study. Among the 284 patients, 215 patients were not treated with IFN (no IFN group). The remaining 69 patients received IFN therapy for HCV infection. In the latter group, HCC had not been detected at the IFN therapy, and was detected after the IFN therapy. The IFN therapy was performed not for HCC, but for HCV-related hepatitis. The administration of IFN therapy was determined based on the informed consent between each physician and patient. The response to IFN therapy was assessed retrospectively based on changes in HCV-RNA. Based on the response, patients were divided into the responders group and non-responders group; 23 patients whose HCV-RNA disappeared after IFN therapy were categorized as the responders group, and 46 patients whose HCV-RNA did not disappear after IFN therapy in non-responders group. Figure 1 summarizes the classification of the enrolled patients. The type, dosage, and duration of IFN administration before surgery varied, though all patients received IFN-α.

Hospital records were retrospectively reviewed for clinical factors including previous history of IFN therapy, tumor- and surgery-related factors. The surgical procedure was selected based on the extent of the tumor and residual liver function. The HCC staging was performed according to the classification system of the Liver Cancer Study Group of Japan [17]. The histological grade of differentiation of HCC was determined according to the Edmondson–Steiner classification, and was based on the areas of the tumor with the highest grade [18]. Non-cancerous lesion of the liver was divided histopathologically into chronic hepatitis CH and liver cirrhosis (LC).

Patients were followed up after hepatic resection at regular intervals of 3–4 months with physical examination, tumor markers including alpha-fetoprotein (AFP), and protein induced by vitamin K absence or antagonists-II (PIVKA-II), liver biochemical tests, abdominal ultrasonography, and abdominal computed tomography (CT) to check for intrahepatic recurrence, and chest radiography and bone scintigraphy for extra-hepatic recurrence. The median duration of clinical follow-up after the initial hepatectomy was 51.2 months.

Data were expressed as mean ± standard deviation. Differences between groups were assessed by the chi-square test, Fisher's exact test, or the Mann–Whitney U test. Survival rates were calculated according to the Kaplan and Meier method and compared using the log-rank test. Statistical analysis was performed using StatView (version 5.0, SAS Institute Inc., Cary, NC). A P-value <0.05 was considered statistically significant. The study protocol was approved by the Human Ethics Review Committee of Osaka University Hospital and a signed consent form was obtained from each patient.

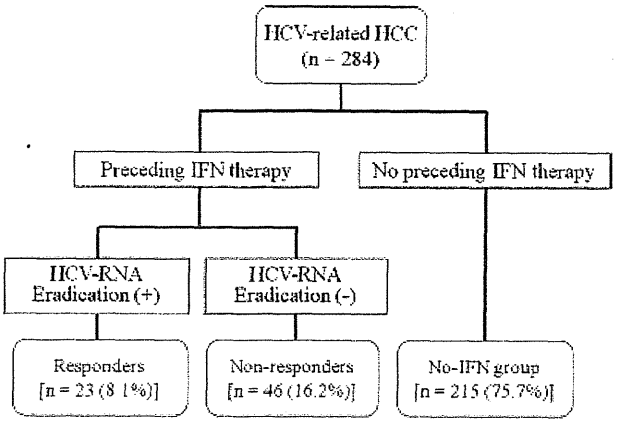

Fig. 1. Distribution of patients enrolled in this study according to the clinical background of preceding IFN therapy. HCV, hepatic C virus; HCC, hepatocellular carcinoma; IFN, interferon.

