bleeding in patients with SGV as inflow vessel.

We also focused on another aspect, a flow direction of SV, and found that FV bleeding was significantly more frequent in patients without forward SV flow than in patients with forward SV flow. Furthermore, diameter, flow velocity and flow volume of SGV were significantly greater in reversed SV cases than in forward SV cases. Thus, reversed SV flow may reflect the presence of advanced blood supply from SGV into FV, and this may be supported by the fact that reversed SV flow was more common in patients with large gastric varices accompanied by chronic portal systemic encephalopathy [13]. Meanwhile, Kim et al. reported that the cumulative bleeding rate of FV was 16% at 1 year, 36% at 3 years, and 44% at 5 years [7], and other studies found bleeding from FV in 23.4% [5], and 25% [4]. However, the FV patients with reversed SV flow in the present study had quite a high risk of bleeding, the cumulative bleeding rate at 3 and 5 years being 38.8% and 59.2%, respectively. Therefore, reversed SV flow demonstrated by Doppler US may be a considerable risky sign for FV bleeding, and hemodynamic evaluation of SGV and SV by Doppler US might be predictive for FV bleeding.

Three patients had a "to and fro" appearance of SV, two patients of which flow direction changed from forward to "to and fro" being bleeders and the other being a non-bleeder. Although the precise mechanism for this flow direction is not clear, the authors have two hypotheses; one is that "to and fro" is a tentative flow direction in the process of change from forward SV flow to reversed SV flow, and the other is that "to and fro" is caused by fewer gradients between portal venous pressure and systemic venous pressure. The former may indicate risky sign, whereas the latter may indicate non-risky sign, though we could not conclude at this time. Further studies should be planed to examine the relationship between SV flow direction and portal venous pressure. Moreover, long-term follow-up with larger numbers of patients might clarify the clinical meaning of this "to and fro" appearance of SV blood flow.

There were three cases of which SV flow direction changed in the clinical course, from forward flow to "to and fro" or reversed flow. It should be noted that one of them bled 25 days after the observation of blood flow change on Doppler US. Repeated observation of portal hemodynamics is the advantage of Doppler US method, and our results suggest that change of flow direction in the SV might also be a risky sign for FV bleeding. An appropriate interval for US examination should be established to practice careful follow-up such FV patients.

Gastrorenal shunt is known as a major drainage pathway from FV and recent study has shown that its hemodynamics may reflect the grading and bleeding FV [13,21]. In fact, blood flow in the FV may be aggregated in this gastrorenal shunt because some FV have multiple inflow routes other than SGV. Our results based on the observation of SGV blood flow alone may be insufficient for the clinical management of all FV. Meanwhile, gastrorenal shunt might include blood flow derived from normal gastric wall other than FV. Further studies may be necessary to measure inflow routes against outflow routes as the preferable parameter reflecting FV bleeding.

There are certain limitations to the present study. First, our results were based on a retrospective study, and the interval of US examinations was not strictly defined in each patient. Practical role of the hemodynamic evaluation of SGV and SV on Doppler US for the management of FV bleeding should be elucidated in a prospective study. Next, our data did not include small-sized FV, because they might have low risk of bleeding. However, changes in the hemodynamics of SGV during the long-term clinical course of small-sized FV would also be an interesting aspect deserving attention in such a study. Third, our results lacked the information of the

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intra-observer variability for measurement results in the SGV. Correspondence of the data of the different date in all subjects may not make sense, because this study had some emergent cases that might have dynamic changes in hemodynamics. However, this variance should be examined in the further studies.

In conclusion, advanced SGV blood flow and reversed SV flow direction may be a hemodynamic features closely related to the FV bleeding. Although, prospective study with a larger population of FV patient may be needed to confirm the clinical significance of our method, evaluation of the hemodynamics in the SGV and SV by Doppler US might be useful for predicting FV bleeding.

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CLINICAL STUDIES

Phylogenetic analysis of hepatitis A virus in sera from patients with hepatitis A of various severities

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Keywords
2B – 2C – 5′NTR – fulminant hepatitis – hepatitis A – hepatitis A virus

Abbreviations
AH, acute hepatitis; AHs, acute hepatitis severe type; FH, fulminant hepatitis; HAV, hepatitis A virus; PT, prothrombin time.

