Table 4 Factors contributing to survival after HCC development

	Univariate analysis		Multivariate analysis	
	RR (95% CI)	*P* value	RR (95% CI)	*P* value
Interferon-related variables				
Application of interferon therapy	3.24 (1.52–11.0)	<0.01*	2.72 (1.29–9.04)	<0.01*
Response to interferon therapy (SVR vs. non-SVR + non-IFN)	10.5 (2.33–121)	<0.01*	–	
Variables at the first treatment of HCC				
Age (<60 years)	0.59 (0.29–1.32)	0.19		
T.Bil (<1.0 mg/dl)	2.68 (1.45–5.02)	<0.01*	1.69 (0.87–3.31)	0.11
ALB (≧3.5 g/dl)	3.45 (1.86–6.55)	<0.01*	2.51 (1.29–4.98)	<0.01*
ALT (<80 IU/l)	0.74 (0.35–1.45)	0.40		
PT (≧70%)	1.48 (0.63–3.06)	0.33		
PLT (≧10 × 10^4/mm^3)	1.63 (0.88–3.07)	0.11		
AFP (<100 ng/ml)	1.42 (0.66–2.81)	0.34		
DCP (<40 mAU/ml)	1.06 (0.56–1.99)	0.84		
Maximum tumor size (<30 mm)	1.48 (0.70–2.87)	0.28		
Number of tumors (single)	0.98 (0.45–1.94)	0.97		

RR risk ratio, *CI* confidence interval, *IFN* interferon, *PEG-IFN* pegylated interferon, *HCV* hepatitis C virus, *HCC* hepatocellular carcinoma, *SVR* sustained virological response, *ALB* albumin, *T.Bil* total bilirubin, *ALT* alanine aminotransferase, *PLT* platelet, *PT* prothrombin time, *AFP* alpha-fetoprotein, *DCP* des-γ-carboxy prothrombin

* *P* values less than 0.05 were considered statistically significant

Table 5 Risk factors contributing to first recurrence of hepatocellular carcinoma (HCC)

	Univariate analysis		Multivariate analysis	
	RR (95% CI)	*P* value	RR (95% CI)	*P* value
Interferon-related variables				
Application of interferon therapy	1.31 (0.97–1.84)	0.07		
Response to interferon therapy (non-SVR + non-IFN vs. SVR)	1.92 (1.01–4.15)	0.04*	1.60 (0.83–3.48)	0.16
Variables at the first treatment of HCC				
Age (≧60 years)	1.29 (0.76–2.37)	0.35		
T.Bil (≧1.0 mg/dl)	1.15 (0.75–1.72)	0.50		
ALB (<3.5 g/dl)	1.55 (1.03–2.29)	0.03*	1.70 (1.11–2.56)	0.01*
ALT (≧80 IU/l)	0.97 (0.63–1.46)	0.91		
PT (<70%)	0.74 (0.41–1.27)	0.30		
PLT (<10 × 10^4/mm^3)	1.26 (0.85–1.85)	0.23		
AFP (≧100 ng/ml)	1.50 (0.91–2.36)	0.11		
DCP (≧40 mAU/ml)	1.45 (0.97–2.17)	0.06		
Maximum tumor size (≧30 mm)	1.71 (1.07–2.65)	0.02*	1.65 (1.02–2.59)	0.04*
Number of tumors (multiple)	1.60 (1.02–2.43)	0.03*	1.66 (1.05–2.56)	0.02*

RR risk ratio, *CI* confidence interval, *IFN* interferon, *PEG-IFN* pegylated interferon, *HCV* hepatitis C virus, *HCC* hepatocellular carcinoma, *SVR* sustained virological response, *ALB* albumin, *T.Bil* total bilirubin, *ALT* alanine aminotransferase, *PLT* platelet, *PT* prothrombin time, *AFP* alpha-fetoprotein, *DCP* des-γ-carboxy prothrombin

* *P* values less than 0.05 were considered statistically significant

However, there have been few trials involving PEG-IFN therapy.

In this study, the overall survival rate of PEG-IFN-treated patients was higher than that of non-IFN patients, and the HCC recurrence rate after curative therapy for HCC in SVR patients was significantly lower than that in non-IFN patients. The survival rates are not different, although the rates of first and second recurrence of the PEG-IFN group (SVR) and PEG-IFN group (non-SVR) were different. The main reason for this discrepancy is that

Table 6 Risk factors contributing to second recurrence of HCC

	Univariate analysis		Multivariate analysis	
	RR (95% CI)	*P* value	RR (95% CI)	*P* value
Interferon-related variables				
Application of interferon therapy	1.97 (0.97–2.15)	0.06		
Response to interferon therapy (non-SVR + non-IFN vs. SVR)	2.77 (1.20–8.05)	0.01*	2.51 (1.06–7.40)	0.03*
Variables at the time of first recurrence of HCC				
Age (≧60 years)	0.81 (0.41–1.77)	0.57		
T.Bil (≧1.0 mg/dl)	1.70 (0.89–3.12)	0.10		
ALB (<3.5 g/dl)	2.81 (1.55–5.09)	<0.01*	2.65 (1.46–4.83)	<0.01*
ALT (≧80 IU/l)	1.36 (0.72–2.69)	0.34		
PT (<70%)	2.47 (0.98–5.46)	0.05		
PLT ($<10 \times 10^4/mm^3$)	0.94 (0.52–1.70)	0.86		
AFP (≧100 ng/ml)	2.13 (0.86–4.54)	0.09		
DCP (≧40 mAU/ml)	1.46 (0.78–2.76)	0.23		
Maximum tumor size (≧30 mm)	1.26 (0.64–2.31)	0.47		
Number of tumors (multiple)	1.21 (0.67–2.13)	0.51		

RR risk ratio, *CI* confidence interval, *IFN* interferon, *PEG-IFN* pegylated interferon, *HCV* hepatitis C virus, *HCC* hepatocellular carcinoma, *SVR* sustained virological response, *ALB* albumin, *T.Bil* total bilirubin, *ALT* alanine aminotransferase, *PLT* platelet, *PT* prothrombin time, *AFP* alpha-fetoprotein, *DCP* des-γ-carboxy prothrombin

* *P* values less than 0.05 were considered statistically significant

few patients died during follow up in both groups. In addition, we observed a significant effect of PEG-IFN (SVR) in the prevention of recurrence by two different analyses (PS score matched analysis and multivariate analysis), although the effect was limited to the prevention of second recurrence, and the term of surveillance was relatively short because PEG-IFN was only available in Japan after 2004. The results were quite similar to those of reports on conventional non-PEG-IFN therapy [17].

We conducted propensity score (PS) matched analysis to adjust the clinical background of the patients in each group. PS in this analysis is a probability of choosing PEG-IFN treatment among the patients that was calculated using seven covariates. By matching the score of the patients in the PEG-IFN group and non-IFN group, we could reconstruct a situation similar to randomization.

PEG-IFN is considered to be more beneficial than non-PEG because it results in the SVR rate being higher and the IFN concentration being maintained at a high level for a longer period [40, 41], which is favorable for its action as a direct anticancer agent. However, there was no difference between conventional IFN and PEG-IFN with regard to the prevention of only late (second) recurrence. We did not compare the effect of PEG-IFN with that of non-PEG-IFN directly, but our results that non-SVR was an independent risk factor for second recurrence but not for first recurrence suggested that IFN treatment after curative treatment of HCC is more beneficial for the suppression of de novo HCC than for preventing the progression of preexisting very small HCC or intrahepatic metastasis, regardless of the type of interferon used.

In the PEG-IFN group, tumor size at HCC recurrence was smaller (13 vs. 16 mm, respectively; $P = 0.03$) and liver function tended to be better (T.Bil, ALB, PLT, PT) than in the non-IFN group. These results suggested that PEG-IFN might inhibit the growth of recurrent tumors as well as preserve liver function, although the inhibitory effect does not appear to be sufficient for complete prevention of recurrence.

PEG-IFN therapy after curative treatment of HCC was generally well tolerated in our study. Among the 37 patients, the PEG-IFN dose had to be reduced for 8 patients (21%); however, only 3 (8%) discontinued treatment with the drug because of adverse events. This rate was similar to that of the non-PEG-IFN group after HCC treatment (8–15%) [17–24]. However, PEG-IFN therapy has fewer side effects than non-PEG-IFN therapy, such as high-grade fever and general fatigue. The good adherence of patients to treatment should be noted, with a low rate of withdrawal as a consequence of adverse events [32]. The number of elderly patients with HCC will increase in the future. Because of fewer side effects and a higher rate of SVR, HCV-related HCC treatment with PEG-IFN should be considered for these elderly patients.

The weak point of this study is that it is a retrospective study and it is difficult to eliminate biases completely even with PS analysis, although no statistical difference was observed between the PEG-IFN group and non-IFN group.

In conclusion, the present study suggests that PEG-IFN therapy after curative treatment of HCC can improve the prognosis and inhibit the recurrence of HCV-related HCC. This work involved a nonrandomized study, so further prospective studies with a larger number of cases are required to reach firm conclusions.

Conflict of interest No author has any conflict of interest.

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HEPATOLOGY

Serum levels of platelet-derived growth factor-BB and vascular endothelial growth factor as prognostic factors for patients with fulminant hepatic failure

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Key words
fulminant hepatic failure, hepatocyte growth factor, platelet-derived growth factor-BB, prognostic factor, vascular endothelial growth factor.

