A sustained virological response (SVR) was defined as HCV-RNA negativity, determined by reverse transcription-polymerase chain reaction, more than 6 months after the termination of IFN therapy. The rest of the patients were considered to have exhibited a nonsustained virological response (non-SVR).

Follow-up of the patients

After curative treatment of primary HCC, all patients underwent liver function tests every 1–2 months, and ultrasonography or three-phase dynamic CT scanning every 3 months. The serum levels of alpha-fetoprotein (AFP), AFP-L3, and des-γ-carboxy prothrombin (DCP) were also determined every 2–3 months. The recurrence of HCC was diagnosed using the same criteria as for the initial development of HCC.

Statistical analysis

Statistical analysis was performed using SAS version 9.1 package and JMP software, version 8.0 (SAS Institute, Cary, NC, USA). Differences between two groups were evaluated using the unpaired Student's t test. The χ^2 test or Fisher's exact probability test was used to compare categorical data. Cumulative incidence curves were determined with the Kaplan–Meier method, and the differences between groups were assessed using the log-rank test. Possible risk factors for survival and HCC recurrence were examined by the Cox proportional hazards regression model with the following 12 variables: interferon-related variables (application of interferon therapy, response to interferon therapy, and HCV genotype), background, liver function, and tumor factors at the first treatment and at recurrence of HCC [age, alanine aminotransferase (ALT), albumin (ALB), total bilirubin (T.Bil), platelet counts (PLT), prothrombin time (PT), AFP, DCP, maximum tumor size, and tumor number]. Parameters that proved to be significant in the univariate analysis were tested by the multivariate Cox proportional hazards regression model.

We also conducted propensity score (PS) matched analysis that can adjust the clinical background of the patients in each group. To calculate PS, we used seven covariates: sex of patients, and variables at the time of development of HCC (age at the time of development of HCC, ALT, ALB, T.Bil, PLT, maximum tumor size, and tumor numbers). The propensity score of choosing the IFN treatment was calculated, followed by matching IFN group and non-IFN group according to a greedy matching technique [34]. The survival and recurrence rates of matched patients were compared by the Kaplan–Meier method and the differences were evaluated by the log-rank test. A P value less than 0.05 was considered statistically significant.

Results

Characteristics of the patients

Table 1 shows the clinical features of the patients in the PEG-IFN and non-IFN (control) groups at the first treatment of HCC, and Table 2 shows their data at the first recurrence of HCC. Clinical and laboratory characteristics were similar in both groups, but those in the PEG-IFN group were slightly younger (63 vs. 67 years old), and

Table 1 Profiles and laboratory tests of the patients

Variables	PEG-IFN	Non-IFN	P value
Number of patients	37	145	
Age (years)	63 (48–77)	67 (43–85)	<0.01*
Sex (male)	29 (78%)	95 (65%)	0.10
HCV genotype (1b high/others/unknown)	23/14/0	55/30/60	0.83
Response to IFN therapy (SVR/non-SVR)	19/18		
Observation period (years)	4.5 (0.8–12.7)	3.3 (0.3–10.8)	0.01*
T.Bil (mg/dl)	0.7 (0.3–2.7)	0.9 (0.2–2.9)	0.04*
ALB (g/dl)	3.9 (2.5–4.7)	3.7 (2.2–4.6)	<0.01*
ALT (IU/l)	75 (17–168)	54 (14–183)	<0.01*
PLT ($\times$1,000/mm^3)	141 (31–307)	96 (34–281)	<0.01*
PT (%)	94 (62–118)	85 (48–145)	0.01*
AFP (ng/ml)	12 (1.6–1,729)	16.9 (0.6–54,535)	0.49
DCP (mAU/ml)	26 (0–5,230)	34 (0–66,700)	0.52
Number of tumors (solitary)	27 (72%)	105 (72%)	0.34
Size of main tumor (mm)	18 (7–55)	20 (9–74)	0.11
Disease stage (I/II/III/IVA)	16/15/6/0	47/48/44/6	0.88

All variables are shown as the median (range in parentheses) unless otherwise noted

IFN interferon, *PEG-IFN* pegylated interferon, *HCV* hepatitis C virus, *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 2 Profiles and laboratory tests of the patients at first recurrence

Variables	PEG-IFN	Non-IFN	*P* value
Number of patients	18	63	
Sex (male)	14 (78%)	40 (63%)	0.24
HCV genotype (1b high/others/unknown)	12/6/0	26/13/24	0.89
Response to IFN therapy (SVR/non-SVR)	8/10		
Treatment method (RFA/ope/PEIT/MCT/other)	15/0/0/1/2	50/4/5/2/2	0.20
T.Bil (mg/dl)	0.7 (0.4–1.4)	0.9 (0.3–2.6)	0.18
ALB (g/dl)	3.7 (2.9–5.0)	3.2 (2.8–4.6)	0.20
ALT (IU/l)	38 (9–295)	50 (16–137)	0.70
PLT ($\times$1,000/mm^3)	105 (39–250)	97 (43–31.2)	0.48
PT (%)	89 (65–117)	83 (35–124)	0.16
AFP (ng/ml)	12 (2.6–144)	11 (1.1–835)	0.40
DCP (mAU/ml)	23 (10–661)	41 (10–28,132)	0.51
Number of tumors (solitary)	11 (61%)	40 (63%)	0.71
Size of main tumor (mm)	13 (6–20)	15 (9–29)	0.16
Disease stage (I/II/III/IVA)	11/5/2/0	36/22/4/1	0.54

All variables are shown as the median (range) unless otherwise noted

IFN interferon, *PEG-IFN* pegylated interferon, *HCV* hepatitis C virus, *RFA* radiofrequency thermal ablation, *ope* operation, *PEIT* percutaneous ethanol injection therapy, *MCT* microwave coagulation therapy, *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

exhibited higher levels of ALB (3.9 vs. 3.7 g/dl), ALT (78 vs. 54 IU/l), and PLT (141 vs. 96 $\times$ 1,000/mm^3) than those in the non-IFN group. The median follow-up was 4.6 years for patients receiving PEG-IFN and 3.6 years for the controls. In the PEG-IFN group, 19 patients exhibited an SVR (12 monotherapy and 7 combination therapy), 2 were biochemical responders, and the other 17 patients were nonresponders.

Adherence and side effects of IFN therapy

Life-threatening adverse events were not observed in this study. In 11 cases of mild to moderate toxicity (5 thrombocytopenia, 3 anemia, and 3 neutropenia), IFN dose was reduced by 50%. Three patients eventually discontinued treatment with the drug because of adverse events: depression and severe malaise ($n = 1$), hemolytic anemia ($n = 1$), and IFN retinopathy ($n = 1$). In 8 cases with moderate toxicity, IFN treatment could be continued.

Cumulative survival rates of hepatocellular carcinoma

In this study, 2 patients in the PEG-IFN group and 39 patients in the non-IFN group died. All the patients who died had recurrence of HCC. The overall survival rate of PEG-IFN patients was higher than that of non-IFN patients (Fig. 1). Five-year survival rates of the PEG-IFN and non-IFN groups were 91% and 65%, respectively ($P < 0.01$).

Recurrence of hepatocellular carcinoma

At the end of the study, recurrence of HCC had occurred in 8 patients (42%) in the SVR group, 10 (55%) in the non-SVR group, and 63 (43%) in the non-IFN group.