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Abstract

Background: We analysed the association of the 5′ nontranslated region (5′NTR), nonstructural proteins 2B and 2C of the hepatitis A virus (HAV) genome, whose mutations have previously been shown to be important for enhanced replication in cell culture systems, in order to align all our data and examine whether genomic differences in HAV are responsible for the range of clinical severities. *Methods:* Our accumulated HAV strains of 5′NTR [nucleotide(nt) 200 and 500], entire 2B and 2C from 25 Japanese patients with sporadic hepatitis A, consisting of seven patients with fulminant hepatitis (FH), five with severe acute hepatitis (AHs) and 13 with self-limited acute hepatitis (AH), in whom the sequences of all three regions were available, were subjected to phylogenetic analysis. *Results:* Fulminant hepatitis patients had fewer nucleotide substitutions in 5′NTR, had a tendency to have more amino acid (aa) substitutions in 2B and had fewer aa substitutions in 2C than AH patients. Four FH and two AHs with a higher viral replication were located in the near parts of the phylogenetic trees, indicating the association between the severity of hepatitis A and genomic variations in 5′NTR, 2B and 2C of HAV. *Conclusions:* Our study suggests that genetic variations in HAV not in one specific region but in 5′NTR, 2B and 2C might cooperatively influence replication of the virus, and thereby affect virulence. Viral factors should be considered and examined when discussing the mechanisms responsible for the severity of hepatitis A.

Hepatitis A is still a major problem worldwide, not only in underdeveloped countries but also in industrialized nations. Because of improvements in sanitation, there have been no hepatitis A epidemics in Japan in recent years. However, sporadic cases of hepatitis A have not been rare of late. Although the majority of hepatitis A cases are self-limited acute hepatitis (AH), some develop into severe forms of hepatitis (1). In fact, in the past several years, there has been an increase in the numbers of patients with sporadic hepatitis A, especially the more severe kind, visiting our hospital. Our analysis of the possible factors responsible for the disease severity in our patients revealed no significant differences in terms of background including age, suggesting that viral factors might be involved in determining the severity of the disease (2, 3).

Hepatitis A virus (HAV) is the sole member of the hepatovirus genus and a member of the Picornavirus family. Virological studies have revealed that HAV is a positive-strand RNA virus comprising approximately 7500 nucleotides and containing a 5′ nontranslated region (NTR), a single long open reading frame encoding a large polyprotein and a 3′NTR. A large polyprotein is cleaved by the viral protease to produce the P1, P2 and P3 regions. The P1 region encodes four structural proteins – VP4, VP2, VP3 and VP1. The P2 and P3 regions encode nonstructural proteins 2A, 2B and 2C, and 3A, 3B, 3C and 3D respectively (4). As far as is known, nonstructural protein 2A participates in virion morphogenesis (5). 2B and 2C play important roles in the replication of viral RNA. 2C is a multifunctional protein and is considered to have helicase and NTPase activities. 2C or 2BC have membrane- and RNA-binding properties (6). 3B is considered to be a genome-linked viral protein (Vpg), 3A a pre-Vpg, 3C a viral protease and 3D an RNA-dependent RNA polymerase.

It was reported that the strains adapted to cell culture systems have mutations in 5′NTR and the P2 region of HAV (7, 8). Zhang *et al.* (9) reported that rapidly replicating, cytopathic variants of HAV isolated from cultured cells required mutations within 5′NTR, 2B and 2C, and these mutations acted cooperatively. Raychaudhuri *et al.* (10) reported that the simian virus 2C gene could confer the phenotype of virulence to an otherwise attenuated virus, and clusters of residues near both ends of the 2C protein were required for virulence using chimeras between human and simian strains of hepatitis A virus in tamarins.

Despite advances in the understanding of HAV, a correlation between the HAV genome and the clinical status of hepatitis A has not been established. Durst *et al.* (11) reported a cluster of fulminant hepatitis A, in which the severity of the infection in three siblings was related to the virulence of HAV. To examine the possibility of differences in hepatitis A viruses in terms of the different categories of hepatitis, we have analysed the viral genomes in sera from hepatitis A patients with a variety of clinicopathological features and reported the associations between some viral regions and clinical severities (3, 12–18).

When analysing the viral genome, rather than focusing on one specific region, perhaps several portions of the HAV genome should be investigated. In the present study, we examined the clinicopathological features of hepatitis A and possible correlations with variations in the three regions of 5′NTR, 2B and 2C of the HAV genome, whose mutations have previously been shown to be important for enhanced replication and virulence in cell culture systems and simians, in the same patients using phylogenetic analysis.

Materials and methods

Patients

Serum samples from 25 patients with hepatitis A in Japan were collected between 1986 and 1999 and stored at −20 °C until analysis. Informed consent was obtained from the patients or appropriate family members. These patients were diagnosed based on the positivity of the IgM antibody to HAV (IgM anti-HAV) in conjunction with compatible symptoms and laboratory findings.

Among the patients seven had fulminant hepatitis (FH), five had severe acute hepatitis (AHs) and 13 had self-limited AH. Patients with a prothrombin time < 40% of control were defined as AHs, and those with hepatic encephalopathy as FH. Patients with significant increases in serum blood urea nitrogen and creatinine (more than three times the upper level of the normal range) were judged to be undergoing acute renal failure. The patients were also investigated for histories of recent exposure to drugs and chemical agents as well as heavy alcohol consumption (> 50 g/day for > 5 years).