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Abstract

Background and Aims: In animal models for acute liver injury, the administration of some angiogenic factors such as vascular endothelial growth factor (VEGF) and granulocyte-colony stimulating factor (G-CSF) are shown to reduce liver injury and improve liver proliferative capacity. The aim of the present study was to assess the role of angiogenic factors in fulminant hepatic failure (FHF).

Methods: Serum levels of nine angiogenic factors (angiopoietin-2, follistatin, G-CSF, hepatocyte growth factor [HGF], interleukin-8, leptin, platelet-derived growth factor [PDGF]-BB, platelet endothelial cell adhesion molecule-1 and VEGF) were measured using the Bio-Plex Protein Array System in 30 patients, 17 of whom were diagnosed with FHF, 13 with acute hepatitis (AH), and 20 controls.

Results: Serum levels of PDGF-BB and VEGF were lower in FHF patients than AH patients and controls (PDGF-BB; 2050 ± 1572 pg/mL *vs* 4521 ± 2419 pg/mL *vs* 8506 ± 5500 pg/mL, VEGF; 39 ± 38 pg/mL *vs* 144 ± 122 pg/mL *vs* 205 ± 121 pg/mL). By using univariate logistic regression models, serum levels of PDGF-BB and VEGF were associated with poor outcomes. Serum PDGF-BB levels were strongly correlated with serum VEGF levels ($r = 0.70$). Furthermore, serum PDGF-BB levels were significantly correlated with platelet counts ($r = 0.79$), PT activity ($r = 0.37$) and D.Bil/T.Bil ratio ($r = 0.50$), while serum VEGF levels were significantly correlated with platelet counts ($r = 0.68$) and PT activity ($r = 0.38$).

Conclusions: We consider that serum levels of PDGF-BB and VEGF are worth investigating as biomarkers for predicting outcomes of FHF patients.

Introduction

Fulminant hepatic failure (FHF) is caused by liver cell death of a critical degree and characterized by hepatic encephalopathy and coagulopathy. The spontaneous survival rate was reported 60–70% in acetaminophen-related FHF and 20–40% in non-acetaminophen-related FHF.[1–3] While in FHF patients with fatal outcomes, post liver transplantation survival ranges between 60% and 80%,[4] and liver transplantation is the most reliable treatment for FHF.

The condition of FHF changes dramatically. If liver transplantation is performed too late, there is increased risk of multiple organ failure and infection. A recent report showed that, in a setting of donor organ shortage, a fourth of the patients listed for emergency liver transplantation were unable to undergo surgery.[5] Thus, in addition to liver transplantation, new therapeutic modalities for promoting liver regeneration are desired.

In a rat model for acute severe liver injury, the administration of vascular endothelial growth factor (VEGF) is reported to promote hepatocyte proliferation and reduce mortality.[6] Furthermore, in a rat model for FHF, granulocyte-colony stimulating factor (G-CSF) is shown to reduce liver injury and improve liver proliferative capacity.[7] Thus, the administration of these angiogenic factors may be effective treatment for FHF.

Clinically, G-CSF administration is shown to induce proliferation of hepatic progenitors in patients with alcoholic steatohepatitis, which leads to liver regeneration.[8] However, the role of these angiogenic factors in FHF patients has been yet to be fully implemented. This study aimed to investigate the associations of serum levels of various angiogenic factors including VEGF and

G-CSF with clinical characteristics and prognosis of FHF patients.

Methods

Patients

The study-subjects consisted of 30 patients, 17 of whom were diagnosed with FHF, 13 with acute hepatitis (AH), and 20 healthy controls.

Patients were diagnosed as having FHF when hepatic encephalopathy of coma grade was greater than II developed within 8 weeks after the onset of disease symptoms with a prothrombin time of less than 40% of the standardized values (Japanese diagnostic criteria).[2] However, patients showing features of chronic liver disease (splenomegaly or varices, collaterals) on computed tomography were excluded.

Etiology of FHF

A diagnosis of fulminant hepatitis A, B and C was made based on the presence of IgM anti-hepatitis A virus antibody, IgM anti-hepatitis B virus core antibody or hepatitis B surface antigen, and hepatitis C virus-RNA identifiable by nested reverse transcription-polymerase chain reaction (RT-PCR), respectively.[9] A diagnosis of autoimmune hepatitis was made according to the criteria revised by the International Autoimmune Hepatitis Group in 1999.[10] A diagnosis of Epstein-Barr virus infection was made based on measurement of Epstein-Barr virus load in whole blood by quantitative PCR amplification assays.[11] A diagnosis of drug-induced liver injury, acute fatty liver of pregnancy and ischemic hepatitis was made based on their distinctive clinical courses. A diagnosis of indeterminate FHF was established when all of the IgM anti-hepatitis A virus antibody, IgM anti-hepatitis B virus core antibody, hepatitis B surface antigen, hepatitis C virus-RNA, antinuclear antibody and anti-smooth muscle antibody were negative with no obvious cause such as drug, acute fatty liver of pregnancy, ischemic hepatitis, Wilson's disease, malignant infiltration, cytomegalovirus infection, Epstein-Barr virus infection and herpes simplex virus infection.

Measurement of angiogenic markers concentration

Serum was collected when patients were admitted to our hospital and before treatment, and stored at −80°C.

Serum levels of angiogenic factors were measured using the Bio-Plex Protein Array System with the Bio-Plex Pro Human Angiogenesis 9-Plex Panel (Bio-Rad Laboratories, Hercules, CA, USA). This panel consisted of angiopoietin-2, follistatin, G-CSF, hepatocyte growth factor (HGF), interleukin 8 (IL-8), leptin, platelet-derived growth factor (PDGF)-BB, platelet endothelial cell adhesion molecule (PECAM)-1 and VEGF. In brief, the Bio-Plex Pro Angiogenesis Standard and samples diluted in Serum Diluent were added to a 96-well filter plate and incubated with the antibody-coupled beads for 1 h with continuous shaking. The beads were washed three times with wash buffer to remove unbound protein and incubated with biotinylated detection antibodies for 30 min with continuous shaking. Following three washes, premixed streptavidin-phycoerythrin was added to each well and incubated for 30 min. After incubation, the beads were washed and re-suspended in assay buffer. The reaction mixture was quantified using the Bio-Plex protein array reader. Each angiogenic marker level was automatically calculated by Bio-Plex Manager software using the appropriate standard curve.

Statistical analysis

SPSS statistical program (release 11.0.1 J, SPSS, Chicago, IL, USA) was used for the statistical analysis.

Dichotomous variables were compared by the Fisher's exact test. Continuous variables were expressed as mean ± standard deviation (SD). The Mann–Whitney *U*-test was used to evaluate differences in the continuous variables between two groups, and the Kruskal–Wallis test was carried out among three groups. The Spearman correlation coefficient was used to evaluate the consistency in the continuous variables between two groups. To identify the association of serum angiogenic factors with poor outcomes, we developed the univariate logistic regression models. The prognostic accuracy of each factor was evaluated based on the area under the curve (AUC) using receiver operating characteristic curve analysis. *P*-values < 0.05 were considered significant.

Results

Characteristics on admission

Of 17 FHF patients, five survived spontaneously, six patients received living donor liver transplantation, and the remaining six patients died without liver transplantation. All 17 AH patients survived spontaneously. Table 1 shows clinical characteristics and laboratory data on admission of FHF patients and AH patients. Direct bilirubin/total bilirubin (D.Bil/T.Bil) ratio and prothrombin (PT) activity were lower in FHF patients than AH patients. Age, gender, etiology and serum levels of T.Bil and transaminase were similar between FHF patients and AH patients.

Serum levels of angiogenic factors

Table 2 shows serum levels of nine angiogenic factors on admission in FHF patients, AH patients and controls. There were significant differences in serum levels of HGF, IL-8, PDGF-BB and VEGF among the three groups. Serum levels of PDGF-BB and VEGF were lower in FHF patients than AH patients and controls (FHF patients *versus* AH patients: $P = 0.002$ and 0.004, respectively; FHF patients *versus* controls: $P = <0.0001$ and 0.0005, respectively). Serum IL-8 levels were lower in FHF patients than AH patients ($P = 0.002$); however, there were no differences in serum IL-8 levels between FHF patients and controls ($P = 0.19$). Serum HGF levels were higher in FHF patients than controls ($P = 0.0004$), while differences in serum HGF levels between FHF patients and AH patients were borderline ($P = 0.069$). On the other hand, there were no differences in serum levels of angiopoietin-2, follistatin, G-CSF, leptin and PECAM-1 among the three groups.