The rate of first HCC recurrence after curative therapy of HCC in SVR patients tended to be lower than that in non-IFN patients (48 vs. 70% at 5 years, respectively, $P = 0.05$; Fig. 2); however, there was no significant difference between non-SVR patients and non-IFN patients (72 vs. 70% at 5 years, respectively; $P = 0.73$). In addition, there was no significant difference between the PEG-IFN group and the non-IFN group (58 vs. 70% at 5 years, respectively; $P = 0.17$). At first HCC recurrence, there was no significant difference in tumor number or liver function between the PEG-IFN and non-IFN groups; however, maximum tumor size in the PEG-IFN group was smaller than that in the non-IFN group (13 vs. 16 mm, respectively; $P = 0.03$). Fifteen of the 17 patients in the PEG-IFN group underwent curative treatment at the first recurrence of HCC.

The rate of second recurrence was not significantly different between the PEG-IFN and non-IFN groups (78 vs. 83% at 3 years, respectively; $P = 0.26$). However, the rate in the SVR group was significantly lower than that in the non-IFN group (65 vs. 83% at 3 years, respectively, $P = 0.03$; Fig. 3). At second HCC recurrence, in the PEG-IFN group, maximum tumor size was smaller (12 vs.

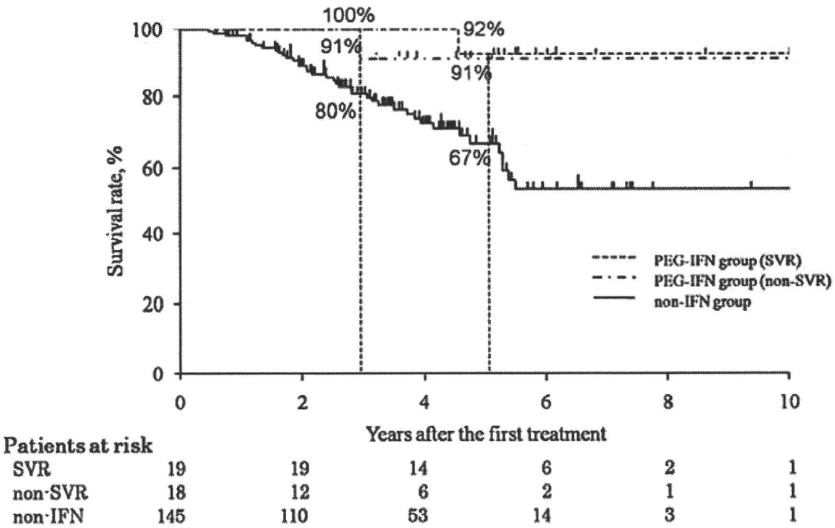

Fig. 1 Cumulative survival rates of pegylated interferon (PEG-IFN) group and non-interferon (non-IFN) group. Two patients in the PEG-IFN group died during the observation period. The survival rate was significantly different between the three groups ($P = 0.01$). *SVR* sustained virological response

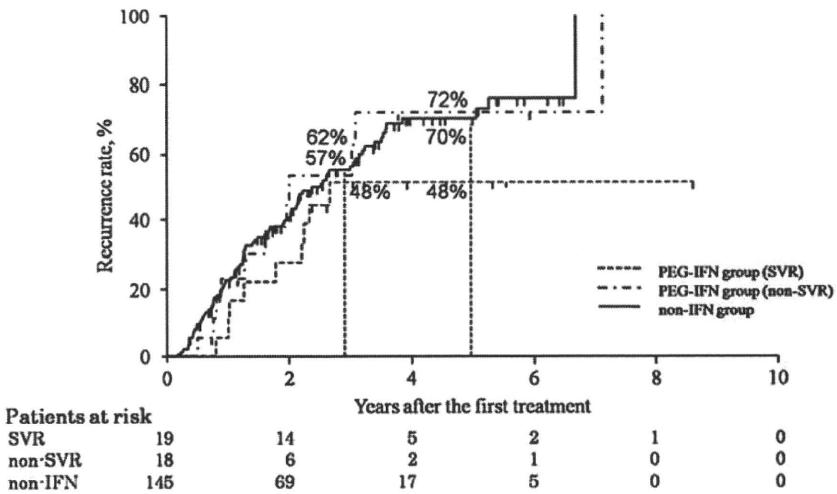

Fig. 2 The rates of first hepatocellular carcinoma (HCC) recurrence. The recurrence rate in SVR patients tended to be lower than that in non-IFN patients (48 vs. 70% at 5 years, respectively; $P = 0.05$); however, there was no significant difference between non-SVR patients and non-IFN patients (72 vs. 70% at 5 years, respectively; $P = 0.73$). *SVR* sustained virological response

15 mm, respectively; $P = 0.02$) and serum ALB was higher (3.3 vs. 3.1 g/dl, respectively; $P = 0.04$) than that in the non-IFN group.

Propensity score matched analysis

To minimize the biases of the PEG-IFN group and non-IFN group, we conducted a propensity score (PS) matched analysis. Thirty-four matched pairs were selected from the PEG-IFN group and non-IFN group by PS. No significant difference in clinical characteristics was observed between the groups (Table 3). Eighteen patients exhibited an SVR [11 monotherapy and 7 combination therapy, 9 (43%) genotype 1b high and 9 (69%) others]. Overall survival rate of the PEG-IFN group was higher than that of the non-IFN group ($P = 0.04$; Fig. 4). Although no significant difference in the first and second HCC recurrence ($P = 0.55$ and 0.62, respectively) was observed between the IFN group and non-IFN group, the rate of second recurrence in the SVR group was significantly lower than that in the non-IFN group (65 vs. 79% at 3 years, respectively, $P = 0.01$; Figs. 5, 6).

Prognostic factors and risk factors of HCC recurrence

To identify the factors that contributed to survival and the recurrence of HCC, a Cox proportional hazard analysis was performed.

Univariate analysis showed that PEG-IFN therapy, low T.Bil, and high serum ALB were independent factors favorably associated with long survival. Among the factors that were significant in the analysis, PEG-IFN therapy [risk ratio = 2.72; 95% confidence interval (CI), 1.29–9.04] and a serum ALB level >3.5 g/dl (risk ratio = 2.51; 95% CI, 1.29–4.98) were shown to be significantly associated with better survival in the multivariate analysis (Table 4).

On the other hand, non-SVR, low ALB, and large and multiple tumors at the initial treatment were significantly

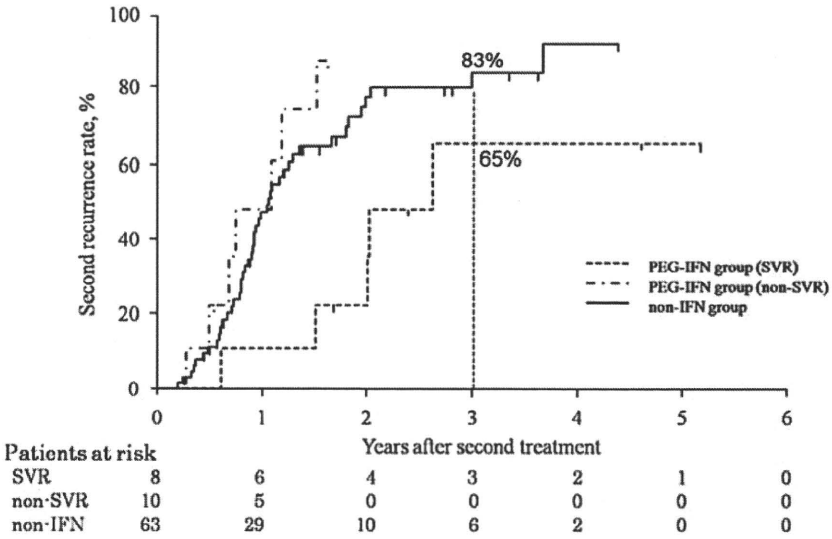

Fig. 3 Rates of second HCC recurrence. The second recurrence rate in the SVR group was significantly lower than that in the non-IFN group (65 vs. 83% at 3 years, respectively; $P = 0.03$. *SVR* sustained virological response