None of the patients had clinical or laboratory evidence of acquired immune deficiency syndrome.

Serological markers

IgM antibody to HBc(IgM anti-HBc), HBsAg and second-generation antibody to hepatitis C virus (HCV) were examined in all cases. IgM anti-HAV, IgM anti-HBc and HBsAg were measured by commercial radioimmunoassay kits (Abbott Laboratories, Chicago, IL, USA); second-generation HCV antibody was measured by the enzyme immunoassay kit (Ortho Diagnostics, Tokyo, Japan). In the FH and AHs patients, HCV RNA, IgM antibody to Epstein–Barr virus (IgM anti-EBV), IgM antibody to herpes simplex virus (IgM anti-HSV), IgM antibody to cytomegalovirus (IgM anti-CMV), anti-smooth muscle antibody, liver kidney microsomal antibody-1 and anti-mitochondrial antibody were also examined. HCV RNA was measured by nested reverse transcriptase-polymerase chain reaction (RT-PCR) as described by the authors (19). IgM anti-EBV, IgM anti-CMV and IgM anti-HSV were examined by enzyme-linked immunosorbent assays. Anti-nuclear antibody, anti-smooth muscle antibody, anti-mitochondrial antibody and anti-liver kidney microsomal-1 antibody were examined by the fluorescent antibody method.

Quantification of hepatitis A virus RNA by real-time reverse transcriptase-polymerase chain reaction

Serum viral RNA was extracted by the High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany). RT-PCR was carried out with a Hepatitis A Virus Quantification Kit (Roche Diagnostics) according to the manufacturer's instructions. Twenty microliters of the PCR mixture contained 15 μl of master mix from the kit and 5 μl of template RNA. The standards of HAV RNA are supplied with this kit. All reactions were performed in a LightCycler (Roche Diagnostics). The C_T values from clinical samples were plotted on the standard curve, and the number of copies wes calculated automatically. This method has a dynamic range of HAV RNA quantification between 0.5 and 5×10^6 copies/μl.

Amplification of serum hepatitis A virus RNA and direct sequencing

Hepatitis A virus RNA was examined by nested RT-PCR and direct sequencing as described previously (14, 17, 18).

Nucleotide sequence accession numbers

The nucleotide sequence data reported herein appear in DDBJ/EMBL/GenBank nucleotide sequence databases with the following accession numbers:

5′NTR

AB045327 for A1, AB045336 for A5, AB045330 for A204, AB045331 for A205, AB045332 for A206, AB045334 for A414, AB045338 for A601, AB045342 for A159, AB045344 for A160, AB045345 for A161, AB045350 for A302, AB045353 for A811, AB045672 for A7, AB045692

for A9, AB045568 for A20, AB045671 for A68, AB045680 for A75, AB045681 for A77, AB045366 for A162, AB045572 for A303, AB045646 for A304, AB045648 for A306, AB045649 for A307, AB045678 for A712 and AB045679 for A713.

2B

AB047652 for A1, AB047671 for A5, AB047660 for A204, AB047661 for A205, AB047662 for A206, AB047669 for A414, AB047673 for A601, AB047654 for A159, AB047655 for A160, AB047656 for A161, AB047663 for A302, AB047680 for A811, AB047675 for A7, AB047681 for A9, AB047658 for A20, AB047674 for A68, AB047678 for A75, AB047679 for A77, AB047657 for A162, AB047664 for A303, AB047665 for A304, AB047666 for A306, AB047667 for A307, AB047676 for A712 and AB047677 for A713.

2C

AB082174 for A1, AB082130 for A5, AB0821323 for A204, AB082133 for A205, AB082134 for A206, A082135 for A414, AB082137 for A601, AB082139 for A159, AB082140 for A160, AB082141 for A161, AB082145 for A302, AB082147 for A811, AB082148 for A7, AB082149 for A9, AB082150 for A20, AB082154 for A68, AB082155 for A75, AB082156 for A77, AB082160 for A162, AB082165 for A303, AB082166 for A304, AB082167 for A306, AB082168 for A307, AB082171 for A712 and AB082172 for A713.

Phylogenetic analysis

To determine the heterogeneity of the viral sequences obtained from the 25 patients, a phylogenetic tree was constructed by the neighbour-joining method. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1000 times. These analyses were conducted using a computer program, GENETYX-MAC version 10.1 (Software Development, Tokyo, Japan).

Statistical analysis

Differences in proportions among the groups were compared by Fisher's exact probability test, Student's *t*-test and Welch's *t*-test (DA STATS version 1.0, Nagata O, Tokyo, Japan).

Results

Clinicopathological characteristics of the patients

The characteristics of the 25 patients with hepatitis A analysed for HAV 5′NTR, 2B and 2C at admission are summarized in Table 1. None of the cases was associated with an epidemic.

