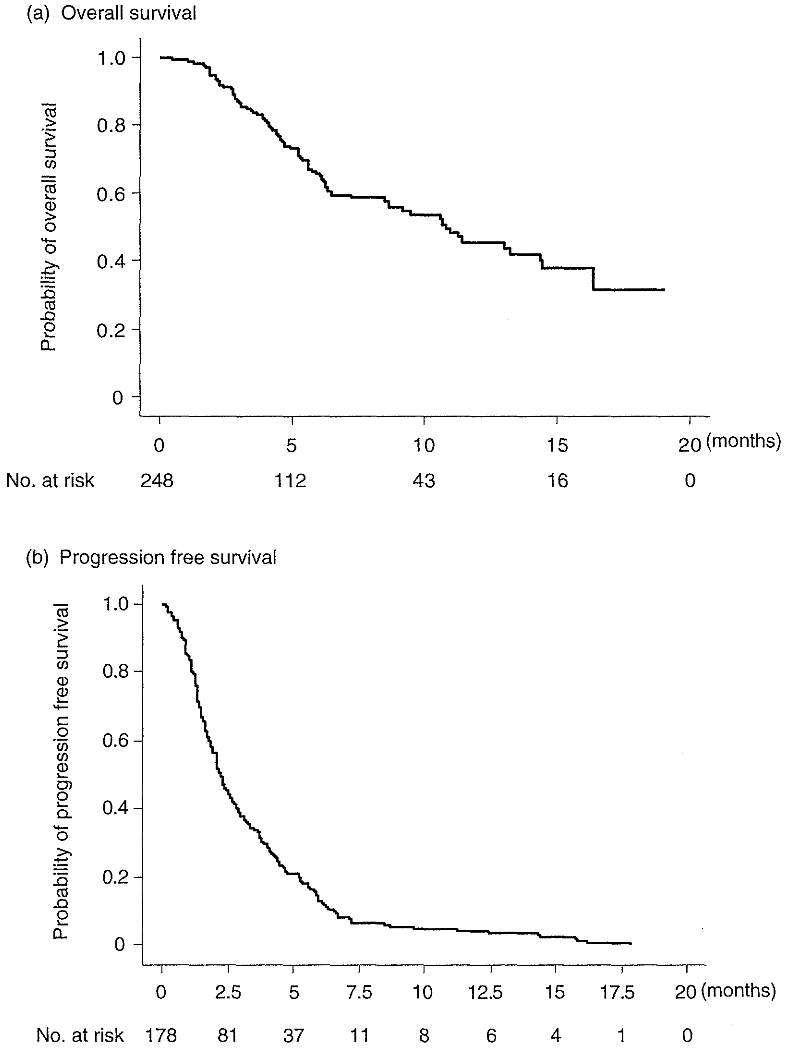

Figure 1 Therapeutic efficacy of sorafenib. (a) Overall survival. (b) Progression free survival.

Progressive disease (PD) was confirmed in 165 patients in the sorafenib group during the study's observation period, of whom a further 23 patients (14%) underwent continued oral administration of sorafenib for ≥1 month after PD confirmation. Comparison of these 23 patients with those in whom therapy was discontinued did not reveal any significant differences in OS, and no data are currently available regarding the efficacy/safety of continued sorafenib administration after confirmation of PD.

Predictors of therapeutic efficacy

CQ1-8 What are the predictors of therapeutic efficacy for sorafenib therapy?

Recommendation Clear predictors of therapeutic efficacy for sorafenib have yet to be established, but the number of intrahepatic lesions and pretreatment levels of tumor markers (AFP, PIVKA-II [DCP]) may be predictors of efficacy.

Scientific statement A study of biomarkers in patients treated with sorafenib has suggested the efficacy of sorafenib is associated with low serum HGF and high c-KIT levels at baseline.[25] Efficacy of sorafenib has also been linked to high levels of ERK expression in tumor tissue.[25,26] However, these reported associations cannot yet be described as established predictors of efficacy, and biomarkers are currently being sought in some prospective clinical trials using sorafenib.

Table 6 Summary of efficacy measures for sorafenib therapy

	NLCT Study ($n=250$)	SHARP Trial[1] ($n=299$)	Asia-Pacific Trial[2] ($n=150$)	Sorafenib phase II[5] ($n=137$)
OS (months)				
Median	11.0	10.7	6.5	9.2
1-year SR (%)	45	44	–	59
6-month SR (%)	65	–	53	–
PFS (months)	†			
Median	2.1	5.5	3.5	4.2/5.5
Antitumor effect (%)	‡			
Complete remission	0	0	0	0
Partial remission	4	2	5	2
Stable	45	71	46	34
Tumor control rate	49	43	53	–

†Patients who died without confirmation of disease progression were excluded.
‡Patients not evaluated for therapeutic response were excluded.
NLCT, New Liver Cancer Therapies; OS, overall survival; PFS, progression-free survival; SHARP, sorafenib hepatocellular carcinoma assessment randomized protocol.

The current results indicate that early AFP response is a useful surrogate marker to predict treatment response and prognosis in patients with advanced HCC who receive anti-angiogenic therapy.[27]

In an attempt to identify predictors of therapeutic efficacy for sorafenib, the NLCT study examined baseline patient characteristics (age, sex, BMI [body mass index], ECOG-PS [Eastern Cooperative Oncology Group – performance status], hepatic functional reserve, prior treatment, cause of hepatic impairment, clinical laboratory values) and tumor factors (presence or absence of intrahepatic/extrahepatic lesions, maximum tumor size, vascular invasion, stage), and consequently found that tumor control rates tended to be higher in patients with <5 intrahepatic lesions compared to those with ≥5 lesions (54% vs. 40%, respectively; $P = 0.058$). In addition, the tumor control rate was significantly higher in patients with a baseline AFP value <10 ng/mL compared with those with values ≥10 ng/mL (68% vs. 43%, respectively; $P = 0.021$). The tumor control rate also tended to be higher in patients with baseline PIVKA-II (DCP) value <40 mAU/mL than in those with a value of ≥40 mAU/mL (60% vs. 42%, respectively; $P = 0.051$) (Table 7).

Hepatic arterial infusion with miriplatin

Indications

CQ2-1 Is miriplatin a platinum preparation that can be used on renal disorder patients?

Recommendation Renal disorder patients can be treated using miriplatin as long as they are capable of undergoing angiography (serum Cre [creatinine] level <2.0 mg/dL) and as long as administration is performed carefully so as to avoid elevation in serum Cre levels after treatment.

Scientific statement Miriplatin remains in the tumor together with Lipiodol, where it slowly releases platinum compounds. This agent is thus believed to gradually increase serum platinum concentration with minimal adverse effect on renal function.

In a randomized phase II trial comparing miriplatin and zinostatin stimalamer (SMANCS) in patients with normal serum Cre levels, renal dysfunction indicated by serum Cre level >1.5 mg/dL was observed in only 2.4% of patients in the miriplatin treatment group (Table 8).[28]

In the NLCT study, median serum Cre prior to miriplatin therapy was 0.8 mg/dL (range, 0.4–10.5 mg/dL), of which patients with a serum Cre level >1.0 mg/dL accounted for 17.7%. Median serum Cre after treatment was 0.8 mg/dL (range, 0.1–12.6 mg/dL), which was unchanged from baseline, and 94.7% of patients experienced an increase of ≤0.5 mg/dL (Table 9). Only 1.8% of patients exhibited renal dysfunction ≥grade 3 as indicated by serum Cre level >3 mg/dL.

Analysis of patients with baseline serum Cre <2.0 mg/dL shows that just 2.5% of patients increased serum Cre >0.5 mg/dL, and no more than 0.6% of patients experienced renal dysfunction ≥grade 3 (Table 9).

In addition, no serious renal dysfunction was observed after miriplatin administration in patients with serum Cre levels around 2.0 mg/dL.

Table 7 Factor analysis of tumor control with sorafenib therapy

	n	Tumor control rate (%)	*P**
Age (years)			
≥65	137	49	0.75
<65	56	46	
Gender			
Male	147	50	0.72
Female	43	47	
ECOG-PS			
0	163	50	0.24
1–3	29	38	
Child–Pugh score			
5	65	48	0.82
6	70	44	
7	23	48	
≥8	10	60	
Child–Pugh class			
A	135	46	0.52
B–C	33	56	
Prior treatment			
Yes	173	48	0.87
None	18	50	
HBs antigens			
Positive	36	50	0.91
Negative	149	49	
HCV antibodies			
Positive	112	50	0.66
Negative	77	47	
Intrahepatic lesions			
Yes	174	47	0.26
None	18	61	
Intrahepatic nodules			
≥5	95	40	0.058
<5	83	54	
Advanced vascular invasion			
Yes	36	50	0.68
None	141	46	
Extrapulmonary lesion(s)			
Yes	105	47	0.64
None	88	50	
Maximum tumor size (mm)			
≥30	108	47	0.79
<30	67	49	
Stage (Japanese Classification of Lung Cancer)			
I–II	15	53	0.41
III	53	57	
IV A	31	39	
IV B	84	46	
Initial dose			
Normal dose	153	48	0.91
Reduction	39	49	
Baseline AFP			
≥10	151	43	0.021
<10	25	68	
Baseline PIVKA-II			
≥40	132	42	0.051
<40	40	60	

*Fisher's exact test.

AFP, α fetoprotein; ECOG-PS, Eastern Cooperative Oncology Group Performance status; HBs, Hepatitis B surface antigen; HCV, hepatitis C virus.

Based on these findings, the Study Group considers that miriplatin therapy can be administered without instigating renal dysfunction in patients with serum Cre <2.0 mg/dL who are capable of undergoing angiography.