RESULTS

The study group comprised 222 (78.2%) men and 62 (21.8%) women, with a mean age of 65 (range, 39–79). Table I summarizes the clinicopathological characteristics of the responders group, the non-responders group, and the no-IFN group. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were significantly lower in the responders group than the non-responders group ($P < 0.001$, $P = 0.001$) and no-IFN group ($P = 0.001$, $P = 0.002$). There were no significant differences in the levels of AST and ALT between the non-responders group and the no-IFN group. Platelet count was significantly higher in the responders than that in the non-responders ($P = 0.008$) and that in the no-IFN group ($P = 0.001$). In the responders group, histopathological status of the non-cancerous liver tissue obtained at surgery was CH in 16 patients (69.6%) and LC in seven patients (30.4%). The percentage of patients of the responders group with LC was significantly lower than that of the non-responders group (28/46, 60.9%; $P = 0.017$) and that of the no-IFN group (123/215, 57.2%; $P = 0.014$). Liver function assessed by Child–Pugh classification was not different among the three groups. Other clinical factors listed in Table I were also not different among the three groups, including tumor- and surgical-related factors. Adjuvant therapy of IFN was administered in a small number of patients (n = 14, 4.9%), and the frequency of such patients was not different among the three groups.

For all the 284 patients, the 1-, 3-, and 5-year disease-free survival (DFS) rates were 70.8%, 36.7%, and 22.8%, respectively. There was no significant difference in DFS between the IFN group (the responders group and the non-responders group) and the no-IFN group ($P = 0.396$). However, the DFS of the responders group (1 year: 89.2%, 3 years: 59.4%, 5 years: 59.4%) was significantly better than that of the no-IFN group (1 year: 70.8%, 3 years: 35.7%, 5 years: 21.6%; $P = 0.039$), and tended to be better than that of the non-responders group (1 -year: 60.4%, 3 years: 32.3%, 5 years: 16.9%; $P = 0.051$; Fig. 2). However, there was no significant difference in DFS between the non-responders group and the no-IFN group ($P = 0.673$). The 1-, 3-, and 5-year overall survival rates for all patients were 94.5%, 80.4%, and 66.9%, respectively. The overall survival rates of the IFN group (responders group and non-responders group) tended to be higher than those of the no-IFN group ($P = 0.093$). The 1-, 3-, and 5-year overall survival rates for the responders group were 100%, 100%, and 100%, respectively, and were significantly higher than the non-responders group (1-year: 94.4%, 3 years: 78.6%, 5 years: 55.4%; $P = 0.026$) and the no-IFN group (1 year: 94.0%, 3 years: 79.0%, 5 years: 66.1%; $P = 0.009$; Fig. 3). There was no significant difference in overall survival between the non-responders group and the no-IFN group ($P = 0.904$).

Univariate analysis was performed between DFS and various clinicopathological factors (Table II). Microscopic vascular invasion (negative vs. positive), tumor stage (I, II vs. III, IV), number of nodules (single vs. multiple), the diameter of largest tumor nodules (<5 cm vs. ≥5 cm), AFP level (<5 ng/m vs. ≥5 ng/m), and preceding IFN therapy (responders vs. non-responders, no-IFN) were significant factors ($P < 0.001$, $P = 0.006$, $P = 0.008$, $P = 0.021$, $P = 0.017$, $P = 0.037$). Multivariate analysis for DFS using the above six factors identified the number of nodules and microscopic vascular invasion as significant independent factors ($P = 0.014$, $P = 0.041$; Table III). In the same analysis, preceding IFN therapy showed a borderline significance with DFS ($P = 0.086$). The diameter of the largest tumor nodules and AFP level also tended to be associated with DFS ($P = 0.090$, $P = 0.098$).

Univariate analysis for overall survival using various clinicopathological factors demonstrated that microscopic vascular invasion (negative vs. positive), preceding IFN therapy (responders vs. non-responders, no-IFN), number of nodules (single vs. multiple), diameter of largest nodules (<5 cm vs. ≥5 cm), and AFP level (<5 ng/m vs. ≥5 ng/m) were significant factors ($P = 0.004$, $P = 0.009$, $P = 0.015$,