Differences in the mean age, sex and presence of chronic liver disease among FH, AHs and AH, and between FH+AHs and AH, were not statistically significant. Serum was sampled 2–17 days after clinical onset. The mean ALT level was higher in AHs than that in AH ($P = 0.002$), and in FH+AHs than that in AH ($P = 0.003$). The mean prothrombin time was prolonged in FH compared with AHs ($P = 0.002$), FH compared with AH ($P < 0.001$), AHs compared with AH ($P < 0.001$) and FH+AHs compared with AH ($P < 0.001$). The mean total bilirubin level was higher in FH than that in AHs ($P = 0.049$).

Table 1. Characteristics of patients

	FH	AHs	AH
n	7	5	13
CLD	1†	1†	3†
Recovery/death	3/4‡	5/0‡	13/0‡
Sex (M/F)	3/4†	5/0†	7/6†
Age*	44.1 ± 13.5†	36.8 ± 12.9†	39.5 ± 9.1†
PT (%)*	16 ± 7§	34 ± 8§	63 ± 20§
ALT (IU/L)*	6337 ± 3838¶	6165 ± 1718¶	2873 ± 1733¶
T-Bil (mg/dl)*	9.4 ± 7.6‖	2.3 ± 0.8‖	5.0 ± 2.3‖

*Mean ± SD.

†Statistically not significant.

‡Statistically significant between FH and AH ($P = 0.007$) by Fisher's exact probability test.

§Statistically significant between FH and AHs ($P = 0.002$) by Student's *t*-test, FH and AH ($P < 0.001$) by Welch's *t*-test, and AHs and AH ($P < 0.001$) by Welch's *t*-test.

¶Statistically significant between AHs and AH ($P = 0.002$) by Student's *t*-test.

‖Statistically significant between AHs and FH ($P = 0.049$) and AHs and AH ($P = 0.002$) by Welch's *t*-test.

AH, acute hepatitis; AHs, severe acute hepatitis; ALT, alanine aminotransferase; CLD, chronic liver disease; FH, fulminant hepatitis; PT, prothrombin time; T-Bil, total bilirubin.

Four of seven patients with FH died of hepatic failure, and all patients with AHs and AH recovered ($P = 0.007$). All seven FH cases needed artificial liver support (plasma exchange and haemodiafiltration). Four (16%) patients – two (28%) with FH and two (15%) with AH – had acute renal failure and were treated by haemodiafiltration.

Two patients with AH were positive for HBsAg and antibody to HBe, and one patient with AH was positive for anti-nuclear antibody, but they showed a typical hepatitis A course. IgM anti-EBV, IgM anti-HSV, IgM anti-CMV, anti-nuclear antibody, anti-smooth muscle antibody, liver kidney microsomal antibody-1 and anti-mitochondrial antibody were negative in all examined cases of FH and AHs. One FH patient and one AHs patient had histories of heavy alcohol consumption. One male patient with AH was homosexual.

Histological examination was performed in all seven FH cases, two of five AHs cases and seven of 13 AH cases in the convalescent phase or postmortem. In the FH cases, liver histology revealed massive necrosis in three patients and submassive necrosis in one. Liver histology in the two patients with histories of heavy alcohol consumption showed pericellular fibrosis, consistent with alcoholic liver disease. The histological findings of the other cases showed AH to be in a residual phase or subsiding.

Phylogenetic analysis

The results of phylogenetic analysis are shown in Figures 1 and 2. Four FH (A204, A601, A414 and A1) and two AHs (A160 and A159) were located in the near parts of the phylogenetic trees (Fig. 2).

The clinical backgrounds, and the biochemical and viral characteristics are shown in Table 2. As described above, none of them were associated with an epidemic. Two of the FH patients died and the others recovered. HAV RNA was quantified by real-time RT-PCR in five of these six patients. Our other recent study of HAV RNA quantification revealed that the mean viral load in > 60 AH at admission was 2.75 ± 1.55 log copies/ml (20), and so these five patients had comparatively higher viral loads (4.35 ± 0.81 log copies/ml) ($P = 0.03$). The HAV genotype was IA in all patients, similar to the majority of Japanese patients in general.

Discussion

Although the severity of hepatitis A varies, it is not clear why it is more severe in some patients than that in others. It is thought that disease severity may be dependent on certain characteristics of the individual patients. It has been reported that ageing and underlying chronic liver disease could be factors that increase hepatitis A severity (21). Vento *et al.* (22) reported that patients with chronic hepatitis C had a substantial risk of FH and death associated with HAV superinfection.

During an urban epidemic in the US, it was described that hepatitis A caused serious illness and death and that complications were more frequent in patients 40 years of age and older, but that young healthy persons were also at risk for severe complications (23). A cluster of fulminant hepatitis A was reported, relating the severity of the infection in three siblings to the virulence of HAV, as the patients were all healthy before the infection and their illness followed a similar course (11).