Table 1 Clinical characteristics and laboratory data on admission

	FHF	AH	*P*-value
Patients, *n*	17	13	
Age, years	37 ± 14	41 ± 17	0.34
Gender, female (%)	12 (71%)	6 (46%)	0.26
Etiology, *n* (%)			
Viral hepatitis	5 (29%)	7 (54%)	0.26
HAV	1 (5%)	4 (31%)	
HBV	4 (24%)	3 (23%)	
AIH	3 (18%)	3 (23%)	
Drug-induced	4 (24%)	1 (8%)	
Indeterminate	5 (29%)	2 (15%)	
Period from initial symptoms to the diagnosis of fulminant hepatic failure, day			
	14 ± 12	–	–
Hepatic coma, n (%)			
II	14 (82%)	–	–
III or IV	3 (18%)	–	–
Laboratory data			
WBC, /mm^3	10 829 ± 6698	7616 ± 2250	0.33
Hemoglobin, g/dL	13.2 ± 1.7	14.2 ± 2.2	0.18
Platelet, ×10^4/mm^3	12.0 ± 6.9	18.0 ± 8.3	0.054
T.Bil, mg/dL	14.3 ± 8.8	11.1 ± 8.15	0.27
D.Bil/T.Bil ratio	0.59 ± 0.14	0.68 ± 0.03	0.027
AST, IU/L	2876 ± 3888	2967 ± 4696	0.98
ALT, IU/L	2817 ± 2563	2728 ± 2573	0.93
Cr, mg/dL	1.0 ± 1.1	0.9 ± 0.5	0.45
PT activity, %	23 ± 10	42 ± 12	0.0005

AH, acute hepatitis; AIH, autoimmune hepatitis; ALT, alanine aminotransferase; AST, aspartate aminotransaminase; Cr, creatinine; D.Bil, direct birilubin; FHF, fulminant hepatic failure; HAV, hepatitis A virus; HBV, hepatitis B virus; PT, prothrombin; T.Bil, total bilirubin; WBC, white blood cell.

Table 2 Serum levels of nine angiogenic factors on admission

	FHF	AH	Control	*P*-value
Patients, *n*	17	13	20	
Angiopoietin-2, pg/mL	1078 ± 689	1440 ± 982	998 ± 462	0.51
Follistatin, pg/mL	728 ± 752	1235 ± 1763	697 ± 207	0.11
G-CSF, pg/mL	129 ± 57	260 ± 222	347 ± 335	0.22
HGF, pg/mL	9958 ± 21 015	3197 ± 2588	1981 ± 791	0.002
IL-8, pg/mL	161 ± 68	710 ± 1167	263 ± 259	0.015
Leptin, pg/mL	3441 ± 2774	3935 ± 2537	4530 ± 1728	0.15
PDGF-BB, pg/mL	2050 ± 1572	4521 ± 2419	8506 ± 5500	< 0.0001
PECAM-1, pg/mL	8340 ± 3347	6842 ± 3366	7573 ± 4328	0.47
VEGF, pg/mL	39 ± 38	144 ± 122	205 ± 121	0.0005

AH, acute hepatitis; FHF, fulminant hepatic failure; G-CSF, granulocyte colony-stimulating factor; HGF, hepatocyte growth factor; IL, interleukin; PDGF, platelet-derived growth factor; PECAM, platelet/endothelial cell adhesion molecule; VEGF, vascular endothelial growth factor.

Association of serum levels of angiogenic factors with prognosis

In the 30 patients with FHF or AH, between 12 patients with good outcomes and 18 patients with poor outcomes (including death and liver transplantation), there were significant differences in serum levels of HGF (3037 ± 2313 pg/mL *vs* 13 014 ± 24 635: $P = 0.004$), PDGF-BB (4099 ± 2413 pg/mL *vs* 1653 ± 1072 pg/mL: $P = 0.002$) and VEGF (120 ± 114 pg/mL *vs* 32 ± 25 pg/mL: $P = 0.010$) (Fig. 1). By univariate logistic regression models, serum levels of PDGF-BB and VEGF were associated with poor outcomes in the 30 patients with FHF or AH (Table 3). The association of serum HGF levels with poor outcomes was borderline. There were no associations between serum levels of the other angiogenic factors and the prognosis.

Table 4 shows the AUC of platelet count, D.Bil/T.Bil ratio, PT activity, and serum levels of HGF, PDGF-BB and VEGF as a prognostic factor. The AUC of PDGF-BB was equal to those of T.Bil/D.Bil ratio and PT activity.

In the 30 patients with FHF or AH, serum PDGF-BB levels were significantly correlated with serum VEGF levels ($r = 0.70$, $P < 0.0001$) (Table 5). Furthermore, serum PDGF-BB levels were correlated with platelet counts ($r = 0.79$, $P < 0.0001$), PT activities ($r = 0.37$, P-value = 0.044) and D.Bil/T.Bil ratio ($r = 0.50$, $P = 0.006$). On the other hand, serum VEGF levels were correlated with platelet counts ($r = 0.68$, $P < 0.0001$) and PT activities ($r = 0.38$, $P = 0.040$).

Discussion

In a setting of donor organ shortage, it is important to accurately identify FHF patients with poor outcomes in order to rescue more patients with liver transplantation. However, biomarkers for predicting accurate prognosis fall short. Previously, serum copy number of transforming growth factor-alpha (TGF-α) mRNA and serum HGF levels were reported as biomarkers for predicting the prognosis of FHF patients.[12,13] However, the specificity (65.5%) of serum copy number of TGF-α mRNA was not sufficient in the original report.[12] Furthermore, in the present study, the association of serum HGF levels with the prognosis of patients with FHF or AH was borderline. So, another biomarker is required. This study suggests that serum levels of PDGF-BB and VEGF may be useful as biomarkers for predicting a poor prognosis of FHF patients. This report is the first concerning serum levels of PDGF-BB and VEGF in FHF patients although sample size of this study was limited. We consider that serum levels of PDGF-BB and VEGF are worth investigating as biomarkers for predicting outcomes of FHF patients. In order to confirm these findings, a further study with a larger sample size is required.

In the present study, serum levels of PDGF-BB and VEGF were well correlated with platelet counts. Platelet release angiogenic factors such as PDGF-BB and VEGF.[14,15] In FHF patients, platelet counts and serum thrombopoietin levels are decreased.[16] Thrombopoietin, produced primarily in the liver but also in the bone marrow and kidney, binds to the thrombopoietin receptor expressed on the surface of stem cells, megakaryocyte progenitor cells, megakaryocytes, and platelets.[17] Thrombopoietin regulates the development and maturation of megakaryocytes and subsequent release of platelets. Additionally,

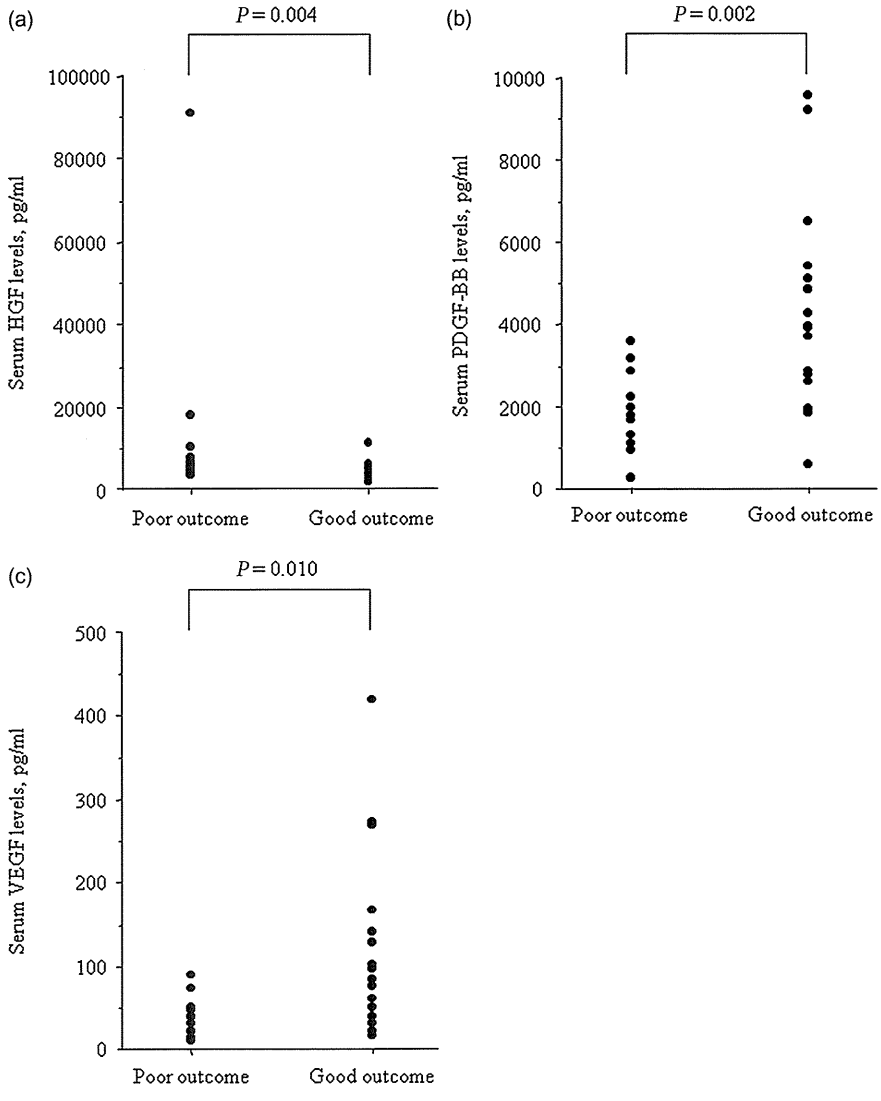

Figure 1 Serum hepatocyte growth factor (HGF), platelet-derived growth factor-BB (PDGF-BB) and vascular endothelial growth factor (VEGF) levels of 30 patients with fulminant hepatic failure (FHF) or acute hepatitis (AH). (a) Serum HGF levels were lower in patients with good outcomes than those with poor outcomes (including death and liver transplantation) ($P = 0.004$). (b, c) Serum PDGF-BB and VEGF levels were higher in patients with good outcomes than those with poor outcomes ($P = 0.002$ and 0.010, respectively).