However, transcatheter arterial infusion (TAI)/TACE with miriplatin simultaneously uses an iodinated contrast medium with drugs that can cause renal dysfunction such as anti-inflammatory analgesics to treat postoperative fever. Sufficient consideration should therefore be given to the risk of drug-induced renal dysfunction, and monitoring of urine volume and fluid replacement should be implemented as necessary.

CQ2-2 Can miriplatin be used safely in patients with Child–Pugh class B?

Recommendation Miriplatin can be used to treat these patients without causing serious complications.

Furthermore, no demonstrable difference in the antitumor effects of miriplatin has been observed between Child–Pugh class A and B patients.

Scientific statement The NLCT study included 281 Child–Pugh class A and 144 Child–Pugh class B patients. In Child–Pugh class B patients, the only SAEs ≥grade 3 were fever and anorexia, at incidences of 0.7% each, with no cases of ascites or hepatic failure ≥grade 3 (Table 10). In a study of TAI with miriplatin, in 17 Child–Pugh class B patients, no significant differences were seen in pre- or posttreatment 15-min retention rates of indocyanine green (ICG_{15}), and no SAEs or increased ascites or hepatic failure necessitating additional therapy or prolonged hospitalization were observed.[30]

Although the retrospective analysis of the NLCT study coupled with differences in characteristics of Child–Pugh class A and B patient effectively precludes simple comparisons of these patients, no significant differences in respective AE incidences were seen, apart from a higher frequency of fever and thrombocytopenia ≥grade 3 among Child–Pugh class B patients (Tables 10 and 11).

In terms of evaluation of antitumor effects according to the RECICL proposed by the Liver Cancer Study Group of Japan, the present study did not reveal any significant differences in therapeutic responses of Child–Pugh class A and B patients (Table 12), while 50% of Child–Pugh class B patients in the aforementioned study of TACE with miriplatin achieved a treatment effect (TE) of "TE3" or "TE4", in which tumor was controlled.[30]

CQ2-3 Is miriplatin effective against cisplatin-resistant HCCs?

Table 8 Abnormal clinical laboratory values with miriplatin therapy

	NLCT Study ($n = 535$)		Phase III Trial[29] ($n = 16$)		Randomized Phase II Trial[28] ($n = 83$)	
	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)
Leukopenia	38.2	5.1	51	0	41.0	1.2
Neutropenia	20.1	5.1	63	19	53.0	8.4
Eosinophilia	14.6	–	100	0	84.3	0
Monocytosis	–	–	–	–	57.8	0
Lymphocytopoenia	–	–	51	0	79.5	0
Thrombocytopenia	32.1	9.3	44	0	50.6	1.2
Increased AST	49.9	12.4	56	44	62.7	26.5
Increased ALT	78.4	26.6	44	19	59	24.1
Increased bilirubin	31.6	3.2	31	19	57.8	12.0
Increased γGTP	16.1	2.0	–	–	49.4	0
Increased ALP	12.3	0.2	44	0	30.1	1.2
Elevated Cre	11.5	1.8	25	0	–	2.4†

CTC-AE v3.0 Japan Society of Clinical Oncology Adverse Drug Reaction Criteria.
†Increased Cre data includes G2 patients.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyltransferase.

Table 9 Incidence of drug-related adverse events with miriplatin therapy (Renal dysfunction)

Elevated Cre	all ($n = 513$)	Baseline Cre <2.0 mg/dL	Baseline Cre ≥2.0 mg/dL
≤0.5 mg/dL	94.7%	97.5%	13.3%
0.6–1.0 mg/dL	2.4%	1.7%	20.0%
1.1–2.0 mg/dL	1.2%	0.2%	33.3%
2.1–3.0 mg/dL	0.6%	0.0%	20.0%
>3.0 mg/dL	1.0%	0.6%	13.3%

Recommendation The clinical usefulness of miriplatin against cisplatin-resistant HCC is not currently known.

Scientific statement Miriplatin is classified as a third-generation platinum drug and a basic research on the drug suggested potential activity in cisplatin-resistant HCCs because cisplatin-resistant HCC cell lines did not show cross-resistance to miriplatin.[31]

A Japanese Phase I trial combining miriplatin and TAI using Lipiodol (Lip-TAI) on HCC refractory to cisplatin/Lip-TAI has reported a treatment success rate of 18.2%.[32]

Table 10 Comparison of adverse events with miriplatin therapy according to Child–Pugh classification

	All ($n = 535$)		Child–Pugh class A ($n = 281$)		Child–Pugh class B ($n = 144$)	
	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)
Fever	81.3	0.2	75.5	0	86.1	0.7*
Biphasic fever	2.8	–	2.5	–	5.1	–
Anorexia	29.7	0.2	31.7	0	34.0	0.7
Administration site pain	21.2	0	25.6	0	15.3	0
Nausea	18.8	0	21.4	0	12.5	0*
Vomiting	13.5	0	11.6	0	6.1	0
Fatigue	9.3	0	12.2	0	10.3	0
Diarrhea	2.0	0	1.8	0	1.0	0
Ascites	1.2	0	0	0	3.0	0
Hepatic failure	0.3	0.3	0.3	0.3	0	0

CTC-AE v3.0.
*$P < 0.05$ (A vs. B).

Table 11 Comparison of clinical laboratory value anomalies with miriplatin therapy according to Child–Pugh classification

	All ($n = 535$)		Child–Pugh class A ($n = 281$)		Child–Pugh class B ($n = 144$)	
	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)
Leukopenia	38.2	5.1	18.2	3.3	25.2	5.8
Neutropenia	20.1	5.1	17.3	3.6	23.4	5.8
Eosinophilia	14.6	–	17.9	–	11.5	–
Thrombocytopenia	32.1	9.3	30.9	5.8	30.2	13.7*(G3)
Increased AST	49.9	12.4	45.2	13.5	50.7	19.4
Increased ALT	78.4	26.6	81.0	28.8	70.3	28.3*
Increased bilirubin	31.6	3.2	26.1	0	46.0	5.8*
Increased γGTP	16.1	2.0	15.8	2.6	14.5	0
Increased ALP	12.3	0.2	12.7	0	10.1	0.7
Elevated Cre	11.5	1.8	11.6	2.2	10.8	1.4

CTC-AE v3.0.
*$P < 0.05$ (A vs. B).
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyltransferase.

However, the study was conducted on a small patient population, so the usefulness of this therapy is yet to be established and future studies are awaited.

Furthermore, no data are currently available regarding the efficacy of miriplatin therapy in patients who are unresponsive to TAI/HAIC using cisplatin.

Method of administration

CQ2-4 What are the effects and AEs of combining embolic materials with miriplatin?

Recommendation Combination therapy of embolic materials and miriplatin is expected to improve antitumor effects compared with miriplatin alone, but there is currently insufficient evidence to support this.

Adverse events associated with combination therapy of embolic materials and miriplatin may not differ noticeably from those of conventional TACE therapy using epirubicin.

Scientific statement Compared with stand-alone therapy, the combination of embolic materials in the hepatic arterial catheterization treatment is generally considered to deliver enhanced antitumor effects based on its blood flow blockage effect,[33] so treatment combined with embolic materials are mostly selected for the treatment of HCC. However, Phase I and II trials using miriplatin have opted not to use embolic materials in combination with miriplatin.[29,32]

Meanwhile, two studies on miriplatin used in combination with embolic materials on a small number of patients have reported high rates of treatment success, with TE3 and TE4 scores obtained in 60.0–77.7% of patients.[30,34]

Table 12 Summary of efficacy measures with miriplatin therapy

	NLCT Study			Phase II Trial[29] ($n = 16$)	Randomized Phase II Trial[28] ($n = 83$)
	All ($n = 535$)	Child–Pugh class A ($n = 281$)	Child–Pugh class B ($n = 144$)		
Anti-neoplastic effect (%)					
TE4	22.8	25.3	23.6	56	26.5
TE3	24.3	26.7	20.8	6	25.3
TE2	26.0	26.0	29.9	19	22.9
TE1	16.6	12.5	17.4	19	20.5
Not evaluated	10.3	9.6	8.3	0	4.8
TE3 + TE4	47.1	52.0	44.4	61	51.8

Response Evaluation Criteria in Cancer of the Liver' (RECICL).

Table 13 Independent factors contributing to effective (TE3/4) achievement with miriplatin therapy

Factor	Category	Risk ratio	95% CI	*P*-value
Embolic material	None	1		<0.001
	Yes	3.66	2.13–6.29	
No. tumors	Single	1		0.017
	2–3	1.01		
	4–9	0.66		
	≥10	0.3	0.13–0.67	
Past history of TAE	None	1		0.018
	Yes	0.48	0.26–0.88	

Cox proportional hazards model.
CI, confidence interval; TAE, transcatheter arterial embolization.

In the NLCT study, embolic material was used in combination with miriplatin on 473 patients (88.4%). Simple comparison of patients undergoing miriplatin/embolic material combination therapy and those who underwent miriplatin alone therapy was not possible due to the retrospective nature of this study, as well as the different patient characteristics of the respective treatment groups. However, antitumor effects were higher in the miriplatin/embolic material therapy group than in the miriplatin therapy group, at 49% and 31%, respectively (Fig. 2). Analysis of independent factors contributing to the achievement of TE3/4 scores in TAI/TACE therapy using miriplatin showed that the use of embolic material had a higher risk ratio of 3.66 ($P < 0.001$) (Table 13).