Table 3 Profiles and laboratory tests of the patients (propensity score matched cases)

Variables	PEG-IFN	Non-IFN	*P* value
Number of patients	34	34	
Age (years)	64 (48–77)	64 (43–85)	0.97
Sex (male)	26 (76%)	29 (85%)	0.48
HCV genotype (1b high/others/unknown)	21/13/0	17/8/9	0.62
Response to IFN therapy (SVR/non-SVR)	18/16		
Observation period (years)	4.6 (0.8–12.7)	3.4 (0.8–10.8)	0.22
T.Bil (mg/dl)	0.7 (0.3–2.7)	0.7 (0.43–1.8)	0.77
ALB (g/dl)	3.9 (2.5–4.7)	3.6 (3.1–4.7)	0.83
ALT (IU/l)	69 (17–168)	61 (17–183)	0.43
PLT ($\times$1,000/mm^3)	147 (31–307)	137 (42–216)	0.49
PT (%)	95 (62–118)	85 (52–110)	0.07
AFP (ng/ml)	11 (1.6–1,729)	10.8 (1.3–11,006)	0.38
DCP (mAU/ml)	29 (0–5,230)	27 (0–66,700)	0.34
Number of tumors (solitary)	25 (74%)	27 (79%)	0.81
Size of main tumor (mm)	19 (7–55)	21 (9–50)	0.06
Disease stage (I/II/III/IVA)	14/14/6/0	12/11/9/2	0.27

All variables are shown as the median (range) unless otherwise noted

IFN interferon, *PEG-IFN* pegylated interferon, *HCV* hepatitis C virus, *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

associated with first recurrence of HCC in univariate analysis. Multivariate analysis showed that low ALB (risk ratio = 1.70; 95% CI, 1.11–2.56) and large (risk ratio = 1.65; 95% CI, 1.02–2.59) and multiple (risk ratio = 1.66; 95% CI, 1.05–2.56) tumors were independent risk factors; however, response to PEG-IFN therapy was not determined to be a significant factor for the first recurrence of HCC (risk ratio = 1.60; 95% CI, 0.83–3.48; Table 5).

Regarding the second recurrence of HCC, non-SVR (risk ratio = 2.51; 95% CI, 1.06–7.40) and low ALB at the first recurrence of HCC (risk ratio = 2.56; 95% CI, 1.46–4.83) were found to be independent risk factors in multivariate analysis as well as univariate analysis (Table 6).

Discussion

Persistent active hepatitis is common in the advanced stage of chronic HCV infection and is a risk factor for the development of HCC. Several reports have shown the inhibitory effects of IFN therapy on the development of HCC. In these reports, the inhibitory effect was considered to be the result of the remission of inflammation, necrosis, and fibrosis in addition to the direct action of IFN on tumor cells [35–39]. Recently, several studies were conducted to show the effect of IFN therapy after curative treatment of HCC, which reduced the risk for recurrence and improved the rate of survival. To date, reports on eight randomized control trials (RCTs) [17–24] and six non-RCTs [25–30] on this effect have been published.

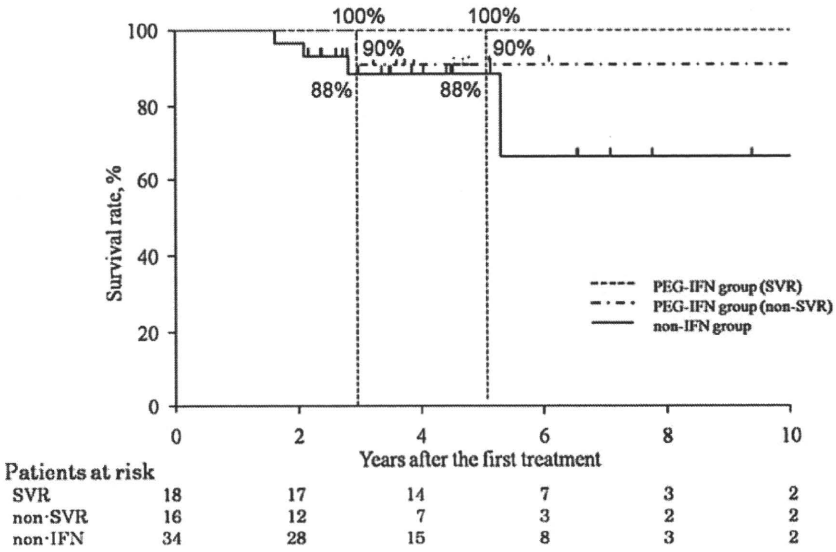

Fig. 4 Cumulative survival rates of PEG-IFN group and non-IFN group after propensity score (PS) matching. Overall-survival rate of the PEG-IFN group was higher than that of non-IFN group ($P = 0.04$). *SVR* sustained virological response

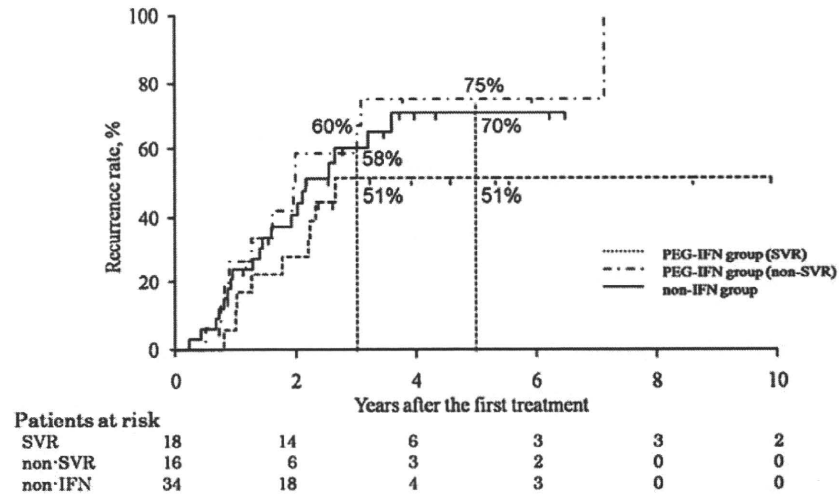

Fig. 5 Rates of first HCC recurrence after PS matching. We found no significant differences between the two groups with respect to first HCC recurrence ($P = 0.55$). *SVR* sustained virological response

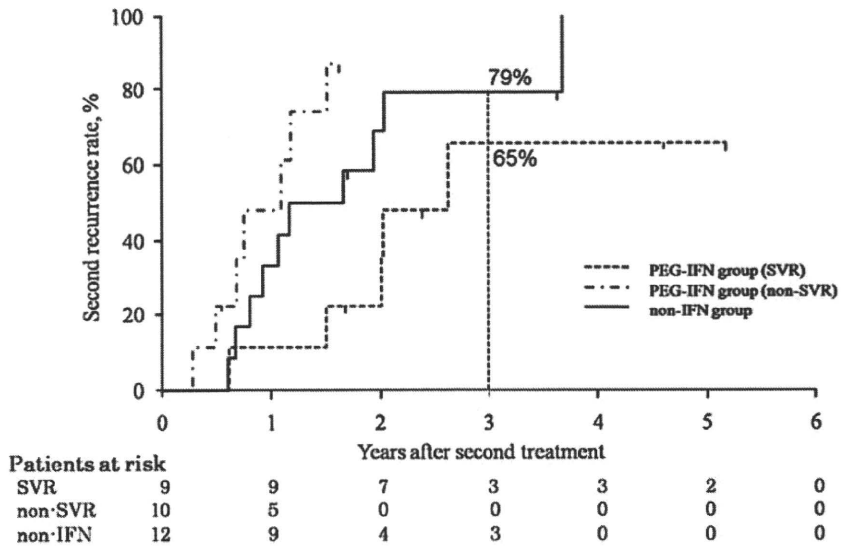

Fig. 6 Rates of second HCC recurrence after PS matching. The second recurrence rate in the SVR group was significantly lower than that in the non-IFN group (65 vs. 79% at 3 years, respectively; $P = 0.01$), although no statistical difference was observed between the IFN group and non-IFN group ($P = 0.62$). *SVR* sustained virological response

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.