Table 3 Associations of serum levels of nine angiogenic factors with poor outcomes in 30 patients with FHF or AH by univariate logistic regression models

	Odds ratio	95% CI	*P*-value
Angiopoietin-2, per 1 pg/mL increase	1.00	0.99–1.00	0.78
Follistatin, per 1 pg/mL increase	1.00	0.99–1.00	0.58
G-CSF, per 1 pg/mL increase	0.99	0.98–1.00	0.15
HGF, per 1 pg/mL increase	1.00	1.00–1.00	0.058
IL-8, per 1 pg/mL increase	0.99	0.99–1.00	0.12
Leptin, per 1 pg/mL increase	1.00	1.00–1.00	0.48
PDGF-BB, per 1 pg/mL increase	0.99	0.99–1.00	0.012
PECAM-1, per 1 pg/mL increase	1.00	1.00–1.00	0.17
VEGF, per 1 pg/mL increase	0.97	0.95–0.99	0.045

AH, acute hepatitis; CI, confidence interval; FHF, fulminant hepatic failure; G-CSF, granulocyte colony-stimulating factor; HGF, hepatocyte growth factor; IL, interleukin; PDGF, platelet-derived growth factor; PECAM, platelet/endothelial cell adhesion molecule; VEGF, vascular endothelial growth factor.

Table 4 Prognostic accuracy by the AUC using receiver operating characteristic curve in 30 patients with FHF or AH

	AUC	95% CI	*P*-value
Platelet	0.76	0.57–0.94	0.020
D.Bil/T.Bil ratio	0.83	0.66–0.99	0.003
PT	0.80	0.64–0.97	0.006
HGF	0.81	0.66–0.97	0.005
PDGF-BB	0.83	0.69–0.98	0.003
VEGF	0.77	0.60–0.94	0.015

AUC, area under the curve; CI, confidence interval; D.Bil, direct birilubin; HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor; PT, prothrombin; T.Bil, total bilirubin; VEGF, vascular endothelial growth factor.

thrombopoietin enhances platelet activation and function. Thus, we speculate that thrombocytopenia due to the decrease of serum thrombopoietin levels may lead to the decrease of serum PDGF-BB and VEGF levels. Furthermore, in the present study,

Table 5 Associations between serum levels of angiogenic factors (HGF, PDGF-BB and VEGF) and laboratory data in 30 patients with FHF or AH

	HGF	PDGF-BB	VEGF
HGF	–	−0.31	−0.29
PDGF-BB	–	–	0.70**
Platelet	−0.26	0.79**	0.68**
T.Bil	0.20	0.09	0.22
D.Bil/T.Bil ratio	−0.34	0.50**	0.35
AST	−0.11	−0.15	−0.39*
ALT	−0.08	−0.13	−0.26
Cr	0.31	−0.10	−0.21
PT activity	−0.36	0.37*	0.38*

*$P < 0.05$; **$P < 0.01$; Cr, creatinine; D.Bil, direct birilubin; HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor; PT, prothrombin; T.Bil, total bilirubin; VEGF, vascular endothelial growth factor. Values shown are the correlation coefficient (r) between the variables.

serum levels of PDGF-BB and VEGF were correlated with PT activities. So, serum levels of PDGF-BB and VEGF may reflect the extent of liver failure.

In the present study, the decrease of serum PDGF-BB and VEGF levels were associated with poor outcomes of FHF patients. Hepatocytes proliferation and liver regeneration are stimulated by VEGF.[6,18] Furthermore, PDGF were reported to increase the expression of pleiotrophin, a potent mitogen for hepatocytes, in sinusoidal hepatic stellate cells.[19] Thus, we speculate that the decrease of serum PDGF-BB and VEGF levels may delay liver regeneration, and this may result in the increased risk of multiple organ failure and infection and the poor outcomes in FHF patients. On the other hand, in human, clinical trials using recombinant VEGF or VEGF gene transfer were already reported.[20,21] Treatment using PDGF-BB and VEGF may provide a new therapeutic strategy for FHF.

Recently, a clinical trial of recombinant human HGF, which stimulates the proliferation of mature hepatocytes and hepatic progenitor cells, in patients with FHF was reported from Japan.[22] In the study, four patients received the administration of recombinant HGF, of whom, two died due to progression of liver failure and two were rescued. However, from the first, serum HGF levels are higher in FHF patients than AH patients or healthy controls. On the other hand, this study indicates that serum levels of PDGF-BB and VEGF are lower in FHF patients. Thus, treatment using PDGF-BB and VEGF may be more reasonable.

We consider that, in order to assess the usefulness of serum PDGF-BB and VEGF levels as biomarkers for predicting outcomes of FHF patients, the relation between the changes of serum PDGF-BB and VEGF levels during the clinical course and the prognosis of FHF patients should be assessed, although, in this study, we could not for lack of the serum collection after the introduction of treatment in AH patients and FHF patients. On the other hand, in this study, 13 of 17 FHF patients (76%) received plasma exchange combined with continuous hemodiafiltration. Serum IL-8 levels were reported to be decreased by plasma exchange combined with continuous hemodiafiltration.[23] However, the effect of plasma exchange combined with continuous hemodiafiltration on serum PDGF-BB and VEGF levels has yet to be fully implemented. Clarification of these points is necessary in the future.

In conclusion, lower serum levels of PDGF-BB and VEGF were associated with poor prognosis of FHF patients in the present study. Thus, we consider that serum levels of PDGF-BB and VEGF are worth investigating as biomarkers for predicting outcomes of FHF patients.

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Loss of runt-related transcription factor 3 expression leads hepatocellular carcinoma cells to escape apoptosis

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Abstract

Background: Runt-related transcription factor 3 (RUNX3) is known as a tumor suppressor gene for gastric cancer and other cancers, this gene may be involved in the development of hepatocellular carcinoma (HCC).

Methods: RUNX3 expression was analyzed by immunoblot and immunohistochemistry in HCC cells and tissues, respectively. Hep3B cells, lacking endogenous RUNX3, were introduced with RUNX3 constructs. Cell proliferation was measured using the MTT assay and apoptosis was evaluated using DAPI staining. Apoptosis signaling was assessed by immunoblot analysis.

Results: RUNX3 protein expression was frequently inactivated in the HCC cell lines (91%) and tissues (90%). RUNX3 expression inhibited 90 ± 8% of cell growth at 72 h in serum starved Hep3B cells. Forty-eight hour serum starvation-induced apoptosis and the percentage of apoptotic cells reached 31 ± 4% and 4 ± 1% in RUNX3-expressing Hep3B and control cells, respectively. Apoptotic activity was increased by Bim expression and caspase-3 and caspase-9 activation.

Conclusion: RUNX3 expression enhanced serum starvation-induced apoptosis in HCC cell lines. RUNX3 is deleted or weakly expressed in HCC, which leads to tumorigenesis by escaping apoptosis.

Background

Hepatocellular carcinoma (HCC)[1] is the sixth most common cancer and responsible for more than half a million deaths worldwide each year [1-3]. Although most HCC cases occur in East Asia and Middle and West Africa, its incidence in some developed countries is increasing [1,4]. In most cases, HCC is fatal because of an incomplete understanding of the pathogenic mechanisms and inadequacies of early detection [1,5].

The activation of proto-oncogenes plays a major role in the development of HCC [1,6-8], and a number of tumor suppressor genes may be associated with the development and progression of HCC [1,9-12]. Although several cancer-related genes are altered in HCC, the frequency of alterations for each individual gene is relatively low. In HCC, the alteration of tumor suppressor genes seems to be more important than that of oncogenes. Established genetic events include the loss of an allele, mutation, or promoter methylation [13-16]. A higher loss of heterozygosity (LOH) frequency was detected at several loci on chromosomes 8p23, 4q22-24, 4q35, 17p13, 16q23-24, 6q27, 1p36, and 9p12-14, suggesting the presence of important tumor suppressor genes at these loci [17]. However, there is little understanding of the several key pathways and the genes involved in these pathways.

Runt-related transcription factor 3 (RUNX3), located on chromosome 1p36, is correlated with tumorigenesis and gastric cancer progression [18,19]. RUNX3 acts as

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an apoptotic factor, downstream of transforming growth factor-β (TGF-β), and as a cell differentiation mediator in intestinal metaplasia of gastric mucosa [19-21]. In gastric cancer cell lines, RUNX3-induced apoptosis depends on Bim expression [22]. RUNX3 protein expression is decreased about 45-60% in human gastric cancer [21] and has been detected in some human malignancies such as those of the colon, lung, pancreas, and bile duct [23-26]. RUNX3 gene expression decreased in 30-80% of HCCs due to LOH and methylation of its promoter [27,28]. The loss or decrease of RUNX3 expression in HCC tissue has been recently reported [29], but the precise function of RUNX3 in HCC needs to be elucidated.