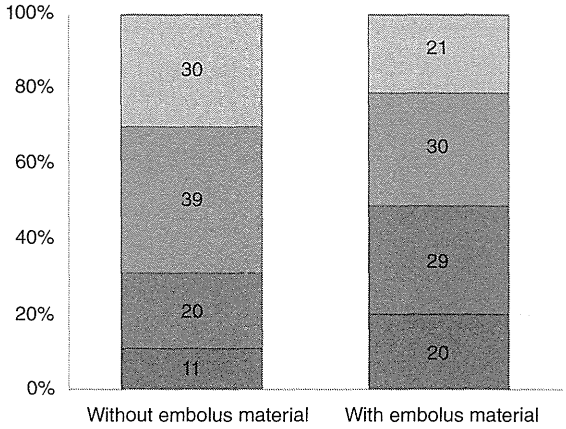

Figure 2 Therapeutic efficacy of miriplatin with or without embolus material.

A Phase III trial of TACE using miriplatin is currently underway, and the results will likely be useful in investigating the efficacy of using miriplatin in combination with embolic materials.

In the NLCT study, patients who underwent combination therapy with embolic material showed a high incidence of fever, suspected to be due to postembolization syndrome. Although high incidences of hematological AEs neutropenia and elevated AST were seen, no significant differences were identified in the incidences of most AEs, and no serious complications such as hepatic failure or ascites were observed (Tables 14 and 15).

Table 14 Comparison of adverse events with or without embolic material during miriplatin therapy

	All ($n = 535$)		TACE patients ($n = 425$)		TAI patients ($n = 54$)	
	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)
Fever	81.3	0.2	84.4	0.2	56.1	0*
Biphasic fever	2.8	–	3.0	–	0	–
Anorexia	29.7	0.2	30.4	0.2	22.4	0
Administration site pain	21.2	0	22.2	0	13.8	0
Nausea	18.8	0	20.1	0	4.0	0
Vomiting	13.5	0	14.2	0	0	0
Fatigue	9.3	0	9.2	0	–	–
Diarrhea	2.0	0	2.1	0	0	0
Ascites	1.2	0	0.9	0	5.6	0
Hepatic failure	0.3	0.3	0.3	0.3	0	0

CTC-AE v3.0.
*$P < 0.05$ (TACE vs. TAI).
TACE, transcatheter arterial chemoembolization; TAI, transcatheter arterial infusion.

Table 15 Comparison of abnormal clinical laboratory values with or without embolic material during miriplatin therapy

	All ($n = 535$)		TACE patients ($n = 425$)		TAI patients ($n = 54$)	
	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)
Leukopenia	38.2	5.1	22.8	5.5	20.4	1.9
Neutropenia	20.1	5.1	21.4	5.5	3.7	0*
Eosinophilia	14.6	–	14.8	–	11.8	–
Thrombocytopenia	32.1	9.3	33.2	10.4	24.1	0
Increased AST	49.9	12.4	52.8	19.3	25.9	8.6*
Increased ALT	78.4	26.6	78	24.5	81.5	44.4*
Increased bilirubin	31.6	3.2	32.1	3.3	27.8	0
Increased γ-GTP	16.1	2.0	16.1	1.8	14.8	3.7
Increased ALP	12.3	0.2	12.6	0.2	9.3	0
Elevated Cre	11.5	1.8	10.7	1.8	18.5	1.9

CTC-AE v3.0.
*$P < 0.05$ (TACE vs. TAI).
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyltransferase; TACE, transcatheter arterial chemoembolization; TAI, transcatheter arterial infusion.

Similarly, a small pilot study (Phase II clinical trial) on miriplatin combined with an embolic material found some mild complications, but none of a serious nature.[34] Another study on the small number of patients did not reveal any serious complications.[30]

CQ2-5 Is standard hydration required prior to administration of miriplatin?

Recommendation Standard hydration is not required except in the case of renal failure.

Scientific statement Sufficient hydration before and after administration of cisplatin (IA-call, Nippon Kayaku, Tokyo, Japan) used in HAIC is necessary to prevent nephrotoxicity.

Miriplatin is highly soluble in Lipiodol and remains in tumor with Lipiodol, where it continuously releases platinum compounds.[35] So only a small amount enters systemic circulation expecting to reduce systemic AEs, including renal dysfunction.

As stated in CQ1, the effect of miriplatin on renal function is considered to be mild. Two of the aforementioned Phase II trials did not perform pretreatment hydration to prevent renal impairment.[28,30] In the NLCT study, patients with advanced renal insufficiency were excluded and no serious renal impairment occurred in patients treated with miriplatin without prior hydration.

Adverse events

CQ2-6 What are the adverse events associated with miriplatin therapy?

Recommendation Post-embolization syndrome characterized mainly by fever is often seen, and biphasic fever is relatively infrequent. Incidences of nausea and vomiting are also low compared with other platinum agents. Complications such as ascites, liver abscess, biloma, and dyspnea have incidences of about 1%.

Scientific statement In the NLCT study, post-embolization syndrome was observed in ≥90% of patients treated with miriplatin. However, the incidence of biphasic fever, which is said to be a characteristic AE associated with miriplatin, was low at 2.8% (Tables 16, 17).

Incidences of nausea and vomiting were low compared with other platinum agents, at 18.8% and 13.5%, respectively.

Hematological AEs were leukopenia at 38.2%, thrombocytopenia at 32.1%, and neutropenia at 20.1%. Incidence of eosinophilia, which is also reported as a characteristic AE of miriplatin, was relatively low at 14.6% (Table 8).[28,29]

Abnormal hepatic function was frequent, with elevated AST and ALT occurring in 49.9% and 78.4% of patients, respectively, of whom a further 12.4% and 26.6% had respective AST and ALT values ≥grade 3. Elevated T-Bil was seen in 31.6% of patients, of whom 3.2% had value ≥grade 3, more than three times the upper limits of normal (ULN).

CQ2-7 What is the extent of deterioration in hepatic function caused by TAI/TACE using miriplatin?

Table 16 Incidence of drug-related adverse events with miriplatin therapy (1)

	NLCT Study ($n = 535$)		Phase II Trial[29] ($n = 16$)		Randomized Phase II Trial[28] ($n = 83$)	
	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)	Total (%)	G3/4 (%)
Fever	81.3	0.2	94	0	96.4	3.6
Biphasic fever	2.8	–	–	–	–	–
Anorexia	29.7	–	–	–	–	–
Abdominal pain	21.2	0	50	0	–	–
Nausea	18.8	0	25	0	–	–
Vomiting	13.5	0	–	–	55.4	1.2
Fatigue	9.3	0	–	–	39.8	0
Chills	–	0	–	–	39.8	0
Administration site pain	21.2	0	50	0	43.4	0
Diarrhea	2.0	0	31	0	–	–
Ascites	1.2	0	–	–	–	–
Hepatic failure	0.3	0.3	–	–	–	–
Vascular injury	–	–	–	–	0	0

CTC-AE v3.0 Japan Society of Clinical Oncology Adverse Drug Reaction Criteria

Recommendation Typically, no deterioration is seen in postoperative ICG_{15}, but prothrobmin time (PT) ratio (%) may display a transient decline.

Scientific statement Hepatic impairment after miriplatin administration has been reported to peak within 2 weeks in 46% of patients, at 3–5 weeks in 23% of patients, and at 9–11 weeks in 31% of patients.[29]

The NLCT study also found that in evaluable patients, ICG_{15} values had not deteriorated at 1–2 weeks after therapy and that PT ratio (%) exhibited a transient decline, but subsequently recovered in the majority of patients.

Child–Pugh class B patients did not find any significant differences in pre- or post-treatment ICG_{15}, and did not find any SAEs or increased ascites or hepatic failure necessitating additional therapy and prolonged hospitalization.[32]

However, the safety of miriplatin used in combination with embolic materials has yet to be established, and a Phase III study on concomitant use of miriplatin and embolizing agents is currently underway.[34]

Table 17 Incidence of drug-related adverse events with miriplatin therapy (2)

	Incidence (%)
Ascites	1.2
Liver abscess	0.6
Biloma	0.3
Dyspnea	0.3

CQ2-8 Does vascular injury occur after intra-arterial administration of miriplatin?

Recommendation Vascular injuries such as hepatic artery occlusion, arterial stenosis and arterioportal shunts, and hepatic lobar atrophy caused by vascular damage are rare.

Scientific statement No reports have described vascular injuries from non-hematological toxicity in previous Japanese Phase I and II trials on miriplatin therapy.[29,32] Likewise, no vascular injuries have been reported in the NLCT study (Table 16). In TAI without the use of embolic materials, the aforementioned randomized phase II trial comparing miriplatin and zinostatin stimalamer (SMANCS) found that vascular injuries occurred in 48.4% of the SMANCS treatment group ($n = 31$), but that no vascular injuries occurred in the miriplatin treatment group ($n = 73$).[28] In a limiting study performing follow-up angiography on nine patients at 2–6 months after treatment, no arterial stenoses, arterial occlusions, or arterioportal shunts were observed.[30]

Evaluation of therapeutic response

CQ2-9 After how many weeks should therapeutic response to miriplatin be evaluated?

Recommendation Non-specific accumulation of Lipiodol appears on dynamic CT at 1 week after administration of miriplatin, so evaluation of therapeutic response should preferably be performed at 4–8 weeks after administration.