TABLE I. Clinicopathological Characteristics of Patients With HCV-Related HCC

	IFN group			*P*-value		
	Responders (n = 23)	Non-responders (n = 46)	No-IFN (n = 215)	Responders versus non- responders	Responders versus no-IFN	Non- responders versus no-IFN
Clinical factors						
Gender (male/female)	19/4	33/13	170/45	0.323	0.793	0.278
Age (years)	66 ± 7	64 ± 7	65 ± 7	0.355	0.653	0.424
Alcohol abuse (+/−)	14/9	27/19	132/83	0.862	0.961	0.733
HCV serotype (1/2/unknown)	19/4/0	35/5/6	166/29/20	>0.999	0.795	0.969
HBs Ag (+/−)	1/22	1/45	7/208	>0.999	0.562	>0.999
AST (IU/L)	28 ± 13	49 ± 27	46 ± 21	<0.001	0.001	0.184
ALT (IU/L)	26 ± 16	52 ± 36	47 ± 29	0.001	0.002	0.233
Platelet count ($\times 10^4/\mu l$)	16.4 ± 3.3	13.0 ± 5.3	13.2 ± 4.5	0.008	0.001	0.867
Albumin (g/dl)	4.0 ± 0.4	3.8 ± 0.6	3.8 ± 0.5	0.142	0.175	0.975
Total bilirubin (mg/dl)	0.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.3	0.114	0.104	0.362
Prothrombin time (%)	77 ± 10	77 ± 8	76 ± 9	0.719	0.888	0.442
Hepaplastin test (%)	81 ± 13	78 ± 12	77 ± 12	0.244	0.217	0.834
Child-Pugh (A/B)	22/1	37/9	187/28	0.148	0.326	0.265
Non-cancerous lesion (CH/LC)	16/7	18/28	92/123	0.017	0.014	0.648
Tumor-related factors						
AFP (ng/ml)	1,791 ± 6,654	545 ± 1,444	851 ± 4,004	0.226	0.332	0.610
PIVKA-II (mAU/ml)	1,773 ± 5,433	1,418 ± 4,733	2,006 ± 4,879	0.786	0.837	0.459
Preoperative TAE (+/−)	10/13	23/23	119/96	0.609	0.278	0.509
Postoperative IFN (+/−)	1/22	1/45	12/203	>0.999	>0.999	0.476
Number of nodules (single/multiple)	17/6	33/13	152/63	0.849	0.747	0.888
Tumor diameter (cm)	3.5 ± 1.9	3.2 ± 2.0	3.6 ± 2.6	0.287	0.725	0.171
Vascular invasion (+/−)	2/21	2/44	18/197	0.596	>0.999	0.543
Stage (I/II/III/IV)	5/12/4/2	12/24/7/3	45/109/49/12	0.967	0.890	0.671
Edmondson-Steiner grade (I, II/III, IV/unknown)	13/10/0	26/16/4	117/89/9	0.672	0.980	0.541
Surgery-related factors						
Hr (0/S/1/2)	13/3/3/4	29/5/7/5	116/41/37/21	0.869	0.615	0.543
Volume of resection (g)	152 ± 118	137 ± 151	165 ± 162	0.393	0.682	0.186
Blood loss (ml)	1,022 ± 1,583	996 ± 702	1,167 ± 1,217	0.770	0.571	0.197
Operation time (min)	253 ± 128	236 ± 99	236 ± 99	0.337	0.940	0.174
Transfusion (+/−)	4/19	7/39	52/163	>0.999	0.465	0.187

Data are expressed as mean ± standard deviation.
IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies.

$P = 0.034$, $P = 0.045$; Table II). Multivariate analysis for overall survival using the above five factors identified number of nodules, microscopic vascular invasion, and preceding IFN therapy as significant independent factors ($P = 0.025$, $P = 0.037$, $P = 0.042$; Table III).