Methods

Cell lines and cell culture

The HCC cell lines HepG2, Hep3B, PLC/PRF/5 (PLC), and SK-Hep1 were obtained from the American Type Culture Collection (Manassas, VA), and the Huh1, Huh7, JHH1, JHH2, JHH4, HLE, and HLF cell lines were obtained from the Health Science Research Resources Bank (Osaka, Japan). Normal human hepatocytes were obtained from Sanko Junyaku Co. Ltd. (Tokyo, Japan). JHH2 and normal human hepatocytes were cultured in William's medium E (Invitrogen, Carlsbad, CA). Other cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen). Media were supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, MO), 1% nonessential amino acids (Sigma), 1% sodium pyruvate (Sigma), and 1% penicillin/streptomycin solution (Sigma). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Quiescence was carried out under restricted serum conditions with 0.1% dialyzed FBS for the indicated time periods.

RNA preparation and reverse transcriptase-polymerase chain reaction

Total RNA was isolated from cells using Trizol™ reagent (Invitrogen). Reverse transcription was performed using random primers and ReverTra Ace™ (Toyobo, Osaka, Japan) reverse transcriptase (RT). Ps-CA and Ps-CB, previously published primer set for RUNX3, were utilized [21]. For each polymerase chain reaction (PCR), 20 μl (total volume) of reaction mixture contained 0.1 μg template DNA, 4 pmol each of the forward and reverse primers, 2 μl deoxynucleoside triphosphates (200 mM each), 1 U pfu Turbo™ DNA polymerase (Stratagene, La Jolla, CA), and 2 μl of 10× pfu reaction buffer. PCR amplification was conducted on an iCycler™ (Bio-Rad, Hercules, CA) with the following cycle conditions: cycle 1, 95°C for 2 min; cycles 2-30, 95°C for 30 s, 58°C for 30 s, and 72°C for 120 s, with a final elongation step of 72°C for 10 min.

Immunoblot analysis

Cells were plated onto 6-well tissue culture plastic dishes and grown to confluence. After cultivating the cells under the indicated conditions, they were washed twice with cold phosphate-buffered saline (PBS) and lysed in 150 μl of sample buffer (100 mM Tris-HCl, pH 6.8, 10% glycerol, 4% sodium dodecyl sulfate [SDS], 1% bromophenol blue, 10% β-mercaptoethanol). The samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P™ polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA), which were blocked using Tris-buffered saline with Tween-20 (TBS-T) (Sigma) containing 5% bovine serum albumin for 1 h. The membranes were incubated with antibodies against RUNX3 (R3-G54; Abcam, Cambridge, MA), poly-histidine (His) (Roche Diagnostics, Basel, Switzerland), Bax, Bcl-2, Bim, cleaved caspase-3 and -9 (Cell Signaling Technology, Beverley, MA), and β-actin (Sigma) overnight at 4°C. We washed the membranes three times with TBS-T and probed with horseradish peroxidase-conjugated secondary antibodies before developing them using an ECL Western blotting detection system (Amersham Biosciences, Piscataway, NJ) by enhanced chemiluminescence.

HCC tissue and immunohistochemistry

Thirty-one patients including 24 men with age ranging from 18 to 71 years (average age, 58 years) and 7 women with age ranging from 59 to 67 years (average age, 63 years) at the time of hepatic resection were included in this study. HCC tissues along with adjacent liver tissues were used for analysis. As per the institutional guidelines, we obtained informed consent from all donors of liver tissue samples, and the study was approved by the Research Ethics Committee of Okayama University.

Immunohistochemistry was performed on formalin-fixed paraffin sections that were dewaxed and dehydrated. After rehydration, endogenous peroxidase activity was blocked for 30 min in a methanol solution containing 0.3% hydrogen peroxide. After antigen retrieval in citrate buffer, the sections were blocked overnight at 4°C. The sections were probed with rabbit polyclonal antibody (ab49117; Abcam) followed by biotinylated anti-rabbit secondary antibody (Dako Japan, Tokyo, Japan). The signal was amplified by avidin-biotin complex formation and developed with diaminobenzidine followed by counterstaining with hematoxylin, after which the sections were dehydrated in alcohol and xylene, and mounted for observation. The sections were scored on a four-tier scale; 0, negative; 1, weak signal; 2, intermediate signal; and 3, strong signal [30]. All sections were scored independently by two observers (Y. K. and K. N.) without prior knowledge. All discrepancies in scoring were reviewed and a consensus was reached.

RUNX3 cloning and transfection

We obtained human RUNX3 cDNA by PCR-based cloning from normal human hepatocytes (Sanko Junyaku). Briefly, cDNA was amplified by PCR using sense (5'-TATGCGTATTCCCGTAGA) and antisense (5'-CTCGAGGCGGCCGCTCAATGGTGATGGTGATGATGACCGGTACGGTAGGGCCGCCACAC; including the six-His tag) oligonucleotide primers with Pfu Turbo™ Hotstart DNA polymerase (Stratagene) and cloned into the PCR II TA cloning vector (Invitrogen). The size of the PCR product was ~1.2 kb. After confirmation by sequencing, RUNX3 cDNA was subcloned into pCEP4 (Stratagene), downstream from a cytomegalovirus promoter. The poly-His tag was replaced with green fluorescent protein (GFP) cDNA from pEGFP-C1 (Clontech, Palo Alto, CA). The human RUNX3 and/or chloramphenicol acetyltransferase (CAT) (control) constructs were transfected into Hep3B cells using FuGENE™6 transfection reagent (Roche), as per the manufacturer's instruction. Cells were selected in complete medium containing 250 μg/ml of hygromycin (Roche). Polyclonal lines consisting of more than 20 colonies were established. At least two independent stably transfected lines were established for each construct.

Transient RUNX3 expression was also conducted using FuGENE™6 in Hep3B, Huh7, HLE, and HLF cells. After transfection, the cells were cultured under serum starved condition for the indicated periods, if needed, and utilized for the following experiments.

MTT assay

Cell proliferative activity was assessed with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells were seeded at 2,000 cells/well in 96-well tissue culture plastic dishes and quiesced for 6 h with 0.1% dialyzed FBS. After 24-120 h of quiescence, the cells were cultured for the indicated periods with or without 10% FBS. At the end of the treatment, 10 μl of MTT (5 mg/ml in PBS) was added to each well, and the wells were incubated for an additional 2 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 200 μl of dimethyl sulfoxide (Sigma). The activity of the mitochondria, reflecting cellular growth and viability, was evaluated by measuring the optical density at 570 nm with a microplate reader (Bio-Rad).

DAPI staining

Cells were plated at 50% confluence on glass chamber slides (Labtek II, Nalgen Nunc, Roskide, Denmark) and quiesced for 6 h with a media containing 0.1% dialyzed FBS. Then, they were treated with 10% FBS, 100 μM caspase inhibitor (caspase inhibitor IV, Calbiochem, Gibbstown, NJ), 1 nM transforming growth factor-α (TGF-α) (Peprotech Inc. Rocky Hill, NJ), 1 nM epidermal growth factor (EGF) (Peprotech), and/or 5 ng/ml platelet derived growth factor (PDGF)-BB (Peprotech). Chromosomal DNA was stained with 4', 6-diamidine-2'-phenylindole dihydrochloride (DAPI) (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, treated cells were washed with PBS and stained with DAPI working solution (1 μg/ml in PBS) for 2 min. The percentage of cells with condensed chromatin and/or fragmented nuclei was established in 300-500 DAPI-stained cells examined under a fluorescence microscope (IX-70, Olympus, Tokyo, Japan).

Flow cytometry analysis

Annexin V and propidium iodide (PI) staining was performed using an annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) to measure apoptosis. Cells were cultured in 10-cm tissue culture plates and quiesced for 6 h with a media containing 0.1% dialyzed FBS. Cells were cultured in medium with or without 10% FBS for 24 h. Then, they were washed twice with PBS, collected, and re-suspended in 85 μl of 1× annexin V-FITC binding buffer. Five microliters of annexin V-FITC conjugate and 10 ml of PI buffer were added, and the cells were incubated at room temperature for 15 min in the dark. After adding 400 μl of 1× annexin V-FITC binding buffer, cells were analyzed using a flow cytometer (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ).

Gene silencing of Bim with small interfering RNA

RUNX3-expressing Hep3B cells were transfected with either scrambled negative control small interfering RNA (siRNA) or Bim siRNA (Applied Biosystems, Foster City, CA). siRNAs were transfected into cells using RNAi-Fect™ transfection reagent (Qiagen, Hilden, Germany). Cells were incubated with scrambled negative control siRNA or Bim siRNA for 24 h before 48 h of serum starvation. The MTT assay and DAPI staining for detecting apoptosis were performed as described above.