In the past several years, increasing numbers of patients with sporadic hepatitis A, especially the more severe forms, have visited our hospital, but our analysis of factors possibly contributing to the severity of the disease failed to reveal any significant differences in patient characteristics including age (2, 3), suggesting that viral factors might determine the severity of the disease. To identify possible differences in hepatitis A viruses for different types of hepatitis, we analysed the HAV genome in sera from hepatitis A patients with various clinicopathological features. Our analysis of whole HAV genomes from three cases of FH and three cases of AH indicated possible associations between the severity of hepatitis A and the nucleotide substitutions in 5′NTR and the amino acid (aa) substitutions in 2B, although there were no unique nucleotide or aa substitutions. On the other hand, it was reported that mutations in 5′NTR, 2B and 2C of HAV were associated with cytopathic variants in cultured cells, and virulence in tamarins, as described above (9, 10). These various observations led us to analyse these three regions of HAV in greater numbers of clinical samples (14, 17, 18).

In our analysis of 5′NTR, FH and AHs patients had fewer nucleotide substitutions than AH in the central part of 5′NTR ($P < 0.001$) (14). Several regions of 5′NTR, including the pyrimidine-rich tract and internal ribosomal entry site, have been examined for possible correlations with replication of HAV RNA *in vitro*, and it has been reported that HAV strains adapted to cell culture systems have mutations in 5′NTR and the P2 region (8), and mutations in 5′NTR significantly enhanced growth of the virus in a cell culture system (24). Thus, nucleotide variations in 5′NTR may influence replication of the virus and thereby affect virulence.

In 2B, there seemed to be more mutations in the strains obtained from FH and AHs patients than in those obtained from AH patients in the central part (18). On the basis of cell culture studies, substitutions in the sequence of 2B protein have been suggested to be associated with the replication capability of the virus. One nucleotide substitution at nt 3889 in 2B, which changed Ala to Val in 2B-216, is responsible for differences in the growth rate of the virus along with the nucleotide substitutions in 2C and/or 5′NTR (25, 26). A substitution at the same nt 3889 appeared from the early stage of replication enhancement in cultured cells, and several HAV strains showed a cytopathic effect (8). An Ala-to-Val substitution in 2B-216 was not observed in our study.

In 2C, FH patients had fewer aa substitutions than AH patients ($P < 0.05$) (17). This indicates that viruses with fewer aa substitutions in 2C may be more virulent in comparison with strains with more aa substitutions. 2C is a multifunctional protein and is involved in replication of the viral genome. Analysis of the primary aa sequence of 2C shows homology with a family of proteins that contains a nucleoside triphosphate (NTP)-binding motif. This motif consists of elements 'A' and 'B'. The residues mutated within the conserved A and B sites of the NTP-binding motif are critical in RNA replication and virus proliferation (27). Elements A and B were conserved in all patients except one of FH. 2C is also suggested to be involved in the rearrangement of cellular membranes (28). The simian HAV 2C gene was reported to be required for virulence in tamarins (10). Thus, subtle substitutions in 2C might influence the replication capability of the virus and thereby affect virulence. We could not find specific nucleotide or aa substitutions in any of the regions.

In the present study, patients with FH had fewer nt substitutions in 5′NTR, and had a tendency to have more aa substitutions in 2B, and fewer aa substitutions in 2C, than patients with AH, and four FH and two AHs were located in the near parts of the phylogenetic trees, indicating the association between severity of hepatitis A and genomic variations in 5′NTR, 2B and 2C of HAV. In these patients, HAV load was higher than that of AH