Scientific statement Evaluation of therapeutic response performed at 1 day or 1 week after starting miriplatin therapy may result in overestimation of response due to the appearance of non-specific Lipiodol deposits. Evaluation of therapeutic response using dynamic CT at 4–8 weeks after therapy is therefore preferable, to allow these non-specific deposits to disappear. In the above-mentioned Phase I clinical trial, therapeutic response to miriplatin was evaluated with dynamic CT at 1 week, 5 weeks, and 3 months after therapy,[32] while the Phase II trial evaluated the antitumor effects of miriplatin using dynamic CT every 3 months.[29]

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Overexpression of gankyrin in mouse hepatocytes induces hemangioma by suppressing factor inhibiting hypoxia-inducible factor-1 (FIH-1) and activating hypoxia-inducible factor-1

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ABSTRACT

Gankyrin (also called p28 or PSMD10) is an oncoprotein commonly overexpressed in hepatocellular carcinomas. It consists of 7 ankyrin repeats and interacts with multiple proteins including Rb, Cdk4, MDM2 and NF-κB. To assess the oncogenic activity *in vivo*, we produced transgenic mice that overexpress gankyrin specifically in the hepatocytes. Unexpectedly, 5 of 7 F2 transgenic mice overexpressing hepatitis B virus X protein (HBX) promoter-driven gankyrin, and one of 3 founder mice overexpressing serum amyloid P component (SAP) promoter-driven gankyrin developed hepatic vascular neoplasms (hemangioma/hemangiosarcomas) whereas none of the wild-type mice did. Endothelial overgrowth was more frequent in the livers of diethylnitrosamine-treated transgenic mice than wild-type mice. Mouse hepatoma Hepa1-6 cells overexpressing gankyrin formed tumors with more vascularity than parental Hepa1-6 cells in the transplanted mouse skin. We found that gankyrin binds to and sequester factor inhibiting hypoxia-inducible factor-1 (FIH-1), which results in decreased interaction between FIH-1 and hypoxia-inducible factor-1α (HIF-1α) and increased activity of HIF-1 to promote VEGF production. The effects of gankyrin were more prominent under 3% O_2 than 1% or 20% O_2 conditions. Thus, the present study clarified, at least partly, mechanisms of vascular tumorigenesis, and suggests that gankyrin might play a physiological role in hypoxic responses besides its roles as an oncoprotein.

1. Introduction

Gankyrin (also called p28, p28^{GANK} or PSMD10) was identified as an oncoprotein commonly overexpressed in hepatocellular carcinomas (HCCs) [1]. Gankyrin was also independently isolated as p28, a supposed component of the 26S proteasome, but recent studies have demonstrated that p28 associates only with free 19S particles of the 26S proteasome or their precursors and functions as a chaperone to guide their assembly [2]. As expected for a protein consisting of 7 ankyrin repeats [3], gankyrin interacts with multiple proteins and shows a variety of activities. For example, gankyrin binds to Rb and Cdk4, and accelerates phosphorylation and degradation of Rb to activate DNA synthesis genes [1]. Gankyrin binds to the E3 ubiquitin ligase MDM2, thereby facilitating ubiquitylation and degradation of p53 [4]. Gankirin binds to NF-κB and suppresses its activity by modulating acetylation via SIRT1 [5]. Gankyrin binds to hepatocyte nuclear factor 4α, which determines hepatocyte differentiation status and enhances its degradation [6]. Gankyrin activates PI3K/AKT/mTOR/hypoxia-inducible factor-1 (HIF-1) signaling [7].

Most solid tumors contain hypoxic regions, and one of the most important cellular factors involved in the hypoxic response which promotes angiogenesis, anaerobic metabolism and resistance to apoptosis is HIF-1 [8,9]. HIF-1 is a heterodimeric transcription factor composed of a constitutively expressed β subunit and an inducibly expressed α subunit (HIF-1α). Under aerobic conditions, HIF-1α is hydroxylated by specific prolyl hydroxylases at two conserved Pro residues in a reaction requiring oxygen. Hydroxylation

Abbreviations: CAD, C-terminal transactivation domain; DEN, diethylnitrosamine; FIH-1, factor inhibiting hypoxia-inducible factor-1; firefly-luciferase, F-Luc; H&E, hematoxylin and eosin; HBX, hepatitis B virus X protein; HCC, hepatocellular carcinoma; HIF, hypoxia-inducible factor; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SAP, serum amyloid P component.

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facilitates binding of von Hippel–Lindau protein, a component of the ubiquitin protein ligase, to HIF-1α, leading to its proteasomal degradation. The ability of HIF-1α to activate transcription is also prevented by factor inhibiting HIF-1 (FIH-1) [8–10]. FIH-1 hydroxylates a specific Asn residue in HIF-1α, and disrupts interaction of HIF-1α with the transcription co-activators p300 and CBP. Under hypoxic conditions, prolyl hydroxylase and FIH-1 activities are inhibited by substrate (O_2) deprivation, resulting in HIF-1α stabilization and binding to the p300/CBP complex, thus allowing HIF transactivation.

Since gankyrin plays important roles in cell proliferation and apoptosis, is overexpressed in most HCCs, and confers tumorigenicity to non-malignant cells, we produced transgenic mice that overexpressed gankyrin specifically in the hepatocytes to assess its oncogenic activity *in vivo*. Unexpectedly, the mice developed vascular tumors (hemangioma/hemangiosarcomas) in the liver, and so we have tried to elucidate the underlying mechanisms for vascularization.

2. Materials and methods

2.1. Transgenic mice

To express gankyrin specifically in the liver, cDNA for the mouse wild-type gankyrin N-terminally tagged with 2× FLAG was cloned into the pBEPBglII expression vector containing the hepatitis B virus X protein (HBX) promoter [11]. Fertilized eggs were obtained from C57BL/6J mice, and transgenic mice were produced at the Center for Animal Resources and Development, Kumamoto University, Japan. A plasmid containing the human serum amyloid P component (SAP) promoter [12] and expressing mouse wild-type gankyrin N-terminally tagged with 3× FLAG was also constructed, and transgenic mice were produced with this at the Genome Information Research Center, Osaka University, Japan, using eggs from D2B6F1 mice. For genotyping, DNA was extracted from the tail of each mouse and analyzed by Southern blotting using gankyrin cDNA as probe.

2.2. Treatment of mice

A single intraperitoneal injection of diethylnitrosamine (DEN, Sigma, 25 mg/kg of body weight) was administered to 14-days-old transgenic and control male mice. Groups of animals were euthanized at 8 months after injection, and the livers were removed, examined for visible lesions, and paraffin embedded after fixation in 10% buffered formalin.

For tumor formation, cells (2×10^6) were suspended in 0.1 ml of PBS and injected subcutaneously into the back of athymic BALB/c mice (Japan SLC Inc.). Each mouse received Hepa1-6 cells on one side and Hepa1-6/GK cells on the other side. All experiments involving mice were approved by the Animal Research Committee of Kyoto University, and conducted in accordance with the institutional and NIH guidelines for the care and use of laboratory animals.

2.3. Human materials

Eighteen specimens of HCC were taken by needle biopsy before initiation of the treatment at Kinki University Hospital, Japan. The study protocol was approved by the institutional review boards, and written informed consent was obtained from all patients for subsequent use of their collected tissues.

2.4. Cell culture and DNA transfection

U-2 OS cells, HEK293 cells, HEK293T cells, mouse hepatoma Hepa1-6 cells and their transfectants were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2 as described [4]. For mild hypoxic conditions, cells were placed in a modular incubator chamber and flushed with a gas mixture containing 1 or 3% O_2, 5% CO_2, and balance N2.

Calcium phosphate-DNA coprecipitation method was used for DNA transfection. Plasmids encoding, gankyrin, shRNA for gankyrin, HIF-1α, FIH-1, and their fusion proteins have been described previously [4,5,10,13].

2.5. Pathologicall analyses

The immunohistochemical staining was performed on 4-µm-thick paraffin sections of tissues fixed in 10% buffered formalin as described [14]. The sections were incubated with the primary antibodies against endothelial cell markers CD31 (dianoba GmbH) and CD34 (Abnova), followed by horseradish peroxidase-conjugated anti-rat immunoglobulin antibody (Santa Cruz Biotechnology), and were developed in Diaminobenzidine colorimetric reagent solution (DAKO). They were counterstained with hematoxylin. To assess the presence of the atypical proliferative lesion of endothelial cells, at least 1 section from 4 lobes were examined under a microscope.

2.6. Analyses of gene expression and protein interactions

Preparation of cell lysates, immunoprecipitation, and Western blot analysis were performed as described [4]. Rabbit polyclonal anti-gankyrin, anti-VEGF-A, anti-HIF-1α, anti-FIH-1, anti-β-actin, and biotin-conjugated anti-HA antibodies (all from Santa Cruz Biotech.), anti-Myc tag antibody (MBL), anti-FLAG and biotin-conjugated anti-FLAG antibodies (Sigma), mouse monoclonal anti-HA antibody (Roche), and rabbit polyclonal antibody raised against recombinant mouse gankyrin were used as the primary antibodies in Western blotting.

For immunoprecipitation, mouse anti-HA antibody (Roche), rabbit anti-FLAG antibody, and biotin-conjugated anti-FLAG and anti-HA antibodies were used.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis was done as described [14]. The relative levels of gankyrin and VEGF-A mRNAs were determined by RT-qPCR using β-actin and GAPDH mRNA for normalization. Primer sequences used were as follows: gankyrin (human, 5′-TCTTCAAGCCATCCTGTGTG-3′ and 5′-TGGTGATGTTGGACTCCTCA-3′), VEGF-A (human, 5′-AAAA CTGCT GGTGTCCCAAG-3′ and 5′-ATTAAACCCAGGCCACCTTT-3′; mouse, 5′-CA GGCTGCTGTAACGATGAA-3′ and 5′-TATGTGCTGGCTTTGGTGAG-3′), β-actin (human, CTACGTCGCCCTGGACTTCGAGC and GATGGAGC CGCCGATCCACACGG), GAPDH (mouse, 5′-ACAACTTTGTC AAGCT-CATTTCCTG-3′ and 5′-TGGTCCAGGGTTTCTTACTCCTTGG-3′).