Results

Loss of RUNX3 expression in HCC cell lines and human HCC tissues

A decreased level or absence of RUNX3 mRNA expression was observed in 10 of 11 HCC cell lines (Figure 1A). RUNX3 mRNA was undetectable in eight cell lines (HepG2, Hep3B, Huh1, Huh7, JHH1, JHH2, JHH4, and HLE). In HLF and SK-Hep1 cells, RUNX3 mRNA was significantly underexpressed (Figure 1A). Normal human hepatocytes expressed RUNX3 mRNA. Sequence analysis was performed in HLF, PLC, and SK-Hep1 cells, and no mutation was detected. In accordance with the mRNA analysis, RUNX3 protein expression was

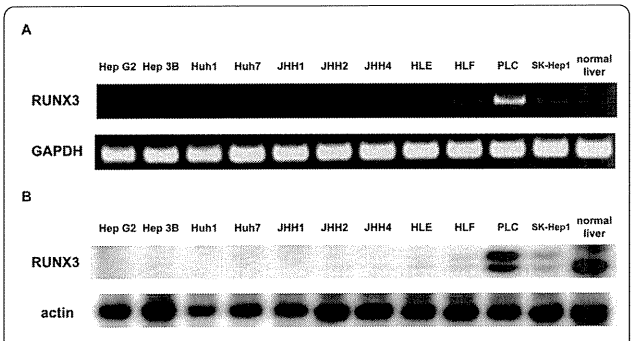

Figure 1 RUNX3 mRNA (A) and protein (B) expression in HCC cell lines. (A) RUNX3 and GAPDH mRNA expression levels were determined by RT-PCR. Shown here are representative gels from three independent experiments. (B) RUNX3 and α-actin protein expression was analyzed by immunoblot using the anti-RUNX3 antibody. Shown here are representative blots from more than three independent experiments.

undetectable in the HepG2, Hep3B, JHH1, JHH2, JHH4, HLE, and HLF cell lines, while the RUNX3 protein was expressed in HLF, PLC, and SK-Hep1 cells (Figure 1B). The RUNX3 protein was significantly underexpressed in HLF and SK-Hep1 cells.

RUNX3 protein expression in human HCC tissue was compared to that in the corresponding tumor-free resection margins using immunohistochemical analysis (Figure 2). Twenty eight (~90%) of these pairs showed a negative or weak signal for RUNX3 expression in HCC tissue, but showed RUNX3 protein expression in tumor-free resection margins (Table 1). In the remaining three pairs, a weak RUNX3 expression signal was detected in the tumor-free resection margins; thus, no negative RUNX3 signal was detected in the tumor-free resection margins.

Ectopic RUNX3 protein expression in Hep3B cells

To assess whether RUNX3 protein expression affected cell survival in the HCC cell lines, a RUNX3 construct was introduced into RUNX3-negative Hep3B cells (Figure 3A). Overall, the clones were expressed at similar

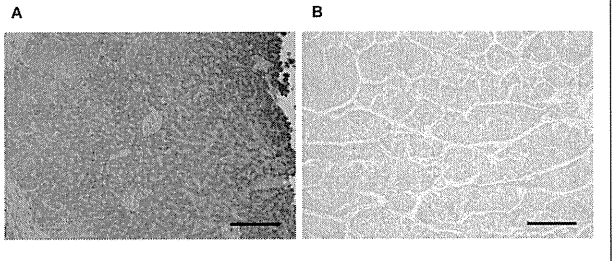

Figure 2 Immunohistochemical staining of RUNX3 in human liver tissues. (A) nontumor liver tissue with a protein score of 3 (B) HCC tissue with a protein score of 0. Bar = 100 μm.

Table 1 RUNX3 expression in HCC samples (n = 31) and the corresponding tumor-free resection margins

RUNX3 protein expression score	HCC samples (n = 31)	Tumor-free sections (n = 31)
0 (negative signal)	13 (41.9%)	0
1 (weak signal)	15 (48.4%)	3 (9.7%)
2 (intermediate signal)	3 (9.7%)	16 (51.6%)
3 (strong signal)	0	12 (38.7%)

levels in all cells, as determined by immunocytochemical analysis (data not shown). RUNX3-expressing Hep3B cells grew slightly slower than normal Hep3B cells in the presence of FBS.

RUNX3 expression inhibited cell growth under serum starvation

RUNX3 has been reported to induce apoptosis in a gastric cancer cell study [21]. The MTT assay was performed to determine whether RUNX3 expression influenced cell growth. RUNX3-expressing Hep3B cells grew slightly slower than CAT-transfected Hep3B cells in the presence of FBS, whereas the growth of RUNX3-expressing Hep3B cells was markedly suppressed in the absence of FBS; growth inhibition could be observed as early as 24 h, and reached 70 ± 12% and 90 ± 8% at 48 and 72 h, respectively (Figure 3B). The inhibition levels were over 4 times than those found in the condition with 10% FBS. This effect was confirmed with GFP-tagged RUNX3-expressing Hep3B cells (70 ± 11% growth inhibition at 72 h).

RUNX3 expression induced apoptosis under serum starvation

The effect of RUNX3 expression on cell survival and the cell cycle with and without FBS was assessed to investigate whether the elicited growth suppression in RUNX3-expressing cells under serum starved conditions was due to an increase in cell death or due to cell cycle inhibition, or both. DAPI staining demonstrated that serum starvation induced apoptosis in RUNX3-expressing Hep3B cells (31 ± 4%) but not in CAT-transfected Hep3B cells (4 ± 1%) in the absence of FBS (Figure 3C). Flow cytometry analysis with annexin V antibody was also performed. RUNX3-expressing Hep3B cells showed a significant increase in a pre-apoptosis population (Annexin V+ PI-) after 24 h of serum starvation compared with CAT-transfected Hep3B cells (Figure 3D).

RUNX3-induced apoptosis through the Bim-caspase pathway

Because a RUNX3-induced apoptotic pathway has been described previously, the effect of altering RUNX3 expression was investigated. Bim protein expression was

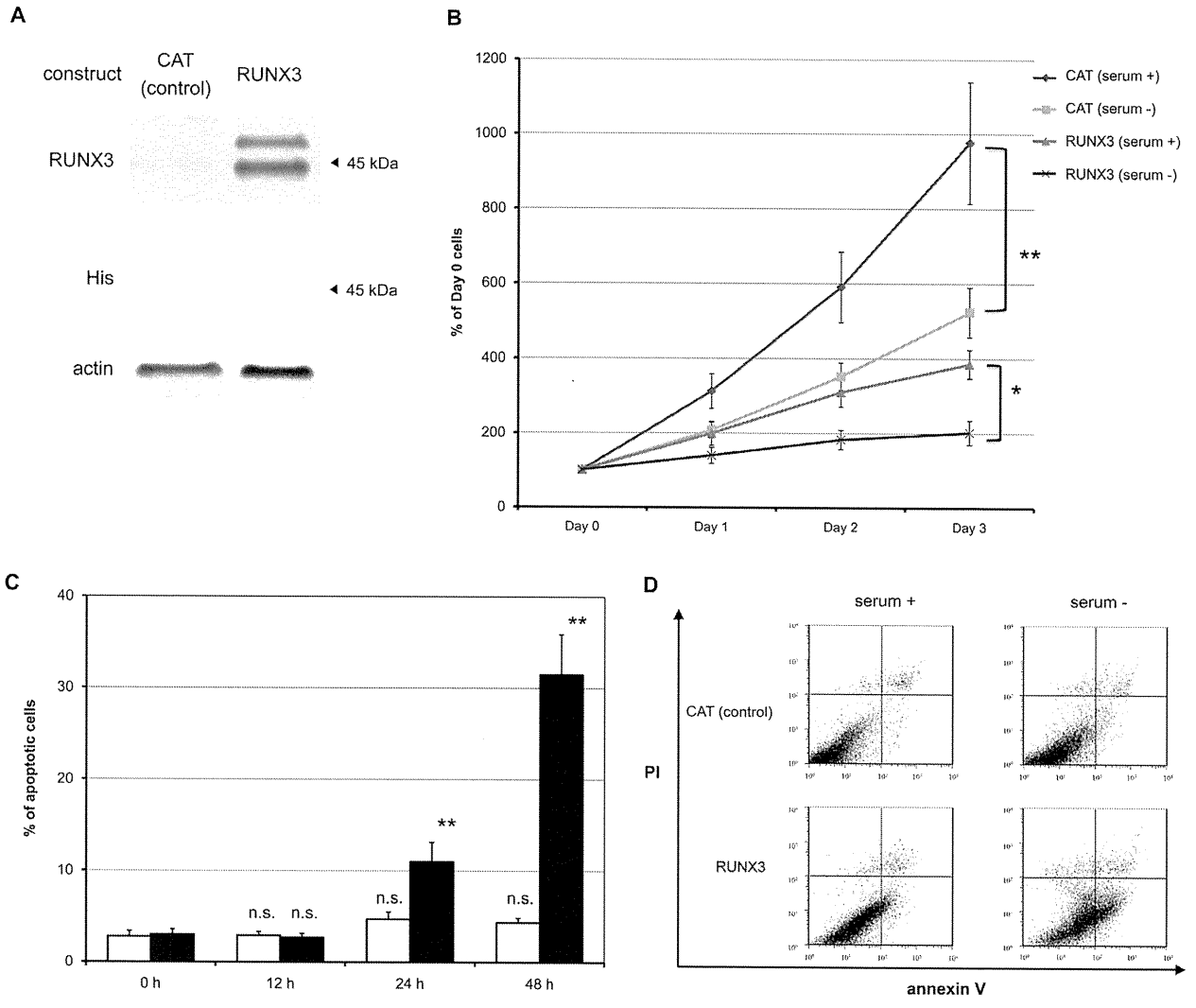

Figure 3 RUNX3 expression in Hep3B cells. CAT (control) and RUNX3 constructs were introduced into Hep3B cells. Polyclonal stably expressing cell lines were selected in the presence of hygromycin. (A) Immunoblot analysis RUNX3 protein expression was analyzed by immunoblot using anti-RUNX3 and anti-His antibodies. Shown here are representative blots from more than three independent experiments. (B) Effect of RUNX3 expression on cell growth Cell proliferative activities were measured by the MTT assay. All results are expressed as ratios to cell number at day 0. Data represent the mean ± S.E. of more than three independent experiments, each with four replicates. *, $P < 0.05$; **, $P < 0.01$ (vs. data at 0 h); Student's *t*-test. (C) Apoptosis determined by DAPI staining Cells were cultured in medium without FBS for the indicated periods. The cells were stained with DAPI, and the percentage of apoptotic cells was examined by fluorescence microscopy (CAT; white bars, RUNX3; black bars). Data represent the mean ± S.E. of more than five independent experiments, each with triplicates. n.s., not significant; **, $P < 0.01$ (vs. data at 0 h); Student's *t*-test. (D) Flow cytometry analysis Cells were cultured in medium with or without 10% FBS for 24 h. They were harvested and washed in PBS without Ca^{2+}/Mg^{2+}, and stained using the Mebcyto™ apoptosis detection kit. The cells were analyzed with a Becton Dickinson FACS Calibur flow cytometer. Shown here are representative plots from more than three independent experiments.