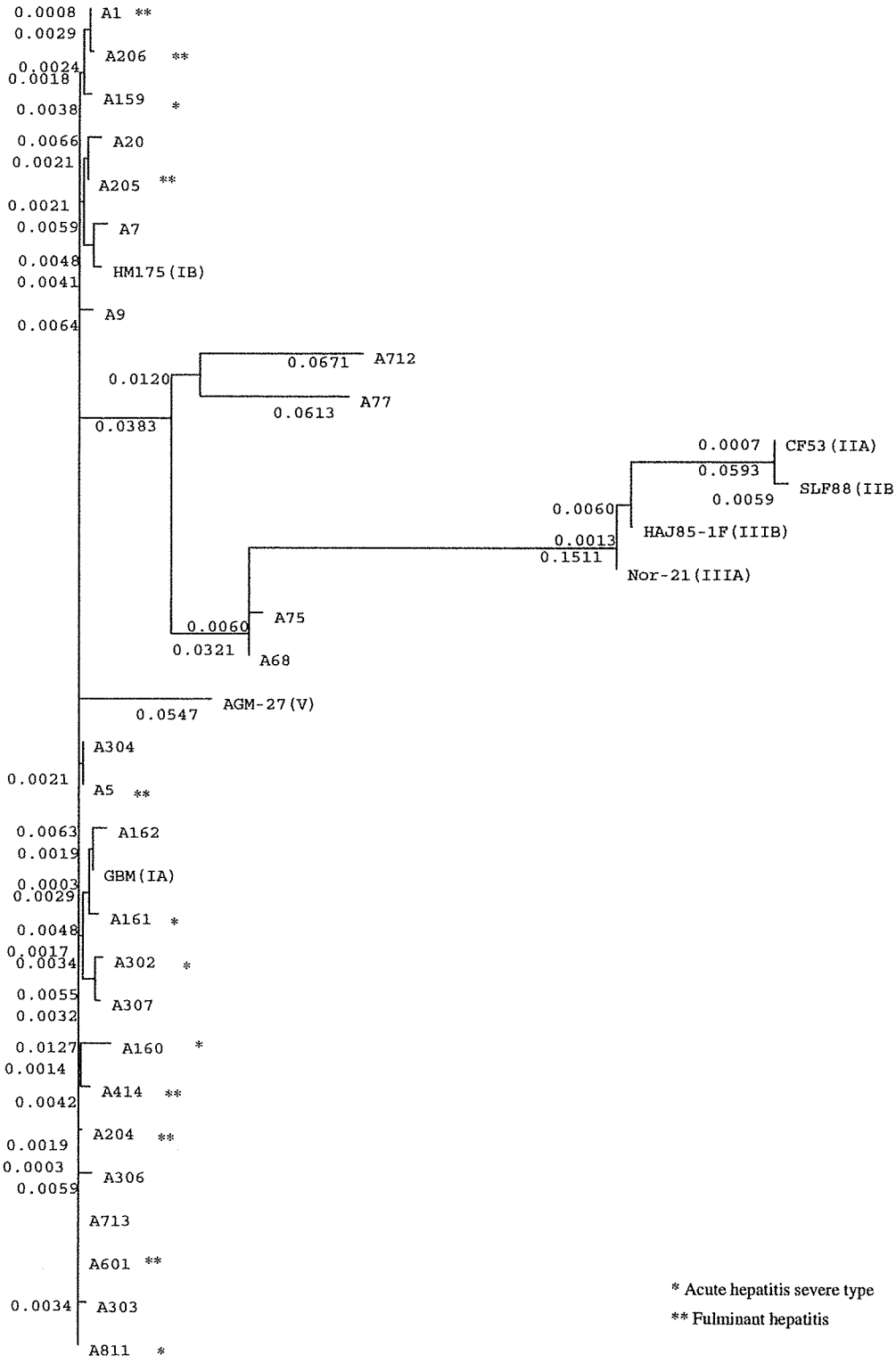

Fig. 1. Genetic relatedness between individual hepatitis A virus (HAV) strains between nucleotides 200 and 500 of the 5′ nontranslated region recovered from 25 patients and HAV reference strains GBM (subgenotype IA), HM175 (subgenotype IB), CF53 (subgenotype IIA), SLF88 (subgenotype IIB), Nor-21 (subgenotype IIIA), HAJ85-1F (subgenotype IIIB) and AGM27 (genotype V). Numbers beside the phylogenetic roots are the results of bootstrap analyses.