References

1. Tsukuma H, Hiyama T, Tanaka S et al (1993) Risk factors for hepatocellular carcinoma among patients with chronic liver disease. N Engl J Med 328:1797–1801
2. Takano S, Yokosuka O, Imazeki F et al (1995) Incidence of hepatocellular carcinoma in chronic hepatitis B and C: a prospective study of 251 patients. Hepatology 21:650–655
3. Shiratori Y, Shiina S, Imamura M et al (1995) Characteristic difference of hepatocellular carcinoma between hepatitis B- and C-viral infection in Japan. Hepatology 22:1027–1033
4. Ikeda K, Saitoh S, Tsubota A et al (1993) Risk factors for tumor recurrence and prognosis after curative resection of hepatocellular carcinoma. Cancer (Phila) 71:19–25
5. Tateishi R, Shiina S, Teratani T et al (2005) Percutaneous radiofrequency ablation for hepatocellular carcinoma. An analysis of 1000 cases. Cancer (Phila) 103:1201–1209
6. Mazziotti A, Grazi GL, Cavallari A (1998) Surgical treatment of hepatocellular carcinoma on cirrhosis: a Western experience. Hepatogastroenterology 45(suppl 3):1281–1287
7. Livraghi T, Goldberg SN, Lazzaroni S et al (1999) Small hepatocellular carcinoma: treatment with radio-frequency ablation versus ethanol injection. Radiology 210:655–661
8. Kubo S, Nishiguchi S, Shuto T et al (1999) Effects of continuous hepatitis with persistent hepatitis C viremia on outcome after resection of hepatocellular carcinoma. Jpn J Cancer Res 90:162–170
9. Kumada T, Nakano S, Takeda I et al (1997) Patterns of recurrence after initial treatment in patients with small hepatocellular carcinoma. Hepatology 25:87–92
10. Shimada M, Takenaka K, Gion T et al (1996) Prognosis of recurrent hepatocellular carcinoma: a 10-year surgical experience in Japan. Gastroenterology 111:720–726
11. Nagasue N, Uchida M, Makino Y et al (1993) Incidence and factors associated with intrahepatic recurrence following resection of hepatocellular carcinoma. Gastroenterology 105:488–494
12. Davis GL, Balart LA, Schiff ER et al (1989) Treatment of chronic hepatitis C with recombinant interferon alfa. A multicenter randomized, controlled trial. Hepatitis Interventional Therapy Group. N Engl J Med 321:1501–1506
13. Di Bisceglie AM, Martin P, Kassianides C et al (1989) Recombinant interferon alfa therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. N Engl J Med 321:1506–1510
14. Nishiguchi S, Kuroki T, Nakatani S et al (1995) Randomised trial of effects of interferon-alpha on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. Lancet 346:1051–1055
15. Yu ML, Lin SM, Chuang WL et al (2006) A sustained virological response to interferon or interferon/ribavirin reduces hepatocellular carcinoma and improves survival in chronic hepatitis C: a nationwide, multicentre study in Taiwan. Antivir Ther 11:985–994
16. Tanaka H, Tsukuma H, Kasahara A et al (2000) Effect of interferon therapy on the incidence of hepatocellular carcinoma and mortality of patients with chronic hepatitis C: a retrospective cohort study of 738 patients. Int J Cancer 87:741–749
17. Shiratori Y, Shiina S, Teratani T et al (2003) Interferon therapy after tumor ablation improves prognosis in patients with hepatocellular carcinoma associated with hepatitis C virus. Ann Intern Med 138:299–306
18. Kubo S, Nishiguchi S, Hirohashi K et al (2001) Effects of long-term postoperative interferon alpha therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. Ann Intern Med 134:963–967
19. Kubo S, Nishiguchi S, Hirohashi K et al (2002) Randomized clinical trial of long-term outcome after resection of hepatitis C virus-related hepatocellular carcinoma by postoperative interferon therapy. Br J Surg 89:418–422
20. Mazzaferro V, Romito R, Schiavo M et al (2006) Prevention of hepatocellular carcinoma recurrence with alpha-interferon after liver resection in HCV cirrhosis. Hepatology 44:1543–1554
21. Lin SM, Lin CJ, Hsu CW et al (2003) Prospective randomized controlled study of interferon-alpha in preventing hepatocellular carcinoma recurrence after medical ablation therapy for primary tumors. Cancer (Phila) 100:376–382
22. Lo CM, Liu CL, Chan SC et al (2007) A randomized, controlled trial of postoperative adjuvant interferon therapy after resection of hepatocellular carcinoma. Ann Surg 245:831–842
23. Ikeda K, Arase Y, Saitoh S et al (2000) Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor: a prospective randomized study of hepatitis C virus-related liver cancer. Hepatology 32:228–232
24. Sun HC, Tang ZY, Wang L et al (2006) Postoperative interferon alpha treatment postponed recurrence and improved overall survival in patients after curative resection of HBV-related hepatocellular carcinoma: a randomized clinical trial. J Cancer Res Clin Oncol 132:458–465
25. Sakaguchi Y, Kudo M, Fukunaga T et al (2005) Low-dose, long-term, intermittent interferon-alpha-2b therapy after radical treatment by radiofrequency ablation delays clinical recurrence in patients with hepatitis C virus-related hepatocellular carcinoma. Intervirology 48:64–70
26. Suou T, Mitsuda A, Koda M et al (2001) Interferon alpha inhibits intrahepatic recurrence in hepatocellular carcinoma with chronic hepatitis C: a pilot study. Hepatol Res 20:301–311
27. Hung CH, Lee CM, Wang JH et al (2005) Antiviral therapy after non-surgical tumor ablation in patients with hepatocellular carcinoma associated with hepatitis C virus. J Gastroenterol Hepatol 20:1553–1559
28. Jeong S, Aikata H, Katamura Y et al (2007) Low-dose intermittent interferon-alpha therapy for HCV-related liver cirrhosis after curative treatment of hepatocellular carcinoma. World J Gastroenterol 13:5188–5195
29. Kudo M, Sakaguchi Y, Chung H et al (2007) Long-term interferon maintenance therapy improves survival in patients with HCV-related hepatocellular carcinoma after curative radiofrequency ablation. A matched case-control study. Oncology 72(suppl 1):132–138
30. Someya T, Ikeda K, Saitoh S et al (2006) Interferon lowers tumor recurrence rate after surgical resection or ablation of hepatocellular carcinoma: a pilot study of patients with hepatitis B virus-related cirrhosis. J Gastroenterol 41:1206–1213
31. Cammà C, Di Bona D, Schepis F et al (2004) Effect of peg-interferon alfa-2a on liver histology in chronic hepatitis C: a meta-analysis of individual patient data. Hepatology 39:333–342
32. Lindsay KL, Trepo C, Heintges T et al (2001) A randomized, double-blind trial comparing pegylated interferon alfa-2b to