enhanced by serum starvation in RUNX3-expressing Hep3B cells but not in control cells (Figure 4A). Activated apoptosis executors, caspase-9 and -3, were found in serum starved RUNX3-expressing Hep3B cells. Expression of the Bim attenuators, Bax and Bcl-2, was not affected by serum starvation. These results imply that Bim plays a major role in serum starvation-induced apoptosis in RUNX3-expressing cells.

Serum starvation-induced apoptosis was abrogated by an apoptosis inhibitor (Figure 4B). Various growth factors were employed to determine whether serum starvation-induced apoptosis was caused by the absence of a growth factor-induced survival signal. As a result, TGF-α, EGF, and PDGF abrogated serum starvation-induced apoptosis in RUNX3-expressing Hep3B cells (Figure 4B).

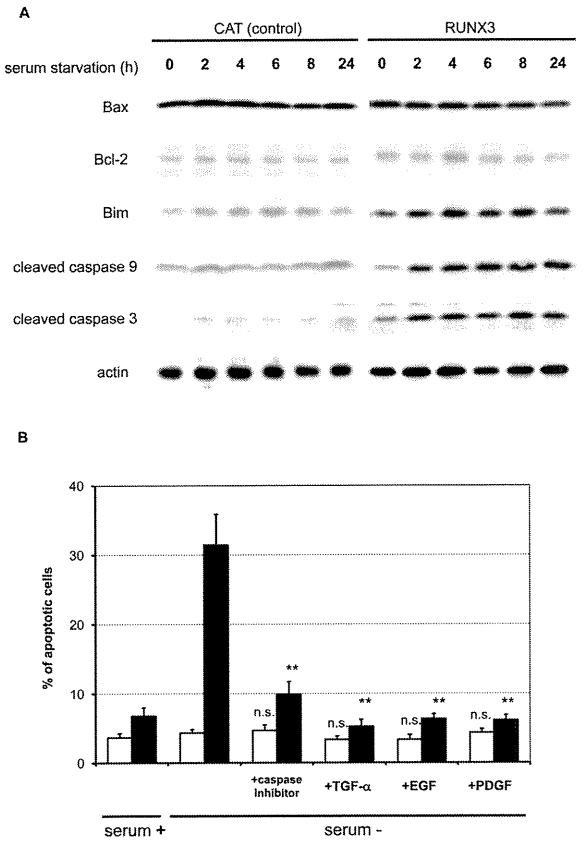

Figure 4 Effect of RUNX3 on apoptosis-related signaling molecules. (A) Immunoblot analysis Equal amounts of cell lysates were size-fractionated with 10% SDS-PAGE and immunoblotted with antibodies against Bax, Bcl-2, Bim, cleaved caspase-9, cleaved caspase-3, and actin. Shown here are representative blots from at least three independent experiments. (B) Apoptosis determined by DAPI staining Cells were cultured in media with or without 10% FBS, caspase inhibitor, and/or TGF-α for 48 h. Cells were stained with DAPI, and the percentage of apoptotic cells was determined under a fluorescent microscope (CAT; white bars, RUNX3; black bars). Data represent the mean ± S.E. of more than five independent experiments, each with triplicates. n.s., not significant; $P > 0.05$; **, $P < 0.01$ (vs. data with no serum); Student's *t*-test.

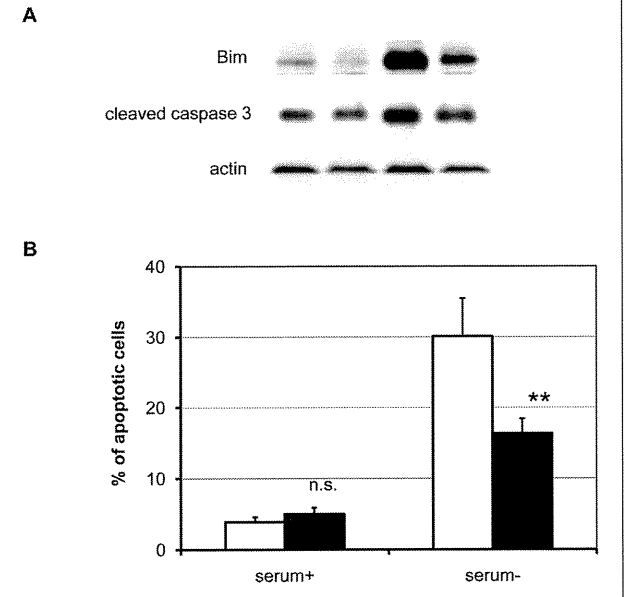

Figure 5 Effect of Bim siRNA gene silencing on cell growth inhibition and apoptosis. After treatment with Bim siRNA duplexes, an immunoblot analysis (A) and DAPI apoptosis detection assay (B) were performed. Shown here are representative blots from more than three independent experiments. All results are expressed as ratios to control siRNA-treated cells (control siRNA, white bars; Bim siRNA, black bars). Data are mean ± S.E. from at least three independent experiments, each performed in triplicate. Statistical analysis was performed with the Student's *t*-test. n.s., not significant; **, $P < 0.01$ (vs. control siRNA-treated cells).

siRNA against Bim reduced serum starvation-induced apoptosis in RUNX3-expressing Hep3B cells

siRNA against Bim was used to knockdown Bim expression in Hep3B cells (Figure 5A). The expression level of cleaved caspase-3, decreased in Bim siRNA-treated cells (Figure 5A). Bim siRNA inhibited serum starvation-induced apoptosis by 46 ± 7% in RUNX3-expressing Hep3B cells (Figure 5B).

Transient ectopic RUNX3 expression in various HCC cell lines

RUNX3 was transiently expressed in various HCC cell lines, including Hep3B, Huh7, HLE, and HLF, introducing a RUNX3 construct-induced RUNX3 protein expression (Figure 6A). Transient RUNX3-expressing cells also showed growth inhibition after 48 h of serum starvation; the inhibition was 50 ± 10%, 46 ± 11%, 60 ± 8%, and 52 ± 9% in Hep3B, Huh7, HLE, and HLF cells, respectively. The RUNX3-expressing HCC cell lines demonstrated enhanced serum starvation-induced apoptosis; the percentage of apoptotic cells determined by DAPI staining was 21 ± 2%, 25 ± 2%, 19 ± 1%, and 20 ± 2% in Hep3B, Huh7, HLE, and HLF cells, respectively (Figure 6B). Serum starvation-induced Bim expression and caspase-3 cleavage were also confirmed in RUNX3-expressing Hep3B, Huh7, HLE, and HLF cells (Figure 6C).

Discussion

The results of the present study demonstrated that RUNX3 is a tumor suppressor gene for HCC. A significant down-regulation of RUNX3 was observed in a high percentage of human HCC cell lines (91%) and tissues (90%) (Figures 1, 2, and Table 1). RUNX3 has been described as a gastric cancer tumor suppressor [21]. In many cancer types, deletion of the RUNX3 locus and reduction of its expression by promoter hypermethylation has been