2.7. Reporter assays

The reporter plasmids p2.1 and p2.4 contain wild-type and mutant copies, respectively, of the hypoxia response element (HRE) from the *ENO1* gene upstream of an SV40 promoter and firefly luciferase (F-Luc) coding sequences [13]. U-2 OS cells were cotransfected with either p2.1 or p2.4, pRL vector expressing Renilla luciferase (pRL-CMV, Promega) and plasmids expressing HA-FIH-1 and FLAG-gankyrin or gankyrin-shRNA [4].

The GAL4 reporter plasmid GAL4E1bLuc containing five GAL4-binding sites upstream of an E1b TATA sequence and the F-Luc gene, and GAL4-expressing plasmids GalA(531–826) and

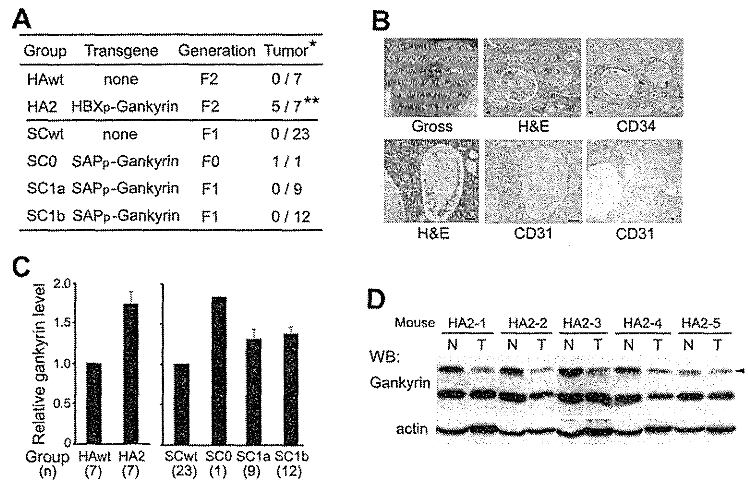

Fig. 1. Vascular tumors in gankyrin-transgenic mice. (A) Incidence of hepatic tumors. Gankyrin was expressed in hepatocytes by using HBX promoter (HBXp) or SAP promoter (SAPp). *number of mice with hepatic tumors at 22 months of age/total number of mice. **$P < 0.05$ compared with HAwt group. (B) Gross and microscopic appearances of hepatic vascular lesions in transgenic mice. H&E, hematoxylin and eosin staining. CD31 and CD34, immunoperoxidase staining for CD31 and CD34, respectively, using diaminobenzidine as substrate. Bar, 50 μm. (C) Gankyrin expression in the non-tumorous liver. Lysates prepared from indicated mice were analyzed by Western blotting and densitometry. Bars are average ± SD of total gankyrin levels normalized with actin levels, and expressed as relative to those of wild-type mice. (D) Expression of endogenous and exogenous (arrowhead) gankyrin in the tumor (T) and non-tumorous portion (N) of the liver from indicated F2 transgenic mice. Western blot analysis.

GalA-N803 expressing the C-terminal transactivation domain (CAD) of the wild-type and FIH-1-insensitive HIF-1α, respectively, fused to the GAL4 DNA-binding domain were described previously [15]. U-2 OS cells were cotransfected with GAL4E1bLuc, pRL-CMV, GAL4-expressing plasmids, and plasmids expressing FLAG-gankyrin or gankyrin-shRNA.

Transfected cells were exposed to mild hypoxia (1 or 3% O_2) for 48 h and harvested for dual luciferase assays (Promega) as described [5].

2.8. Statistical analysis

To determine whether the means of two groups are significantly different from each other, the Student's *t*-test and chi-square test were used. All statistical analyses including Fisher's exact probability test were performed using the JMP software (SAS Institute). A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Vascular neoplasms developed in the liver of gankyrin-transgenic mouse

The HBX promoter [11] was first used to direct hepatocyte-specific expression of the wild-type gankyrin in transgenic mice. Two founder (F0) mice were obtained and subsequently mated with wild-type mice to produce F1 offspring containing the transgene. F1 mice were then mated with wild-type mice to produce F2 offspring. When F0, F1 and F2 mice were sacrificed at 9–13 months

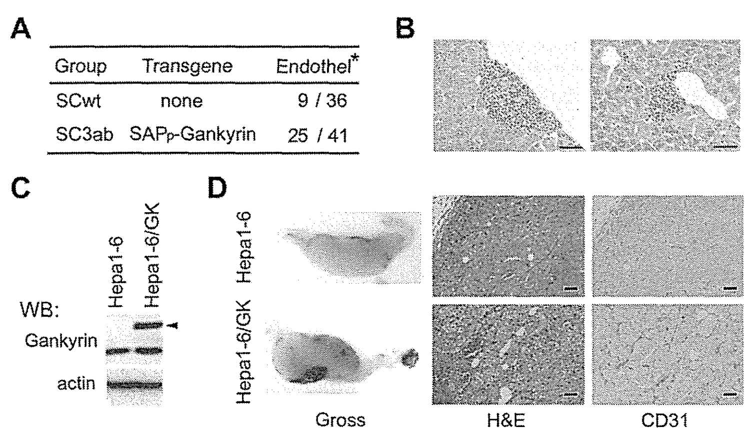

Fig. 2. Increased vascularity of hepatic tumors overexpressing gankyrin. (A) Vascularity in the livers of gankyrin-transgenic (SC3ab) and wild-type (SCwt) mice with diethylnitrosamine (DEN)-induced HCCs. Eight months after DEN treatment, mice were sacrificed and tumor vascularity was evaluated microscopically. *Number of mice with endothelial overgrowth in the liver/number of mice administered DEN. $P < 0.05$ between these groups. (B) Typical examples of atypical proliferation of endothelial cells in (A). H&E stain. Bar, 50 μm. (C) Expression of endogenous and exogenous (arrowhead) gankyrin in Hepa1-6 and Hepa1-6/GK cells analyzed by Western blotting. (D) Vascularity of Hepa1-6 and Hepa1-6/GK tumors in nude mouse skin. Typical gross and microscopic (H&E and immunoperoxidase staining of CD31) appearances of formalin-fixed paraffin-embedded tumors. Bar, 50 μm.

of age, no hepatic tumor was observed (data not shown). At 22 months of age, however, 5 of the remaining 7 male F2 developed hepatic vascular tumors, whereas no tumor was found in the control mice (Fig. 1A and B). The protein level of gankyrin in the non-tumorous liver of transgenic mice was about 1.7-fold compared with that of wild-type mice (Fig. 1C). The tumors consisted of large somewhat irregular vascular channels lined by endothelial cells. In some areas elongated or spindle-shaped endothelial cells lined vascular spaces, formed solid sheets, with an atypical nucleus, suggesting malignancy (Fig. 1B). Immunohisochemical analysis demonstrated that the tumor cells expressed the endothelial cell markers CD31 and CD34. The expression of gankyrin was less in the vascular tumors than non-tumorous liver tissues (Fig. 1D). Taken together, these results indicated that the observed tumors were hemangioma/hemangiosarcomas.

To increase the expression level of transgene in the liver, we next produced gankyrin-transgenic mice using the SAP promoter [12]. At the age of 22 months, one of the 3 F0 mice developed hemangioma/hemangiosarcomas, but none of its F1 offspring and other 2 F0 mice (Fig. 1A). In the F0 with vascular tumors, the transgene was integrated into more than one locus, resulting in inheritance of less integration sites and lower levels of gankyrin expression in the offspring (Fig. 1C and data not shown).

3.2. Increased vascularity in tumors overexpressing gankyrin

To evaluate the effect of gankyrin on angiogenesis in the liver, we used the DEN-induced hepatocarcinogenesis model. At 8 months after DEN treatment, 100% of wild-type mice and SAP promoter-driven gankyrin-transgenic mice (F4) developed hepatocarcinomas. Multiplicity of tumors was not different between the two groups, but the incidence of microscopic vascular lesions characterized by angiectasis and atypically proliferating endothelial cells was significantly higher in the transgenic mice than wild-type mice (Fig. 2A and B).

To further examine the effect of gankyrin on neovasculization, we stably overexpressed FLAG-tagged gankyrin in mouse Hepa1-6 hepatoma cells (Hepa1-6/GK cells, Fig. 2C). Two weeks after inoculation, both Hepa1-6 and Hepa1-6/GK cells formed tumors, and tumor vascularity was grossly more prominent in the Hepa1-6/GK tumors compared with Hepa1-6 tumors in all 6 mice inoculated (Fig. 2D). Immunohistochemical staining with anti-CD31 endothelial marker antibody demonstrated increased blood vessel density in Hepa1-6/GK tumors compared with Hepa1-6 tumors. Thus, overexpression of gankyrin increased the neovascularization.

3.3. Increased VEGF expression induced by gankyrin

Since HIF-1-mediated expression of VEGF stimulates angiogenesis [8,9], we analyzed expression of HIF-1α and VEGF-A. As shown in Fig. 3A, expression levels of VEGF-A protein and mRNA were higher in the livers of gankyrin-transgenic mice compared to wild-type mice. Overexpression of gankyrin in Hepa1-6 cells also increased protein level of VEGF-A, although the HIF-1α level was not increased. (Fig. 3B).