interferon alfa-2b as initial treatment for chronic hepatitis C. Hepatology 34:395–403
33. Kumada H, Okanoue T, Onji M et al (2010) The Study Group for the Standardization of Treatment of Viral Hepatitis Including Cirrhosis, Ministry of Health, Labour, Welfare of Japan. Guidelines for the treatment of chronic hepatitis, cirrhosis due to hepatitis C virus infection for the fiscal year 2008 in Japan. Hepatol Res 40:8–13
34. Parsons LS, Ovation Research Group (2001) Reducing bias in a propensity score matched-pair sample using greedy matching techniques. In: Proceedings of the Twenty-Sixth Annual SAS Users Group International Conference. SAS Institute Inc., Cary, pp 214–226
35. Hisaka T, Yano H, Ogasawara S et al (2004) Interferon-alpha-Con1 suppresses proliferation of liver cancer cell lines in vitro and in vivo. J Hepatol 41:782–789
36. Ogasawara S, Yano H, Momosaki S et al (2007) Growth inhibitory effects of IFN-beta on human liver cancer cells in vitro and in vivo. J Interferon Cytokine Res 27:507–516
37. Nakaji M, Yano Y, Ninomiya T et al (2004) IFN-alpha prevents the growth of pre-neoplastic lesions and inhibits the development of hepatocellular carcinoma in the rat. Carcinogenesis (Oxf) 25:389–397
38. Yano H, Ogasawara S, Momosaki S et al (2006) Growth inhibitory effects of pegylated IFN alpha-2b on human liver cancer cells in vitro and in vivo. Liver Int 26:964–975
39. Miki A, Yano Y, Kato H et al (2008) Anti-tumor effect of pegylated interferon in the rat hepatocarcinogenesis model. Int J Oncol 32:603–608
40. Chung RT, Andersen J, Volberding P et al (2004) Peginterferon Alfa-2a plus ribavirin versus interferon alfa-2a plus ribavirin for chronic hepatitis C in HIV-coinfected persons. N Engl J Med 351:451–459
41. Carrat F, Bani-Sadr F, Pol S et al (2004) Pegylated interferon alfa-2b vs standard interferon alfa-2b, plus ribavirin, for chronic hepatitis C in HIV-infected patients: a randomized controlled trial. JAMA 292:2839–2848

Nakanishi et al. BMC Cancer 2011, 11:3
http://www.biomedcentral.com/1471-2407/11/3

BMC Cancer

RESEARCH ARTICLE Open Access

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

Yutaka Nakanishi[1], Hidenori Shiraha[1*], Shin-ichi Nishina[1], Shigetomi Tanaka[1], Minoru Matsubara[1], Shigeru Horiguchi[1], Masaya Iwamuro[1], Nobuyuki Takaoka[1], Masayuki Uemura[1], Kenji Kuwaki, Hiroaki Hagihara, Junichi Toshimori, Hideki Ohnishi[1], Akinobu Takaki[1], Shinichiro Nakamura[1], Yoshiyuki Kobayashi[1], Kazuhiro Nouso[1,2], Takahito Yagi[3], Kazuhide Yamamoto[1]

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

* Correspondence: hshiraha@md.okayama-u.ac.jp
[1]Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan
Full list of author information is available at the end of the article

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

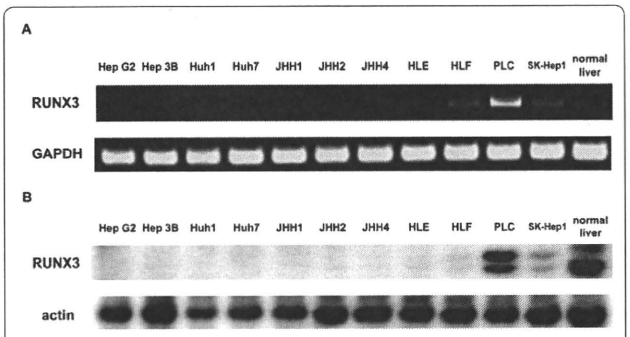

Figure 1 RUNX3 mRNA (A) and protein (B) expression in HCC cell lines. (A) RUNX3 and GAPDH mRNA expression levels were determined by RT-PCR. Shown here are representative gels from three independent experiments. (B) RUNX3 and α-actin protein expression was analyzed by immunoblot using the anti-RUNX3 antibody.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

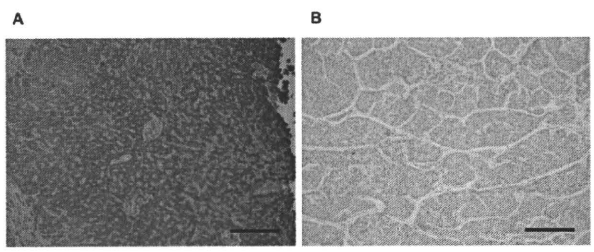

Figure 2 Immunohistochemical staining of RUNX3 in human liver tissues. (A) nontumor liver tissue with a protein score of 3 (B) HCC tissue with a protein score of 0. Bar = 100 μm.