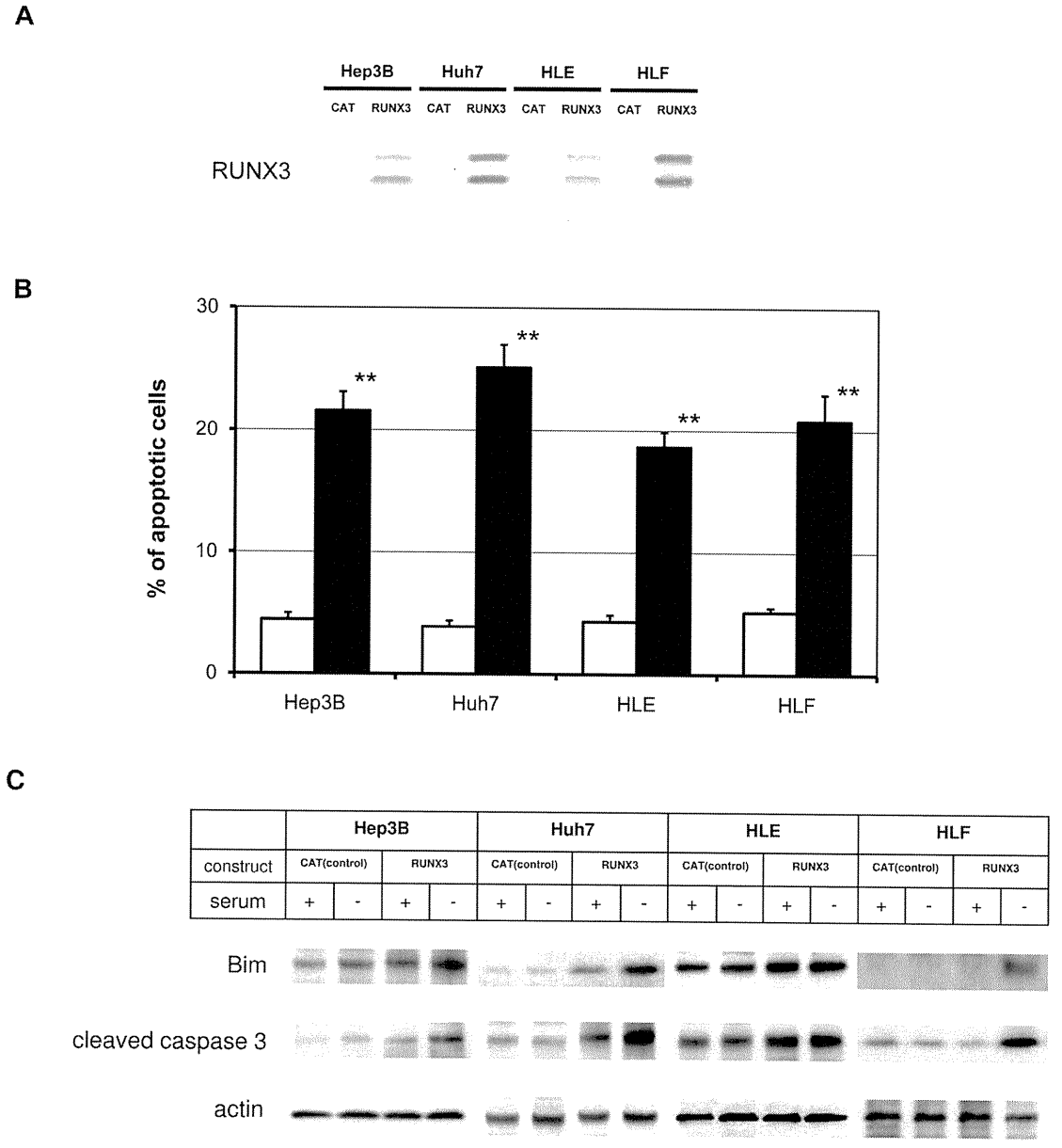

Figure 6 Effect of transient ectopic RUNX3 expression. CAT (control) and RUNX3 constructs were introduced into HCC cell lines. After a 48-h incubation period, an immunoblot analysis for RUNX3 expression (A), a DAPI apoptosis detection assay (B), and an immunoblot analysis for Bim and caspase (C) were performed. Shown here are representative blots from more than three independent experiments. All results are expressed as ratios to control CAT-transfected cells (CAT; white bars, RUNX3; black bars). Data represent the mean ± S.E. of more than five independent experiments, each with triplicates. **, $P < 0.01$ (vs. data at 0 h); Student's *t*-test.

reported [23-26]. However, little is known about the role of RUNX3 in HCC tumor suppression. We hypothesized that loss of RUNX3 expression contributes the development of HCC by escaping apoptosis. The results of the present study provide clear evidence that RUNX3 elicits serum starvation-induced apoptosis in HCC cells by activating the Bim-caspase pathway.

Stable expression of RUNX3 protein was established in Hep3B cells (Figure 3A), and they showed apoptosis under serum starved conditions (Figure 3B). This effect was reproducible in the Hep3B, Huh7, HLE, and HLF HCC cell lines transiently expressing RUNX3. The inhibition of cell growth in transient RUNX3-expressing cells was generally lower than that in stable RUNX3-

expressing Hep3B cells, probably due to low transfection efficiency.

Serum starvation-induced apoptosis is caused by caspase activation in ectopic RUNX3-expressing Hep3B cells (Figures 3C and 3D). To explore the signaling molecule responsible for apoptosis, Bim protein expression was induced in serum starved RUNX3-expressing Hep3B cells (Figure 4A). This is the first report demonstrating that RUNX3 enhances Bim expression under serum starved conditions in HCC cells, which appears to be consistent with the important role of Bim in previous studies on other types of cells. Bim expression was induced by the cooperation of RUNX3 and TGF-β in a study of gastric epithelial cells [21,31]. Bim protein also plays an important role in cell death [32]. Bim induces sequential activation of caspase-9 and -3 [32]. The potency of Bim as a cell death inducer is attenuated by Bax and Bcl-2 subfamily proteins [33]. The expression of Bax and Bcl was not affected by RUNX3 expression (Figure 4A). The expression of Bad (data not shown), a Bcl-2 antagonist known as a serum starvation-induced apoptosis initiator [34], increased with serum starvation but was not attenuated by RUNX3 expression (Figure 4A). Bim siRNA was used to evaluate whether Bim expression regulates serum starvation-induced apoptosis in RUNX3-expressing cells. As a result, Bim siRNA successfully knocked down Bim expression in RUNX3-expressing Hep3B cells (Figure 5A). Knockdown of Bim expression abrogated serum starvation-induced apoptosis in RUNX3-expressing Hep3B cells (Figure 5B). Consequently, RUNX3 expression enhanced serum starvation-induced apoptosis through the Bim-caspase pathway in Hep3B cells. This effect was reproducible in the Huh7, HLE, and HLF HCC cell lines transiently expressing RUNX3 (Figure 6).

Serum starvation triggered apoptosis in RUNX3-expressing HCC cells. As this leads to the question of how serum prevents apoptosis in RUNX3-expressing cells, RUNX3-expressing Hep3B cells were treated with TGF-α, EGF, or PDGF (Figure 4C). These growth factors reduced apoptosis in RUNX3-expressing Hep3B cells by activating the PI3/Akt signaling pathway (data not shown), which is consistent with a previous report [34].

RUNX3 induces apoptosis in the presence of TGF-β [21]. In a study of gastric epithelial cells, RUNX3 enhanced Bim expression during TGF-β-induced apoptosis [21,31]. In a study of a gastric and esophageal cancer cell lines, RUNX3 expression made cancer cells sensitive to TGF-β-induced apoptosis [21,35-38]. These reports suggest that TGF-β is required for RUNX3-related apoptosis. In the present study, ectopic RUNX3 expression enhanced serum starvation-induced apoptosis in the absence of TGF-β. This discrepancy may be explained by the autocrine action of TGF-β in Hep3B cells, which have an intact TGF-β signaling pathway [39]. Furthermore, some HCC cell lines, including Hep3B, produce TGF-β [40]. Further study is required to establish whether TGF-β is involved in the enhanced apoptosis of HCC.

It has been reported that p53, Rb, p16, phosphatase, and tensin homolog (PTEN) are altered in HCC. The p53 gene is the most extensively studied gene of solid tumors. Alteration of this gene occurs at a relative low frequency (28-42%) in HCC compared to other solid tumors [11,17,41,42]. The Rb gene is another well-studied tumor suppressor gene in HCC and other solid tumors. Rb mutations are found in only 15% of HCCs [42]. The LOH of chromosome 13q, where Rb gene is located, is more frequent in HCC (25-48%) [43,44]. The p16 gene, also known as the cyclin-dependent kinase inhibitor 2A gene, regulates the Rb pathway and is found in 64% of HCCs [9]. PTEN negatively regulates the PI3K/Akt signaling pathway, which is involved in the regulation of cell survival [45]. Alteration of PTEN was found in ~40% of HCCs [10]. The frequency of alteration of each individual gene was relatively low, while RUNX3 expression was frequently down-regulated in both human HCC cell lines (91%) and tissues (90%).

Alterations in some tumor suppressor genes are due to LOH in HCC [17]. Similar to other tumor suppressor genes, some of the alterations in RUNX3 are due to the LOH of chromosome 1p36, where RUNX3 is located. Perhaps another mechanism for RUNX3 down-regulation is hypermethylation of the RUNX3 promoter region [13-16]. In a previous report, 30-40% of HCCs showed LOH of the RUNX3 gene and 40-80% showed promoter hypermethylation [28]. In agreement with these reports, RUNX3 down-regulation was detected in ~90% of HCC tissue specimens.

Conclusions

RUNX3 expression elicits serum starvation-induced apoptosis in HCC cells via the Bim-caspase pathway. Because RUNX3 expression is generally suppressed in HCC cell lines and tissues, loss of RUNX3 expression leads to tumorigenesis by escaping apoptosis.

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Authors' contributions

HS conceived the design and drafted the manuscript. YN performed experiments. NT, ST, SN, MU, MM, MI and AT helped performing experiments for YN. SN, YK, KN, KK, HH, JT, HO and TY contributed for the collection of HCC tissues. YN performed immunohistochemical study. KY provides financial supports and participates in the discussion of the results. All authors read and approved the final manusctipt.

Competing interests

The authors declare that they have no competing interests.

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