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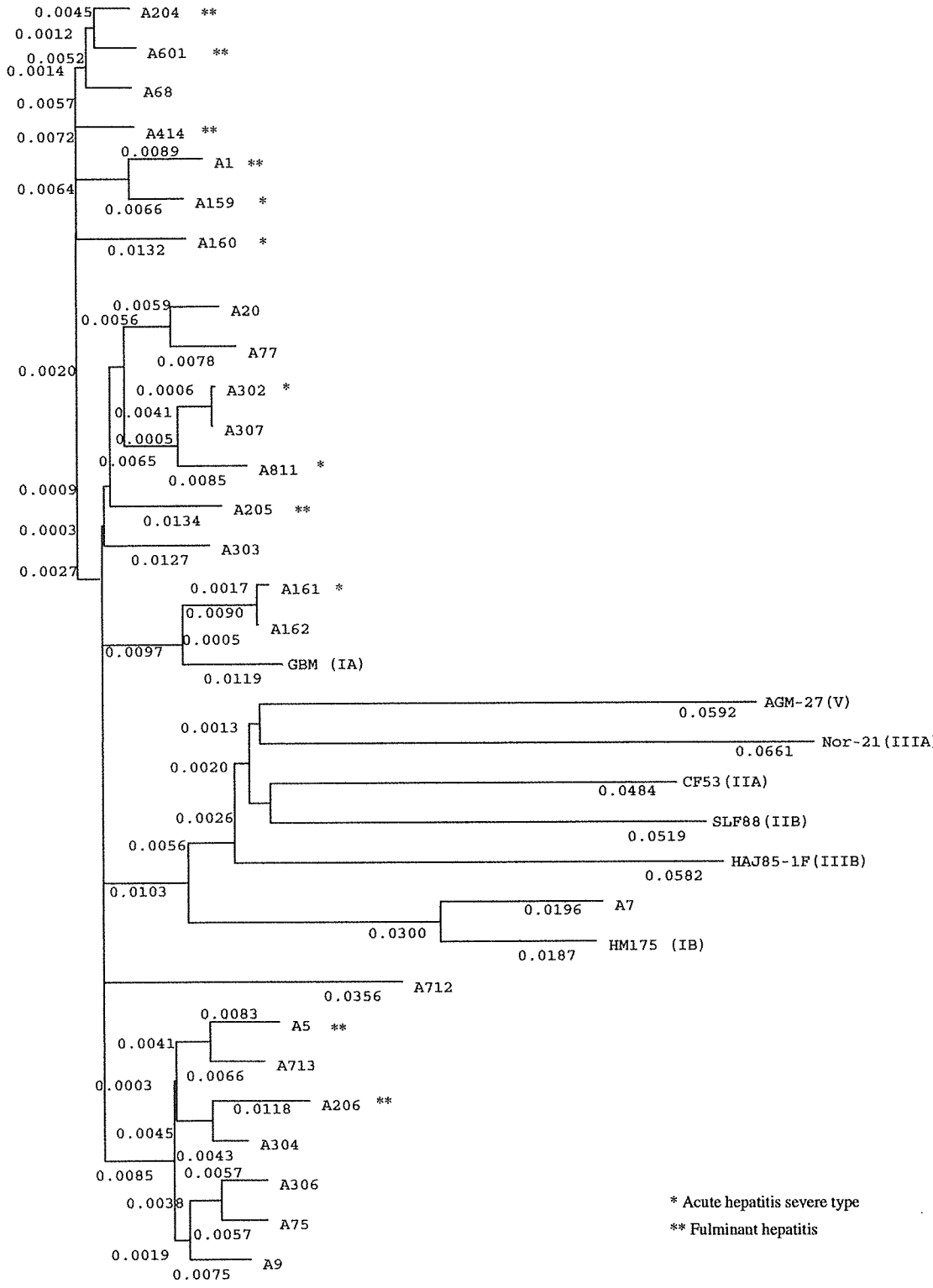

Fig. 2. Genetic relatedness between individual hepatitis A virus (HAV) of entire 2B and 2C recovered from 25 patients and HAV reference strains GBM (subgenotype IA), HM175 (subgenotype IB), CF53 (subgenotype IIA), SLF88 (subgenotype IIB), Nor-21 (subgenotype IIIA), HAJ85-1F (subgenotype IIIB) and AGM27 (genotype V). Numbers beside the phylogenetic roots are the results of bootstrap analyses.

Table 2. Clinical, biochemical and viral characteristics of six patients with fulminant and severe hepatitis located in the near parts of the phylogenetic tree

Patient	Diagnosis	Age/sex	Origin	Onset	Outcome	ALT (IU/L)	T-Bil (mg/dl)	PT (%)	IgM-HA (cut-off index)	Viral load (log copies/ml)	Days from onset
A204	FH	39/F	Tohoku	February 1990	Death	4470	5.3	10	3.8	ND	3
A601	FH	64/F	Shinetsu	January 1997	Death	12 500	7.0	13	2.9	3.7	9
A414	FH	49/M	Shinetsu	January 1989	Recovery	5276	26.3	13	+	5.0	7
A160	AHs	39/M	Kanto	June 1998	Recovery	9164	1.6	38	3.1	5.1	4
A1	FH	29/M	Kanto	March 1992	Recovery	1175	7.3	17	5.1	3.3	6
A159	AHs	50/M	Kanto	May 1998	Recovery	5655	2.5	20	4.6	4.6	5

Patient	Genotype	5′ NTR homology (%)	2B nt homology (%)	2C nt homology (%)
A204	IA	99.0	93.8	90.0
A601	IA	99.3	94.3	89.3
A414	IA	98.7	95.0	88.6
A160	IA	97.7	95.2	88.3
A1	IA	98.7	96.0	88.5
A159	IA	98.7	96.9	88.8

Homology, sequences were compared with wild-type HAV genotype IA strain GBM.
AH, acute hepatitis; AHs, acute hepatitis severe type; ALT, alanine aminotransferase; FH, fulminant hepatitis; 5′NTR, 5′-nontranslated region; ND, not done; nt, nucleotide; Ti-Bil, total bilirubin.

patients. Rezende *et al.* (29) reported that HAV-related liver failure is because of an excessive host response associated with a marked reduction in viral load, and there is a discrepancy between their data and ours. But they did not show the time points of serum sampling that represent critical data about viraemia in AH, and so we cannot discuss the discrepancy.