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

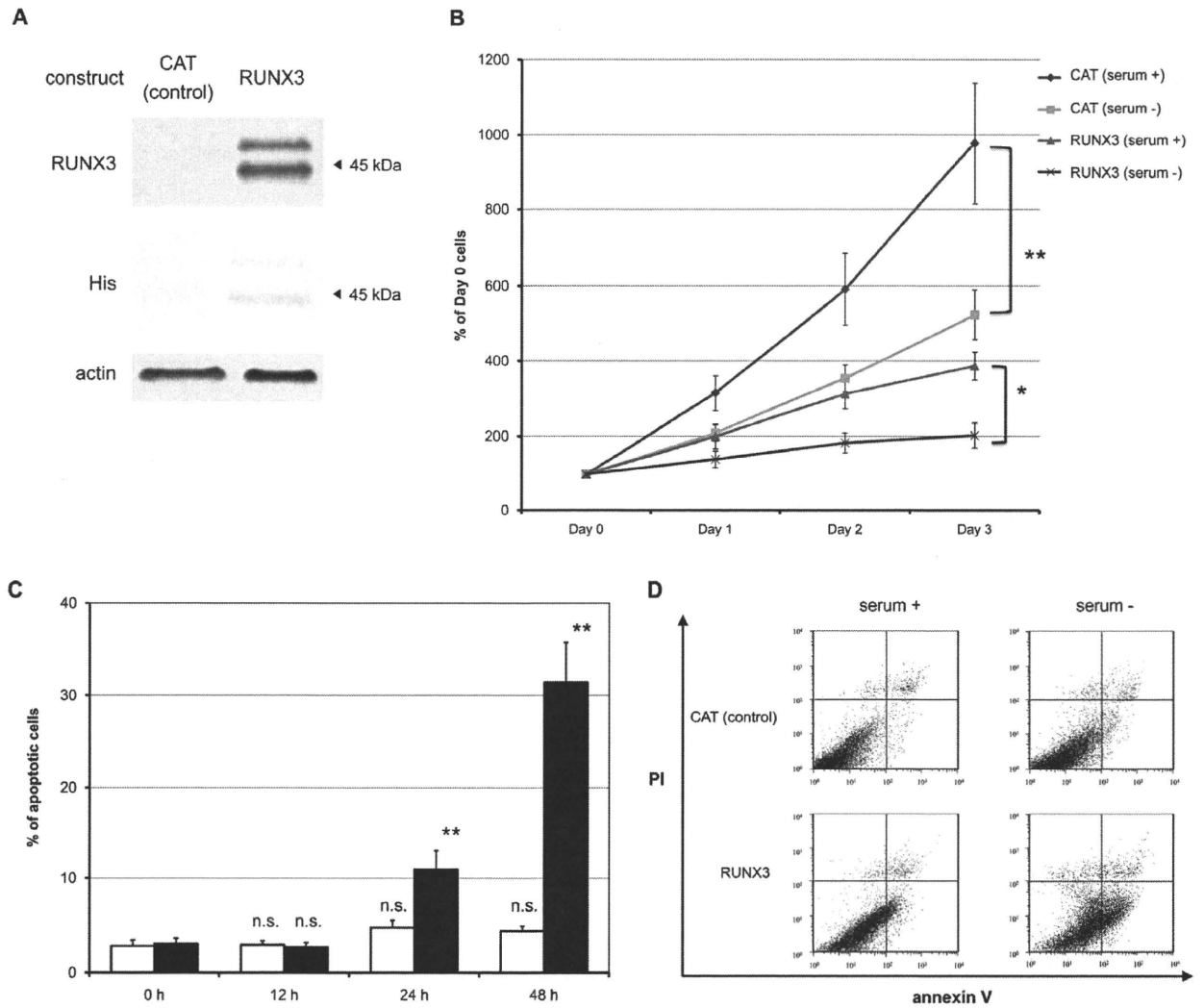

Figure 3 RUNX3 expression in Hep3B cells. CAT (control) and RUNX3 constructs were introduced into Hep3B cells. Polyclonal stably expressing cell lines were selected in the presence of hygromycin. (A) Immunoblot analysis RUNX3 protein expression was analyzed by immunoblot using anti-RUNX3 and anti-His 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).

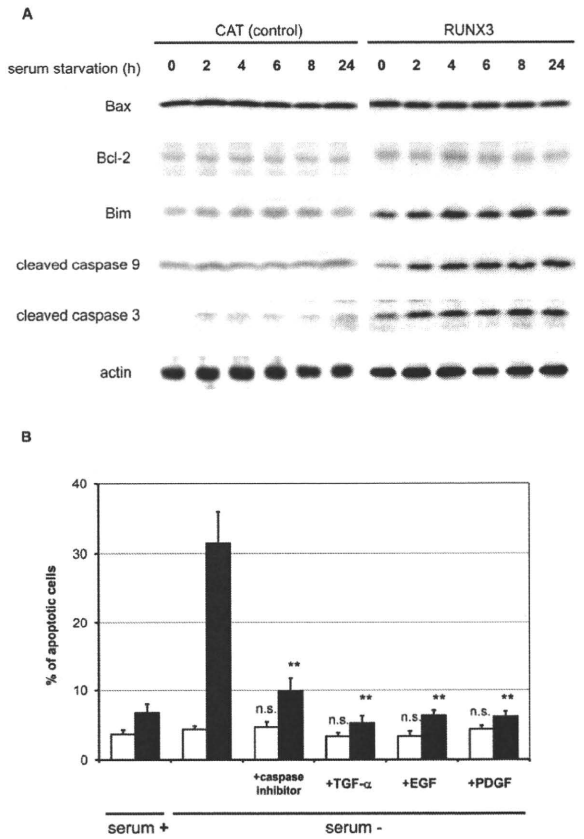

Figure 4 Effect of RUNX3 on apoptosis-related signaling molecules. (A) Immunoblot analysis Equal amounts of cell lysates were size-fractionated with 10% SDS-PAGE and immunoblotted with antibodies against Bax, Bcl-2, Bim, cleaved caspase-9, cleaved caspase-3, and actin. Shown here are representative blots 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.

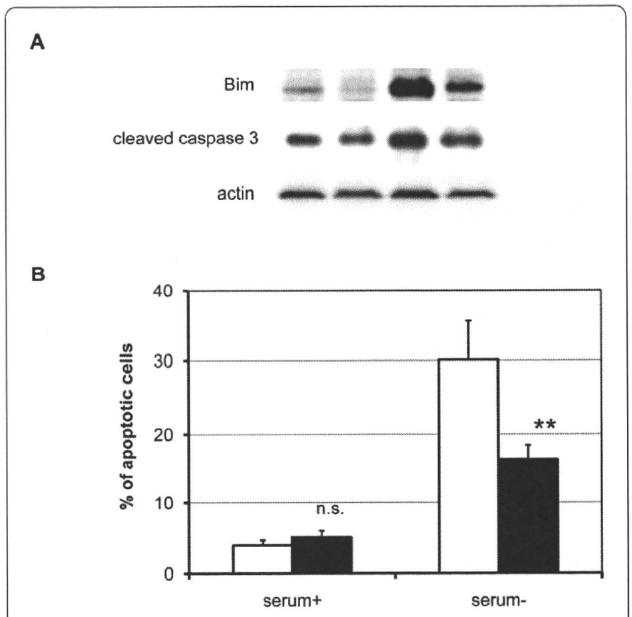

Figure 5 Effect of Bim siRNA gene silencing on cell growth inhibition and apoptosis. After treatment with Bim siRNA duplexes, an immunoblot analysis (A) and DAPI apoptosis detection assay (B) were performed. Shown here are representative blots from more than three independent experiments. All results 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