HCC recurred postoperatively in nine (39.1%) patients of the responders group, 29 (63.0%) of the non-responders group, and in 157 (73.0%) of the no-IFN group. Table IV summarizes the clinical characteristics of patients with recurrent HCC at diagnosis of the recurrence. AST and ALT levels in the responders group were

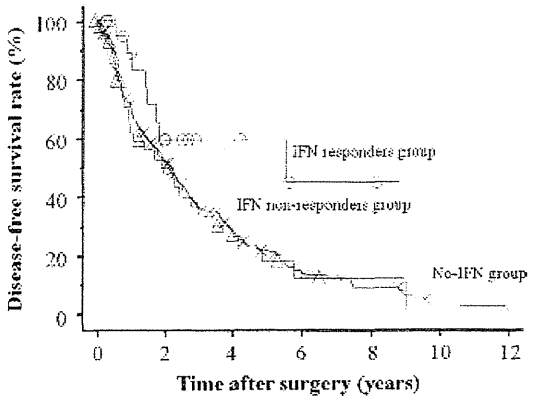

Fig. 2. Disease-free survival after initial surgery for HCC in the responders group, the non-responders group, and the no-IFN group. Open circles: responders (n = 23), open triangles: non-responders (n = 46), crosses: no-IFN (n = 215). IFN: interferon.

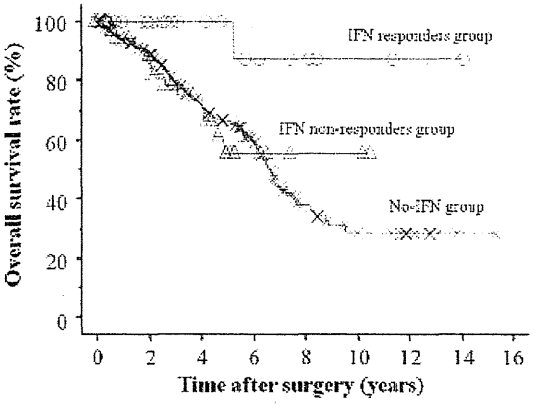

Fig. 3. Overall survival calculated from the initial surgery for HCC for the responders group, the non-responders group, and the no-IFN group. Open circles: responders (n = 23), open triangles: non-responders (n = 46), crosses: no-IFN (n = 215). IFN: interferon.

TABLE II. Univariate Analysis of Disease-Free Survival and Overall Survival of Patients With HCV-Related HCC

	Number of patients	Disease-free survival	Overall survival
Clinical factors			
Gender (male/female)	222/62	0.732	0.789
Age, years (<66/≥67)	143/141	0.682	0.842
Alcohol abuse (+/−)	172/112	0.955	0.572
HCV genotype (1/2/unknown)	220/40/25	0.612	0.427
AST (IU/L) (<40/≥40)	126/158	0.496	0.547
ALT (IU/L) (<40/≥40)	122/162	0.216	0.301
Total bilirubin (mg/dl) (<1.0/≥1.0)	252/32	0.890	0.587
Albumin (g/dl) (<3.5/≥3.5)	114/170	0.174	0.171
Prothrombin time (%) (<70/≥70)	77/207	0.693	0.875
Hepaplastin test (%) (<70/≥70)	75/209	0.427	0.398
Platelet count ($\times 10^4/\mu l$) (<10/≥10)	83/201	0.176	0.123
Child-Pugh (A/B)	246/38	0.866	0.594
Non-cancerous lesion (CH/LC)	126/158	0.247	0.177
Tumor-related factors			
AFP (ng/ml) (<5/≥5)	54/230	0.021	0.045
PIVKA-II (mAU/ml) (<400/≥400)	190/83	0.130	0.142
Preceding IFN (responders/non-responders, no-IFN)	23/261	0.037	0.009
Preoperative TAE (+/−)	152/132	0.863	0.562
Postoperative IFN (+/−)	14/270	0.222	0.253
Number of nodules (single/multiple)	202/82	0.008	0.015
Tumor diameter (cm) (<5/≥5)	232/52	0.017	0.034
Vascular invasion (+/−)	22/262	<0.001	0.004
Stage (I, II/III, IV)	207/77	0.006	0.197
Edmondson-Steiner grade (I, II/III, IV)	156/115	0.328	0.265
Surgery-related factors			
Hr (0/S, 1, 2)	158/126	0.313	0.893
Intraoperative blood loss (L) (<1/≥1)	151/133	0.289	0.270
Operation time (min) (<240/≥240)	141/143	0.221	0.493
Transfusion (+/−)	63/221	0.756	0.180

IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies.

significantly lower than those in the non-responders group ($P = 0.047$, $P = 0.045$) and those in the no-IFN group ($P = 0.028$ and $P = 0.034$). There were no significant differences in AST and ALT levels between the non-responders and no-IFN groups. Platelet count was significantly higher in the responders group than that in the no-IFN group ($P = 0.029$) and tended to be higher than that in the non-responders group ($P = 0.079$). Figure 4A shows the distribution of interval between initial hepatectomy and recurrence. In most patients, HCC recurred within 2 years in the three groups, and the distribution of the interval was not different among the three groups. In all groups, the

TABLE III. Multivariate Analysis of Disease-Free Survival and Overall Survival of Patients With HCV-Related HCC

	OR	95% CI	*P*-value
Disease-free survival			
AFP (ng/ml) (<5/≥5)	1.427	0.937–2.174	0.098
Preceding IFN (responders/non-responders, no-IFN)	1.809	0.919–3.561	0.086
Number of nodules (single/multiple)	1.707	1.022–2.850	0.041
Tumor diameter (cm) (<5/≥5)	1.391	0.951–2.037	0.090
Vascular invasion (−/+)	2.331	1.186–4.587	0.014
Stage (I, II/III, IV)	1.287	0.715–2.315	0.401
Overall survival			
AFP (ng/ml) (<5/≥5)	1.689	0.847–3.367	0.137
Preceding IFN (responders/non-responders, no-IFN)	7.750	1.076–55.798	0.042
Number of nodules (single/multiple)	1.622	1.062–2.476	0.025
Tumor diameter (cm) (<5/≥5)	1.381	0.842–2.268	0.200
Vascular invasion (−/+)	2.247	1.049–4.808	0.037

IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies. OR, odds ratio, 95% CI, 95% confidence interval.

TABLE IV. Clinicopathological Characteristics of Patients With Recurrent HCC in the Responders Group, the Non-Responders Group, and the No-IFN Group

	IFN group			*P*-value		
	Responders (n = 9)	Non-responders (n = 29)	No-IFN (n = 157)	Responders versus non-responders	Responders versus no-IFN	Non-responders versus no-IFN
Clinical factors						
Gender (male/female)	9/0	22/7	126/31	0.164	0.212	0.590
Age (years)	67 ± 7	66 ± 6	67 ± 7	0.641	0.971	0.378
AST (IU/L)	30 ± 25	50 ± 28	55 ± 28	0.047	0.028	0.786
ALT (IU/L)	32 ± 26	53 ± 33	54 ± 34	0.045	0.034	0.902
Platelet count ($\times 10^4/\mu l$)	14.8 ± 3.3	12.2 ± 3.7	11.8 ± 3.5	0.079	0.029	0.720
Albumin (g/dl)	3.9 ± 0.3	3.7 ± 0.4	3.6 ± 0.4	0.122	0.085	0.782
Total bilirubin (mg/dl)	0.7 ± 0.2	0.7 ± 0.2	0.8 ± 0.3	0.216	0.242	0.757
Prothrombin time (%)	76 ± 8	75 ± 12	75 ± 11	0.894	0.942	0.918
Hepaplastin test (%)	75 ± 11	74 ± 11	73 ± 13	0.872	0.817	0.907
Child-Pugh (A/B)	8/1	25/4	130/27	>0.999	>0.999	0.791
Tumor-related factor						
AFP (ng/ml)	51 ± 112	60 ± 98	81 ± 305	0.983	0.848	0.757
PIVKA-II (mAU/ml)	90 ± 83	258 ± 712	200 ± 696	0.491	0.640	0.744
Latency to recurrence (years)	2.6 ± 2.8	2.0 ± 2.0	2.2 ± 2.1	0.497	0.561	0.707
Recurrence site (intrahepatic/extrahepatic)	8/1	29/0	150/7			
Intrahepatic recurrence (single/multiple)	6/2	11/18	57/93			