Thus, genetic variations not in one specific region but in 5′NTR, 2B and 2C might cooperatively influence replication of the virus and thereby affect virulence. Our findings are in accordance with the basic reports that the pathogenicity of HAV could be related to cooperative mutations within 5′NTR and P2 in cultured cells and simians, and the clinical finding that there has been only one report about a cluster of fulminant hepatitis A, unlike the many reports of clusters of fulminant hepatitis B.

Our current study suggests that both viral and host factors should be considered and examined when discussing the mechanisms responsible for the severity of hepatitis A. Further, we should examine several portions of the HAV genome including 5′NTR, 2B and 2C rather than focus on one specific region when analysing viral factors. Our study also suggests that vaccination should be considered all the more if HAV itself is involved in the pathogenicity of hepatitis A, because safe and extremely effective inactivated HAV vaccines are available.

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CLINICAL STUDIES

Ultrasound-guided treatments under low acoustic power contrast harmonic imaging for hepatocellular carcinomas undetected by B-mode ultrasonography

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contrast agent – hepatocellular carcinoma – liver – Sonazoid™ – ultrasound

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Abstract

Background/Aims: The aim was to examine the efficacy of contrast-enhanced ultrasound (US) with Sonazoid™ to demonstrate ultrasonically unrecognizable hypervascular hepatocellular carcinoma (HCC) and apply percutaneous US-guided treatments. *Methods:* The subjects of this prospective study were 44 cirrhotic patients with 55 hypervascular lesions (12.7 ± 4.5 mm) found by contrast-enhanced computed tomography but unrecognized by non-contrast US. Contrast-enhanced US was performed to demonstrate these hepatic lesions after an intravenous injection of Sonazoid™ (0.0075 ml/kg). The sonograms in both the early phase (for 1 min after injection) and the late phase (5–10 min after) were taken in the harmonic imaging mode under a low mechanical index (0.24–0.3). *Results:* Fifty-three lesions were demonstrated by contrast-enhanced US, 52 with positive enhancement in the early phase and 44 with negative enhancement in the late phase. Percutaneous US-guided treatments were successfully performed for 42 lesions (ethanol injection in 20 and radiofrequency ablation in 22) in 32 patients with reference to contrast-enhanced US findings. Six patients were treated by transarterial chemoembolization alone because they had more than three lesions in the liver. In the remaining seven lesions in six patients, six were diagnosed as non-HCC lesions: five with vascular abnormalities such as arterioportal or arteriovenous communication and the other one with benign lesion in alcoholic liver disease. These six lesions and one HCC lesion with severe liver damage were followed up without any treatment. *Conclusions:* As the detectability of ultrasonically unrecognizable hypervascular HCC improved by contrast-enhanced US with Sonazoid™, a wider application of percutaneous US-guided treatments may be possible.

Hepatocellular carcinoma (HCC) is increasing worldwide and is one of the most common carcinomas in the eastern part of Asia (1). As the prognosis of cirrhotic patients depends on the occurrence and progression of HCC, management of this neoplasm is a major issue in clinical practice. However, surgical treatment is not always an appropriate choice, as the majority of HCC patients have poor liver function and recurrence is not rare (2–4).

Real-time observation is the most significant point to be emphasized in the clinical use of ultrasound (US), and the percutaneous US-guided technique is a reasonable procedure for the treatment of HCC with minimal invasiveness (5–9). Although these methods require demonstration of focal hepatic lesions by US, this is not always easy because of deformity and/or coarse parenchymal echo in cirrhotic liver, and modified echo patterns as a result of previous treatments (10, 11). Contrast-enhanced US with Levovist facilitated the application of percutaneous US-guided treatments by successful localization in about 75% of ultrasonically invisible hypervascular HCCs (12). However, the utility of second-generation microbubble contrast agents for the localization of such focal hepatic lesions has not been established.

Second-generation microbubble contrast agents have acquired stability of microbubble by homogenization of particle size distribution in comparison with earlier agents (13, 14). The combination of second-generation contrast agents with harmonic imaging mode under lower mechanical index (MI) may provide US images with an improved signal-to-noise ratio, and a higher detection rate of focal lesions in the liver is expected (15, 16).

Sonazoid™ is a newly developed perflubutane US contrast agent (17–19). The microbubble has the characteristic property of accumulation in the Kupffer cell, and it is the largest difference between this agent and SonoVue, a popular agent in Europe. Application of Sonazoid-enhanced sonograms produced by accumulated microbubble as well as circulating microbubble may contribute greatly to the detection of focal hepatic lesions. With this background, the present study was designed to examine the efficacy of contrast-enhanced US with the new perflubutane microbubble agent Sonazoid™ in the visualization of ultrasonically unrecognized hepatic lesions that had a hypervascular appearance on contrast-enhanced computed tomography (CT), for the application of percutaneous US-guided treatments in cirrhotic patients.

Material and methods

Patients

Between February 2007 and May 2008, a prospective study was performed to examine the efficacy of contrast-enhanced US with Sonazoid™ (GE Healthcare, Oslo, Norway) to