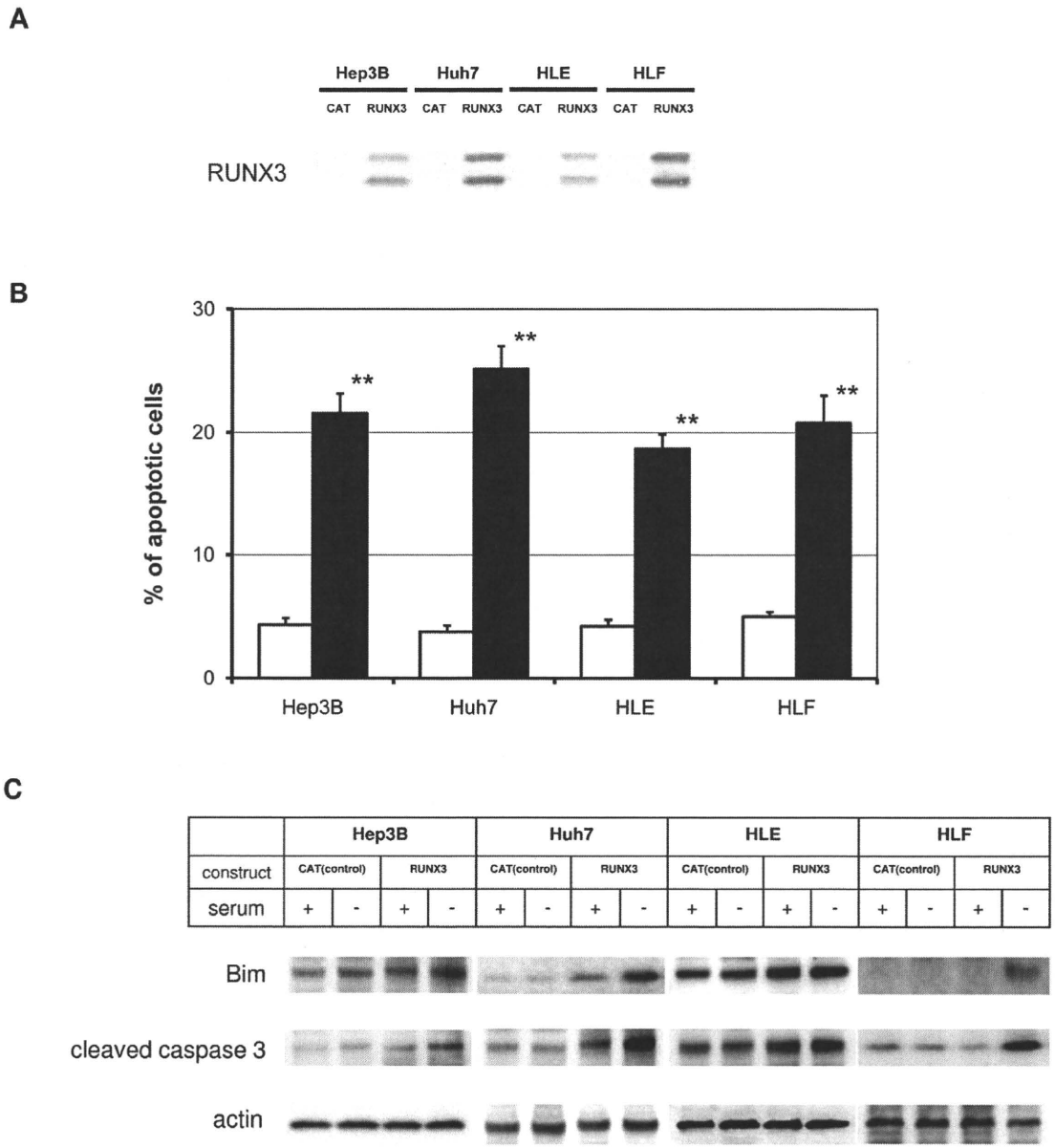

Figure 6 Effect of transient ectopic RUNX3 expression. CAT (control) and RUNX3 constructs were introduced into HCC cell lines. After a 48-h incubation period, an immunoblot analysis for RUNX3 expression (A), a DAPI apoptosis detection assay (B), and an immunoblot analysis for Bim and caspase (C) were 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.

Acknowledgements

We thank Tatsuya Fujikawa and Naoki Ueda for their valuable suggestions, and Noriaki Tanaka for providing the HCC tissues.

Author details

[1]Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. [2]Department of Molecular Hepatology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan. [3]Department of Gastroenterological Surgery, Transplant, and Surgical Oncology, Okayama University Graduate School of Medicine and Dentistry, 2-5-1 Shikata-cho, Okayama 700-8558, Japan.

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.