Transcriptional activation of the VEGF gene in response to hypoxia is mediated by binding of HIF-1 to HRE [13]. To examine whether gankyrin affects transcriptional activity mediated by HRE, we transfected U-2 OS cells with HRE-Luc reporter plasmid. Gankyrin enhanced the luciferase activity induced by mild hypoxia (3% O_2) by 4-fold, but only 1.5-fold or no enhancement at 1% or 20% O_2 concentration, respectively (Fig. 3C and data not shown). Conversely, suppression of gankyrin expression by shRNA reduced the luciferase activity. When HRE was mutated, the luciferase activity was not increased by hypoxia, and the enhancing effect of gankyrin was not observed, indicating that the effect was mediated by HRE.

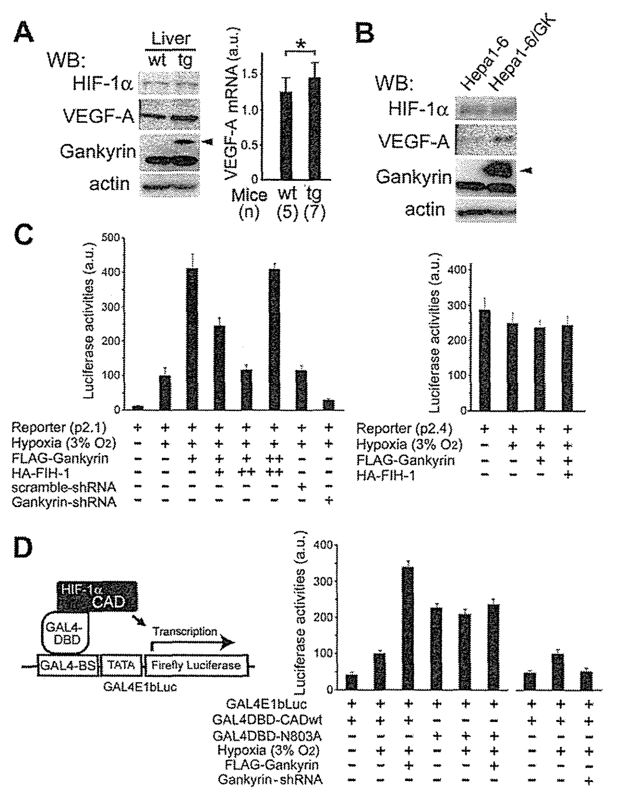

Fig. 3. Increased VEGF expression induced by gankyrin. (A) VEGF-A and HIF-1α expression in the livers of wild-type (wt) and gankyrin-transgenic (tg) mice. Western blotting (left) and RT-qPCR (right). Arrowhead, FLAG-Gankyrin. VEGF-A transcript levels were normalized with GAPDH levels. Values are average ± SD. $^*P < 0.05$. a.u., arbitrary unit. (B) Effects of gankyrin on expression of VEGF-A. Hepa1-6 cells and Hepa1-6 transfectants overexpressing FLAG-Gankyrin (Hepa1-6/GK) were analyzed by Western blotting. Arrowhead, FLAG-Gankyrin. (C) HRE-dependent transcriptional activation. U-2 OS cells were cotransfected with *ENO1*-F-luciferase (Luc) reporter plasmids (p2.1) or mutated reporter plasmids lacking the HIF-1 recognition sequence (p2.4), and plasmids expressing R-Luc, FLAG-Gankyrin, HA–FIH-1, gankyrin-shRNA, and scrambled-shRNA as indicated. 48 h later, some dishes were transferred to hypoxic conditions. After further 48-h incubation, cell lysates were analyzed for Luc activity. F-Luc activity was normalized with R-Luc activity. Values are average ± SD from 3 independent experiments. a.u., arbitrary unit. (D) Asn803-dependent increase in HIF-1α transcriptional activity. U-2 OS cells were cotransfected with Gal4–F-Luc reporter plasmids (GAL4E1bLuc), plasmids expressing GAL4 DNA-binding domain (DBD) fused to wild-type (wt) or N803A mutant C-terminal half (CAD) of HIF-1α, R-Luc, and FLAG-Gankyrin and gankyrin-shRNA as indicated. After 48 h of hypoxic incubation, Luc activities were measured and expressed as in (C). GAL4-BS, GAL4-binding sites. a.u., arbitrary unit.

We next examined whether gankyrin affects transcriptional activity of HIF-1α. We employed a reporter system composed of the F-Luc gene whose expression is controlled by GAL4-binding elements (GAL4E1bLuc, Fig. 3D) and the HIF1α-CAD fused to the GAL4 DNA-binding domain [13]. Compared with normoxia (20% O_2), luciferase activity was 2.5-fold higher at 3% O_2 concentration (Fig. 3D). Overexpression of gankyrin further increased the HIF-1α activity by 3-fold, whereas suppression of gankyrin reduced it. At 1% or 20% O_2 concentration, however, these effects of gankyrin were not observed (data not shown). When fusion protein of HIF-1α-CAD mutated at Asn803 was used, gankyrin showed no effect. Since Asn803 is the critical residue for FIH-1 to inhibit HIF-1α activity, these results suggest that the effect of gankyrin was mediated by FIH-1.

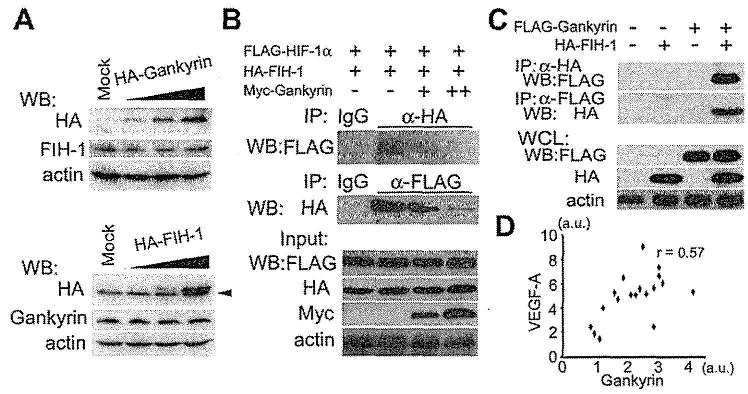

Fig. 4. Binding of gankyrin to FIH-1. (A) Effect of gankyrin on FIH-1 protein level. HEK293 cells were transfected with increasing amounts of plasmids expressing HA-gankyrin or HA-FIH-1, or empty vector (Mock) as indicated. 48 h later, cell lysates were analyzed by Western blotting. Arrowhead, non-specific bands. (B) Effect of gankyrin on binding of HIF-1α to FIH-1. HEK293T cells were cotransfected with plasmids expressing FLAG–HIF-1α, HA–FIH-1, and Myc-tag-gankyrin, and cultured at 3% O_2 for 48 h. Cell lysates were immunoprecipitated (IP), and precipitants and inputs were analyzed by Western blotting (WB) using the indicated antibodies. (C) Interaction of gankyrin with FIH-1. HEK293T cells were cotransfected with plasmids expressing FLAG-Gankyrin and HA-FIH-1, and cultured at 3% O_2 for 48 h. Cell lysates were immunoprecipitated, and precipitants and inputs were analyzed by WB as in (B). WCL, whole cell lysates. Experiments were repeated three times with similar results. (D) Scatter plot of mRNA levels of gankyrin and VEGF-A in human hepatocellular carcinoma specimens. a.u., arbitrary unit.

3.4. Sequestration and inhibition of FIH-1 by gankyrin

We examined whether or not gankyrin suppresses FIH-1 expression. Overexpression of gankyrin did not affect FIH-1 level (Fig. 4A). When FIH-1 was overexpressed, the gankyrin level did not change, either. Thus, we suspected that gankyrin might affect the interaction of FIH-1 and HIF-1α. As shown in Fig. 4B, binding of FIH-1 and HIF-1α was suppressed by gankyrin. As FIH-1 binds ankyrin repeat domain proteins [16] and gankyrin contains 7 ankyrin repeats [3], we checked the possibility that gankyrin binds to FIH-1. HA-FIH-1 and FLAG-gankyrin were coimmunoprecipitated by either anti-HA or anti-FLAG antibody from cells cultured at 3% O_2, but not or only slightly at 1% or 20% O_2 concentration, respectively (Fig. 4C and data not shown). These results demonstrate that gankyrin binds to and sequester FIH-1, resulting in decreased interaction between FIH-1 and HIF-1α and increased activity of HIF-1 under mild hypoxic conditions. When we further examined the mRNA levels of gankyrin and VEGF-A in biopsy specimens of human HCC, a moderate positive correlation ($r = 0.57$, $P < 0.02$) was found (Fig. 4D), suggesting that the gankyrin–FIH-1 interaction might have some clinical relevance.

4. Discussion

Hemangioma/hemangiosarcomas are occasionally seen in mouse liver with incidences less than 3% [17]. In the present study, 71% of the F2 transgenic mice overexpressing HBX promoter-driven gankyrin developed hepatic hemangioma/hemangiosarcomas, whereas none of the wild-type mice did. This phenotype was probably not due to random insertional mutagenesis in the transgenic mice as it was also observed in F0 mice expressing SAP promoter-driven gankyrin. In the subsequent generations of this F0, however, gankyrin levels were decreased and no hemangioma/hemangiosarcoma developed. The finding that mice with 70% increase, but none with 35% increase in the protein level of hepatic gankyrin developed hemangioma/hemangiosarcomas (Fig. 1C) suggests that there is a critical level of gankyrin to show this phenotype.