Received: 10 August 2010 Accepted: 4 January 2011
Published: 4 January 2011

References

1. El-Serag HB, Rudolph KL: **Hepatocellular carcinoma: epidemiology and molecular carcinogenesis.** *Gastroenterology* 2007, **132(7)**:2557-2576.
2. Garcia M, Jernal A, Ward EM, M CM, Hao Y, Siegel RI, Thun MJ: **Global Cancer Facts & Figures 2007.** Society AC. Atlanta, GA; 2007.
3. Parkin DM, Bray F, Ferlay J, Pisani P: **Estimating the world cancer burden: Globocan 2000.** *Int J Cancer* 2001, **94(2)**:153-156.
4. El-Serag HB, Mason AC: **Rising incidence of hepatocellular carcinoma in the United States.** *N Engl J Med* 1999, **340(10)**:745-750.
5. Kremer-Tal S, Reeves HL, Narla G, Thung SN, Schwartz M, Difeo A, Katz A, Bruix J, Bioulac-Sage P, Martignetti JA, *et al*: **Frequent inactivation of the tumor suppressor Kruppel-like factor 6 (KLF6) in hepatocellular carcinoma.** *Hepatology* 2004, **40(5)**:1047-1052.
6. Kaposi-Novak P, Lee JS, Gomez-Quiroz L, Coulouarn C, Factor VM, Thorgeirsson SS: **Met-regulated expression signature defines a subset of human hepatocellular carcinomas with poor prognosis and aggressive phenotype.** *The Journal of clinical investigation* 2006, **116(6)**:1582-1595.
7. Shachaf CM, Kopelman AM, Arvanitis C, Karlsson A, Beer S, Mandl S, Bachmann MH, Borowsky AD, Ruebner B, Cardiff RD, *et al*: **MYC inactivation uncovers pluripotent differentiation and tumour dormancy in hepatocellular cancer.** *Nature* 2004, **431(7012)**:1112-1117.
8. Sicklick JK, Li YX, Melhem A, Schmelzer E, Zdanowicz M, Huang J, Caballero M, Fair JH, Ludlow JW, McClelland RE, *et al*: **Hedgehog signaling maintains resident hepatic progenitors throughout life.** *American journal of physiology* 2006, **290(5)**:G859-870.
9. Azechi H, Nishida N, Fukuda Y, Nishimura T, Minata M, Katsuma H, Kuno M, Ito T, Komeda T, Kita R, *et al*: **Disruption of the p16/cyclin D1/retinoblastoma protein pathway in the majority of human hepatocellular carcinomas.** *Oncology* 2001, **60(4)**:346-354.
10. Hu TH, Huang CC, Lin PR, Chang HW, Ger LP, Lin YW, Changchien CS, Lee CM, Tai MH: **Expression and prognostic role of tumor suppressor gene PTEN/MMAC1/TEP1 in hepatocellular carcinoma.** *Cancer* 2003, **97(8)**:1929-1940.
11. Tannapfel A, Busse C, Weinans L, Benicke M, Katalinic A, Geissler F, Hauss J, Wittekind C: **INK4a-ARF alterations and p53 mutations in hepatocellular carcinomas.** *Oncogene* 2001, **20(48)**:7104-7109.
12. Yamada T, De Souza AT, Finkelstein S, Jirtle RL: **Loss of the gene encoding mannose 6-phosphate/insulin-like growth factor II receptor is an early event in liver carcinogenesis.** *Proc Natl Acad Sci USA* 1997, **94(19)**:10351-10355.
13. Fujimoto Y, Hampton LL, Wirth PJ, Wang NJ, Xie JP, Thorgeirsson SS: **Alterations of tumor suppressor genes and allelic losses in human hepatocellular carcinomas in China.** *Cancer Res* 1994, **54(1)**:281-285.
14. Kawai H, Suda T, Aoyagi Y, Isokawa O, Mita Y, Waguri N, Kuroiwa T, Igarashi M, Tsukada K, Mori S, *et al*: **Quantitative evaluation of genomic instability as a possible predictor for development of hepatocellular carcinoma: comparison of loss of heterozygosity and replication error.** *Hepatology* 2000, **31(6)**:1246-1250.
15. Nishida N, Nagasaka T, Nishimura T, Ikai I, Boland CR, Goel A: **Aberrant methylation of multiple tumor suppressor genes in aging liver, chronic hepatitis, and hepatocellular carcinoma.** *Hepatology* 2008, **47(3)**:908-918.
16. Yang B, Guo M, Herman JG, Clark DP: **Aberrant promoter methylation profiles of tumor suppressor genes in hepatocellular carcinoma.** *Am J Pathol* 2003, **163(3)**:1101-1107.
17. Buendia MA: **Genetics of hepatocellular carcinoma.** *Semin Cancer Biol* 2000, **10(3)**:185-200.
18. Ito K, Liu Q, Salto-Tellez M, Yano T, Tada K, Ida H, Huang C, Shah N, Inoue M, Rajnakova A, *et al*: **RUNX3, a novel tumor suppressor, is frequently inactivated in gastric cancer by protein mislocalization.** *Cancer Res* 2005, **65(17)**:7743-7750.
19. Ito Y, Miyazono K: **RUNX transcription factors as key targets of TGF-beta superfamily signaling.** *Curr Opin Genet Dev* 2003, **13(1)**:43-47.
20. Hanai J, Chen LF, Kanno T, Ohtani-Fujita N, Kim WY, Guo WH, Imamura T, Ishidou Y, Fukuchi M, Shi MJ, *et al*: **Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Calpha promoter.** *J Biol Chem* 1999, **274(44)**:31577-31582.
21. Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB, *et al*: **Causal relationship between the loss of RUNX3 expression and gastric cancer.** *Cell* 2002, **109(1)**:113-124.
22. Yamamura Y, Lee WL, Inoue K, Ida H, Ito Y: **RUNX3 cooperates with FoxO3a to induce apoptosis in gastric cancer cells.** *J Biol Chem* 2006, **281(8)**:5267-5276.
23. Araki K, Osaki M, Nagahama Y, Hiramatsu T, Nakamura H, Ohgi S, Ito H: **Expression of RUNX3 protein in human lung adenocarcinoma: Implications for tumor progression and prognosis.** *Cancer Sci* 2005, **96(4)**:227-231.
24. Ku JL, Kang SB, Shin YK, Kang HC, Hong SH, Kim IJ, Shin JH, Han IO, Park JG: **Promoter hypermethylation downregulates RUNX3 gene expression in colorectal cancer cell lines.** *Oncogene* 2004, **23(40)**:6736-6742.
25. Li J, Kleeff J, Guweidhi A, Esposito I, Berberat PO, Giese T, Buchler MW, Friess H: **RUNX3 expression in primary and metastatic pancreatic cancer.** *J Clin Pathol* 2004, **57(3)**:294-299.
26. Wada M, Yazumi S, Takaishi S, Hasegawa K, Sawada M, Tanaka H, Ida H, Sakakura C, Ito K, Ito Y, *et al*: **Frequent loss of RUNX3 gene expression in human bile duct and pancreatic cancer cell lines.** *Oncogene* 2004, **23(13)**:2401-2407.
27. Mori T, Nomoto S, Koshikawa K, Fujii T, Sakai M, Nishikawa Y, Inoue S, Takeda S, Kaneko T, Nakao A: **Decreased expression and frequent allelic inactivation of the RUNX3 gene at 1p36 in human hepatocellular carcinoma.** *Liver Int* 2005, **25(2)**:380-388.
28. Xiao WH, Liu WW: **Hemizygous deletion and hypermethylation of RUNX3 gene in hepatocellular carcinoma.** *World J Gastroenterol* 2004, **10(3)**:376-380.
29. Li X, Zhang Y, Qiao T, Wu K, Ding J, Liu J, Fan D: **RUNX3 Inhibits Growth of HCC Cells and HCC Xenografts in Mice in Combination With Adriamycin.** *Cancer Biol Ther* 2008, **7(5)**.
30. Ng IO, Chung LP, Tsang SW, Lam CL, Lai EC, Fan ST, Ng M: **p53 gene mutation spectrum in hepatocellular carcinomas in Hong Kong Chinese.** *Oncogene* 1994, **9(3)**:985-990.
31. Yano T, Ito K, Fukamachi H, Chi XZ, Wee HJ, Inoue K, Ida H, Bouillet P, Strasser A, Bae SC, *et al*: **The RUNX3 tumor suppressor upregulates Bim in gastric epithelial cells undergoing transforming growth factor beta-induced apoptosis.** *Mol Cell Biol* 2006, **26(12)**:4474-4488.
32. O'Connor L, Strasser A, O'Reilly LA, Hausmann G, Adams JM, Cory S, Huang DC: **Bim: a novel member of the Bcl-2 family that promotes apoptosis.** *Embo J* 1998, **17(2)**:384-395.
33. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A: **The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex.** *Molecular cell* 1999, **3(3)**:287-296.
34. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME: **Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor.** *Cell* 1999, **96(6)**:857-868.
35. Guo C, Ding J, Yao L, Sun L, Lin T, Song Y, Fan D: **Tumor suppressor gene Runx3 sensitizes gastric cancer cells to chemotherapeutic drugs by downregulating Bcl-2, MDR-1 and MRP-1.** *Int J Cancer* 2005.
36. Osaki M, Moriyama M, Adachi K, Nakada C, Takeda A, Inoue Y, Adachi H, Sato K, Oshimura M, Ito H: **Expression of RUNX3 protein in human gastric mucosa, intestinal metaplasia and carcinoma.** *Eur J Clin Invest* 2004, **34(9)**:605-612.
37. Torquati A, O'Rear L, Longobardi L, Spagnoli A, Richards WO, Daniel Beauchamp R: **RUNX3 inhibits cell proliferation and induces apoptosis by reinstating transforming growth factor beta responsiveness in esophageal adenocarcinoma cells.** *Surgery* 2004, **136(2)**:310-316.
38. Jin YH, Jeon EJ, Li QL, Lee YH, Choi JK, Kim WJ, Lee KY, Bae SC: **Transforming growth factor-beta stimulates p300-dependent RUNX3**

acetylation, which inhibits ubiquitination-mediated degradation. *J Biol Chem* 2004, **279(28)**:29409-29417.

39. Li G, Wang S, Gelehrter TD: **Identification of glucocorticoid receptor domains involved in transrepression of transforming growth factor-beta action.** *J Biol Chem* 2003, **278(43)**:41779-41788.
40. Mouri H, Sakaguchi K, Sawayama T, Senoh T, Ohta T, Nishimura M, Fujiwara A, Terao M, Shiratori Y, Tsuji T: **Suppressive effects of transforming growth factor-beta1 produced by hepatocellular carcinoma cell lines on interferon-gamma production by peripheral blood mononuclear cells.** *Acta Med Okayama* 2002, **56(6)**:309-315.
41. Bressac B, Kew M, Wands J, Ozturk M: **Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa.** *Nature* 1991, **350(6317)**:429-431.
42. Ozturk M: **Genetic aspects of hepatocellular carcinogenesis.** *Semin Liver Dis* 1999, **19(3)**:235-242.
43. Higashitsuji H, Itoh K, Nagao T, Dawson S, Nonoguchi K, Kido T, Mayer RJ, Arii S, Fujita J: **Reduced stability of retinoblastoma protein by gankyrin, an oncogenic ankyrin-repeat protein overexpressed in hepatomas.** *Nature medicine* 2000, **6(1)**:96-99.
44. Hsia CC, Di Bisceglie AM, Kleiner DE Jr, Farshid M, Tabor E: **RB tumor suppressor gene expression in hepatocellular carcinomas from patients infected with the hepatitis B virus.** *Journal of medical virology* 1994, **44(1)**:67-73.
45. Li DM, Sun H: **PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells.** *Proc Natl Acad Sci USA* 1998, **95(26)**:15406-15411.

Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2407/11/3/prepub

doi:10.1186/1471-2407-11-3
Cite this article as: Nakanishi *et al.*: Loss of runt-related transcription factor 3 expression leads hepatocellular carcinoma cells to escape apoptosis. *BMC Cancer* 2011 **11**:3.