How does overexpression of gankyrin in hepatocytes induce endothelial cell-derived tumors? As gankyrin induces dedifferentiation of HCCs [6], it may also induce transdifferentiation of hepatocytes into endothelial cells. A more feasible explanation, however, would be that gankyrin facilitates a sustained release of angiogenic growth factors, providing the milieu leading to hemangiosarcoma formation [18]. Consistent with this notion, endothelial overgrowth was more frequent in the HCCs of gankyrin-transgenic mice than wild-type mice after DEN treatment. Furthermore, mouse hepatoma transfectants overexpressing gankyrin induced more neovascuralization than parental cells when subcutaneously inoculated into nude mice. VEGF-A was the first identified member of the VEGF family, and mice with transgenic VEGF-A expressed in the liver have increased vascularization and vascular permeability [19]. When myoblasts overexpressing VEGF-A are transplanted into limb or heart muscle of mice, they induce hemangiomas [20]. In the present study, VEGF-A level was higher in the liver of gankyrin-transgenic mice compared with wild-type mice, and gankyrin increased VEGF-A expression in cultured hepatoma cells. Thus, VEGF-A probably contributed to formation of hemangioma/hemangiosarcomas in the gankyrin-transgenic mice.

HIF-1 is a major factor regulating the level of VEGF, and despite induction of multiple angiogenic target genes such as adrenomedullin and placental growth factor, VEGF is essential for HIF-1 mediated neovascularization [21]. Hypoxia induces changes in the hydroxylation status of well-conserved Pro and Asn residues of HIF-1α, resulting in protein stabilization and transcriptional activation [8,9]. Signaling through receptor tyrosine kinases induce HIF-1 expression by increasing the rate of HIF-1α protein synthesis via PI3K/Akt/mTOR pathway [8], and gankyrin activates this to promote VEGF expression [7]. In the present study, the HIF-1α protein level was not increased in cells overexpressing gankyrin. Reporter assays indicated, however, that HIF-1 transcriptional activity was increased by gankyrin, and that it was dependent on Asn803 of HIF-1α. FIH-1 hydroxylates this residue and inhibits transcriptional activity [8–10]. In addition to HIF-1α, proteins containing ankyrin repeat domains are common targets for hydroxylation by FIH-1, and IκBα as well as Notch-1 block the FIH-1-mediated HIF-1α repression by sequestering FIH-1 [22]. In this case, the recognition of each substrate and their relative affinity for FIH-1 is an important determinant of FIH-1 sequestration and consequently HIF regulation. Consistent with the recent study using recombinant proteins [16], gankyrin and FIH-1 were co-immunoprecipitated from cell lysates. Furthermore, overexpression of gankyrin reduced the amount of HIF-1α bound to FIH-1, suggesting a higher affinity for FIH-1 of gankyrin than HIF-1α at mild hypoxia. As expected, gankyrin increased the HIF-1 transcriptional activity in reporter assays, which was dependent on FIH-1. Interestingly, the binding of gankyrin to FIH-1 and enhancement of HIF-1 activity were dependent on the O_2 concentration.

We have demonstrated in this study that sustained overexpression of gankyrin in hepatocytes, although at a low level, can induce liver hemangioma/hemangiosarcomas in mice. Gankyrin sequesters FIH-1 from HIF-1α to activate HIF-1 and increase production of VEGF, which at least partly contributes to hemangioma/hemangiosarcoma formation. Further studies will clarify why spontaneous hemangioma/hemangiosarcomas are extremely rare in humans in contrast to experimental animals [18], and shed light on mechanisms of vascular tumorigenesis as well as hepatocarcinogeneis. The present study also suggests that gankyrin might play a physiological role in hypoxic responses besides its roles as an oncoprotein.

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Original Article

Perihepatic lymph node enlargement is a negative predictor for sustained responses to pegylated interferon-α and ribavirin therapy for Japanese patients infected with hepatitis C virus genotype 1

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Aim: Although perihepatic lymph node enlargement (PLNE) is reportedly associated with the negative outcome of interferon therapy for chronic hepatitis C, there were limitations in that the results were obtained in patients with various genotypes, viral loads and treatment regimens. We aimed to precisely clarify the significance of PLNE in interferon therapy for chronic hepatitis C.

Methods: Between December 2004 and June 2005, 112 patients with hepatitis C virus (HCV) genotype 1 and HCV RNA of more than 100 KIU/mL were enrolled, who underwent pegylated interferon-α plus ribavirin therapy thereafter. PLNE was defined as a perihepatic lymph node of more than 1 cm in the longest axis by ultrasonography.

Results: The sustained virological response (SVR) rate was lower in patients with PLNE (4/22, 18.2%) than in those without (37/90, 41.1%; $P = 0.045$) and viral load decline was smaller in patients with PLNE than in those without ($P = 0.028$). The proportion of PLNE positive patients was the smallest in the SVR group ($P = 0.033$) among the patient groups divided by the treatment outcome. PLNE was retained as a negative predictor for SVR by multivariate logistic regression analysis ($P = 0.012$). Furthermore, PLNE was not significantly associated with the mutations at HCV core protein and at interferon sensitivity-determining region, or interleukin-28B polymorphism in 45 patients with HCV genotype 1, enrolled between December 2011 and March 2012.

Conclusion: PLNE is a negative predictor for SVR in patients with HCV genotype 1 and HCV RNA of more than 100 KIU/mL treated with pegylated interferon-α plus ribavirin, independent of other known predictors for SVR.

Key words: chronic hepatitis C, hepatitis C virus core protein, interferon sensitivity-determining region, interleukin-28B

INTRODUCTION

PERIHEPATIC LYMPH NODE enlargement (PLNE) is frequently observed in patients with chronic liver disease,[1] especially in those with hepatitis C.[2,3] Although it has been shown that PLNE is associated with inflammatory activity, stage of liver fibrosis or hepatitis C viral load,[3–8] the reported findings were inconsistent,[2,9] suggesting that the clinical significance of PLNE has not been fully established yet. We have recently reported that PLNE is a negative predictor for hepatocellular carcinoma (HCC) development in chronic hepatitis C patients.[10]

Regarding PLNE and efficacy of interferon (IFN) therapy for chronic hepatitis C, PLNE was reportedly more frequently found in non-responders.[11,12] Dietrich *et al.* reported that perihepatic lymph node volume before IFN treatment was significantly larger in non-responders than in sustained virological responders,

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based on a study in which the patients had various genotypes and viral loads and were treated with IFN-α with or without ribavirin (RBV).[11] Soresi *et al.* reported that PLNE was more frequent in non-responders to IFN-α with RBV, although this association did not reach statistical significance by logistic regression analysis, in which the patients also had various genotypes.[12] We also reported that the sustained virological response (SVR) rate in patients who received IFN therapy was significantly lower in patients with PLNE than in those without, although our patients also had various genotypes and viral loads, and treatment regimens were various including IFN-α or pegylated IFN-α (PEG IFN) with or without RBV.[10] It is well known that patients with hepatitis C virus (HCV) genotype 1 and high baseline viral load are most difficult to treat with IFN[13] and that PEG IFN plus RBV has been the most effective standard of care for chronic hepatitis C until telaprevir emerged.[14] Thus, the previous results regarding PLNE and efficacy of IFN therapy for chronic hepatitis C had limitations, because they were analyzed with various genotypes, viral loads and treatment regimens.

In this study, in order to precisely clarify the potential association between PLNE and efficacy of IFN therapy for chronic hepatitis C, we analyzed the patients with HCV genotype 1 and HCV RNA of more than 100 KIU/mL at the start of therapy by PEG IFN with RBV in the well-characterized chronic hepatitis C cohort, in which liver stiffness values were found to be a risk for HCC development.[15]

METHODS

Subjects

THE PREVIOUS COHORT in which we originally analyzed the risk of liver stiffness for HCC development was employed; 866 chronic hepatitis C patients were enrolled between December 2004 and June 2005 at the Department of Gastroenterology, The University of Tokyo Hospital.[15] Among these patients, 112 patients, who had HCV genotype 1 and HCV RNA >100 KIU/mL, underwent PEG IFN plus RBV therapy after the enrollment. The potential association between PLNE and efficacy of PEG IFN plus RBV therapy was examined with these patients. When each subject was screened for HCC with ultrasonography at or immediately after the enrollment, the presence of PLNE was evaluated. The criteria to identify PLNE were previously described, and PLNE was defined as a lymph node at perihepatic area which was at least 1 cm in the longest axis.[10]

Moreover, between December 2011 and March 2012, 45 chronic hepatitis C patients with genotype 1 were enrolled at the Department of Gastroenterology, The University of Tokyo Hospital, to assess the potential association between PLNE and the known factors to predict the response to IFN therapy, namely, the mutations at position 70 of HCV core protein, those at IFN sensitivity-determining region (ISDR) of NS5A protein or interleukin (IL)-28B polymorphism.

The present study was carried out in accordance with the ethical guidelines of the Declaration of Helsinki and was approved by the Institutional Research Ethics Committee of the authors' institution. Informed consent was obtained for the use of the samples in this study.

Laboratory tests

Sequences of ISDR and the core region of HCV were determined by direct sequencing after amplification by reverse transcription and polymerase chain reaction as reported previously.[16,17] Genetic polymorphism in one tagging single nucleotide polymorphism located near the *IL-28B* gene (rs8099917) was determined by direct sequencing.[18] Homozygosity (GG) or heterozygosity (TG) of the minor sequence was defined as having the IL-28B minor allele, whereas homozygosity for the major sequence (TT) was defined as having the IL-28B major allele. Null virological response (NVR) was defined as detectable HCV RNA by qualitative polymerase chain reaction with a lower detection limit of 50 IU/mL (Amplicor; Roche Diagnostic Systems, Pleasanton, CA, USA) during the therapy. SVR was defined as undetectable HCV RNA 24 weeks after the completion of therapy. Relapse was defined as reappearance of HCV RNA after the completion of therapy. HCV RNA was quantitated using Amplicore HCV ver. 2.0 (Roche, Tokyo, Japan) or Cobas Ampliprep/Cobas TaqMan assay system (Roche, Tokyo, Japan). HCV genotype was determined based on the serotyping assay (SRL, Tokyo, Japan) or direct sequence analysis.[19]

Statistical analysis

Data were expressed as the mean ± standard deviation (SD) unless otherwise indicated. The categorical variables were compared by χ^2-test or Fischer's exact test, whereas continuous variables were compared by unpaired Student's *t*-test (parametric), Mann–Whitney *U*-test (non-parametric), Kruskal–Wallis rank sum test (non-parametric) or Wilcoxon rank sum test (non-parametric). For comparing group means, we used ANOVA. In the analysis of predicting factors for the responses to PEG IFN plus RBV therapy, the following variables were tested in univariate and multivariate logistic regression analysis: age, sex, serum albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (GGT), alkaline phosphatase, total bilirubin, α-fetoprotein (AFP) levels, prothrombin activity (%), liver stiffness values, platelet count, HCV viral load and the presence of PLNE. Factors that had a P-value of less than 0.4 in univariate analysis were subsequently included in multivariate logistic regression analysis. A P-value of less than 0.05 was considered significant. Data processing and analysis were performed using StatView ver. 5.0 (SAS Institute, Cary, NC, USA) and SPSS ver. 14.0 (SPSS, Chicago, IL, USA) software.

RESULTS

Association between PLNE and efficacy of PEG IFN plus RBV therapy

THE CLINICAL FEATURES of patients are summarized according to the presence or absence of PLNE in Table 1. All the patients were infected with HCV genotype 1 and had a high viral load (HCV RNA >100 KIU/mL). No patients were co-infected with hepatitis B virus. PLNE was observed in 22 of 112 (20.0%) patients analyzed.

PEG IFN-2a was administrated in 24 patients, while PEG IFN-2b in 88 patients. This ratio between patients treated with PEG IFN-2a and those with PEG IFN-2b was not different between patients with PLNE and those without (data not shown). Most of the patients were treated when the extended treatment of PEG IFN plus RBV therapy for more than 48 weeks was not generally performed in Japan; 27 of 112 (24.1%) patients received PEG IFN plus RBV for more than 48 weeks up to 72 weeks. After PEG IFN plus RBV therapy, SVR was achieved in 41 of 112 (36.6%) patients in this cohort. Between patients with PLNE and those without, the SVR rate was significantly lower in the former (4/22, 18.2%) than in the latter (37/90, 41.1%) ($P = 0.045$; Table 1). Of note, liver stiffness values as well as serum albumin levels, prothrombin activity and platelet count, which may be suggestive of liver fibrosis, were not different between patients with PLNE and those without (Table 1). Only serum GGT levels among the blood parameters were significantly lower in patients with PLNE than in those without.

To examine whether PLNE may influence virological response, the viral loads before treatment and at week 4 of treatment are depicted according to the presence or absence of PLNE in Figure 1. Viral decline between the start of treatment and week 4 was smaller in patients with PLNE than in those without ($P = 0.028$ by Wilcoxon rank sum test).

Patient characteristics according to the virological responses are shown in Table 2. In six patients, the

Table 1 Patient characteristics according to the presence or absence of PLNE

Parameter	PLNE positive, $n = 22$	PLNE negative, $n = 90$	P-value
Age (years)†	60.5 ± 8.8	57.2 ± 8.9	0.12
Male/female	14/8	40/50	0.10
Albumin (g/dL)†	4.01 ± 0.40	4.04 ± 0.33	0.75
AST (U/L)†	62.6 ± 41.2	61.0 ± 38.9	0.87
ALT (U/L)†	76.0 ± 62.2	72.4 ± 56.6	0.81
GGT (U/L)†	37.2 ± 24.6	53.5 ± 39.1	0.018
Alkaline phosphatase (U/L)†	211.7 ± 80.9	199.0 ± 66.3	0.50
Total bilirubin (mg/dL)†	0.82 ± 0.29	0.87 ± 0.47	0.48
Prothrombin time activity (%)	85.8 ± 12.5	85.9 ± 14.7	0.97
AFP (ng/mL)†	22.8 ± 44.0	13.0 ± 21.7	0.32
Liver stiffness (kPa)†	10.8 ± 6.9	12.7 ± 11.9	0.32
Platelet count ($\times 10^4/\mu L$)†	15.2 ± 8.2	15.5 ± 5.3	0.85
HCV viral load (KIU/mL)‡	731 (358–1070)	636 (151–1045)	0.51
SVR rate (%)§	18.2	41.1	0.045

†Data are presented as mean ± standard deviation, and compared by Student's t-tests.
‡Data are presented as median (25–75% range), and compared by Mann–Whitney U-tests.
§Data are compared by χ^2-tests.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; HCV, hepatitis C virus; IL, interleukin; PLNE, perihepatic lymph node enlargement; SVR, sustained virological response.

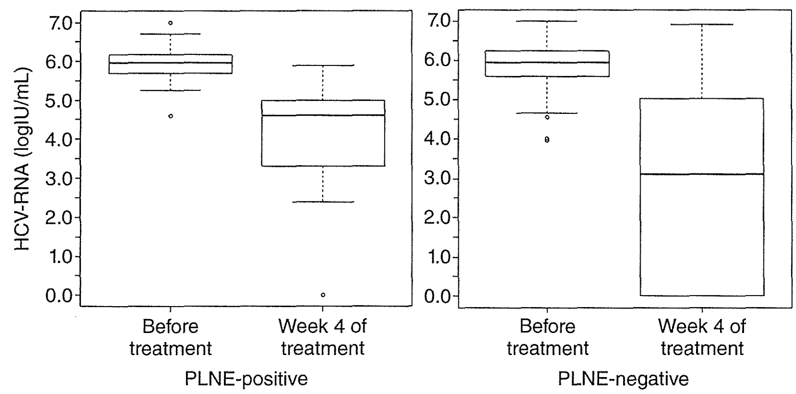

Figure 1 Changes in viral load in patients treated by PEG IFN plus RBV according to the presence or absence of PLNE. The viral loads before treatment with PEG IFN plus RBV and at week 4 of treatment were analyzed in patients with PLNE (PLNE positive, $n = 22$) and without PLNE (PLNE negative, $n = 90$). Viral decline was smaller in patients with PLNE than in those without ($P = 0.028$ by Wilcoxon rank sum test). HCV, hepatitis C virus; PEG IFN, pegylated interferon-α; PLNE, perihepatic lymph node enlargement; RBV, ribavirin.

effect of PEG IFN plus RBV therapy was lost during the treatment. Among the three groups of patients divided by the treatment outcome, namely, SVR, relapse or NVR, the rate of PLNE positive patients was least (4/41, 9.8%) in the SVR group ($P = 0.033$). Regarding other parameters, liver stiffness values were lowest ($P = 0.033$) in the SVR group, whereas serum albumin levels were lowest in the NVR group ($P = 0.004$), in line with the well-known evidence that patients with advanced liver fibrosis are difficult to treat with IFN.[20]

Then, the predicting factors for SVR were analyzed, and the results are shown in Table 3. Nine factors that had a *P*-value of less than 0.4 in univariate analysis were subsequently included in multivariate logistic regression analysis. As a result, ALT, GGT, liver stiffness and PLNE were retained as independent predicting factors for SVR (Table 3). These results suggest that the presence of PLNE is a negative predictor for achievement of SVR by PEG IFN and RBV therapy in chronic hepatitis C patients with genotype 1 and HCV RNA of more than 100 KIU/mL, independent of liver fibrosis.

Table 2 Patient characteristics according to the virological responses

Parameter	SVR, $n = 41$	Relapse, $n = 29$	NVR, $n = 36$	*P*-value
Age (years)†	57.3 ± 8.9	57.3 ± 9.1	58.8 ± 8.9	0.71
Male/female§	18/23	16/13	17/19	0.55
Albumin (g/dL)†	4.11 ± 0.28	4.10 ± 0.35	3.87 ± 0.37	0.004
AST (U/L)†	61.2 ± 43.8	56.6 ± 33.9	65.2 ± 34.1	0.67
ALT (U/L)†	81.6 ± 70.6	63.2 ± 40.1	71.4 ± 37.1	0.36
GGT (U/L)†	42.6 ± 30.3	48.5 ± 33.4	62.8 ± 45.6	0.056
Alkaline phosphatase (U/L)†	187.2 ± 73.9	211.7 ± 56.6	203.0 ± 73.7	0.33
Total bilirubin (mg/dL)†	0.78 ± 0.22	0.86 ± 0.32	0.99 ± 0.67	0.11
Prothrombin time (%)†	86.1 ± 17.1	84.8 ± 12.4	87.1 ± 12.3	0.80
AFP (ng/mL)†	9.6 ± 18.5	12.0 ± 27.0	23.2 ± 35.5	0.082
Liver stiffness (kPa)†	8.9 ± 4.7	13.5 ± 11.7	15.4 ± 14.9	0.033
Platelet counts ($\times 10^4/\mu L$)†	16.1 ± 4.4	16.8 ± 7.7	13.7 ± 5.8	0.077
HCV viral load (KIU/mL)‡	641 (150–1260)	670 (286–1168)	615 (168–951)	0.86
PLNE negative/positive§	37/4	21/8	28/8	0.033
Rate of PLNE positive (%)	9.8	27.6	22.2	

†Data are presented as mean ± standard deviation and compared by Student's *t*-tests.
‡Data are presented as median (25–75% range) and compared by Kruskal–Wallis rank sum tests.
§Data are compared by Fischer's exact tests.
AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase; HCV, hepatitis C virus; IL, interleukin; NVR, null virological response; PLNE, perihepatic lymph node enlargement; SVR, sustained virological response.