Data are expressed as mean ± standard deviation.
IFN, interferon; HCV, hepatic C virus; HBs Ag, hepatitis B surface antigen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CH, chronic hepatitis; LC, liver cirrhosis; AFP, alpha-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonists-II; TAE, transcatheter arterial chemoembolization; Hr, hepatic resection; 0: partial resection; S, subsegmentectomy; 1, one segmentectomy; 2, two segmentectomies.

majority of first recurrence sites were residual liver [responders group: 89% (8/9), non-responders group: 100% (29/29), no-IFN group: 94% (150/157)] (Fig. 4B). In the responders group, among eight patients with intrahepatic recurrence, solitary recurrence was seen in six patients (75.0%). On the other hand, the percentage of solitary intrahepatic recurrence was 37.9% (11/29) in the non-responders group and 38.0% (57/150) in the no-IFN group. In the responders group, surgery, percutaneous therapy, and transarterial chemoembolization (TACE) was selected in three, four, and two patients for treatment of recurrence, respectively (Fig. 4C). The proportion of patients in whom surgery or percutaneous therapy was selected for treatment in the responders group (7/9, 77.8%) was significantly higher than that of the non-responders group (7/29, 24.1%, $P = 0.006$) and the no-IFN group (28/157, 17.8%, $P < 0.001$).

Figure 5 shows the overall survival from diagnosis of the first HCC recurrence in the three groups. The overall survival rate of the

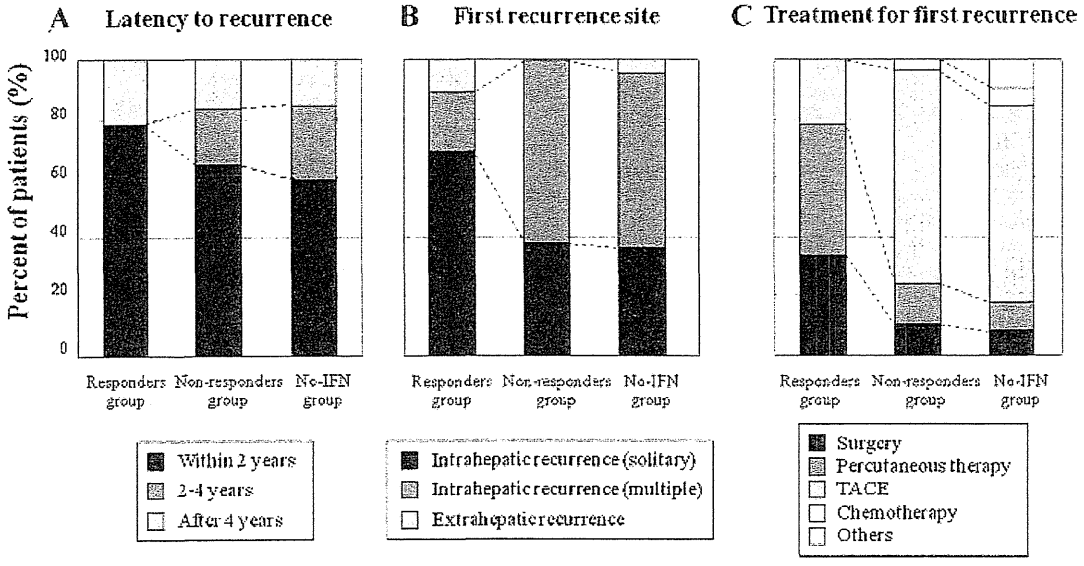

Fig. 4. **A**: Distribution of the latency from the initial hepatectomy to HCC recurrence for the responders, the non-responders, and the no-IFN group. **B**: Distribution of the first recurrence site in patients with HCC recurrence of the responders, the non-responders, and the no-IFN group. **C**: Distribution of selected treatment for first HCC recurrence in the responders, the non-responders, and the no-IFN group. IFN: interferon, TACE: transcatheter arterial chemoembolization.

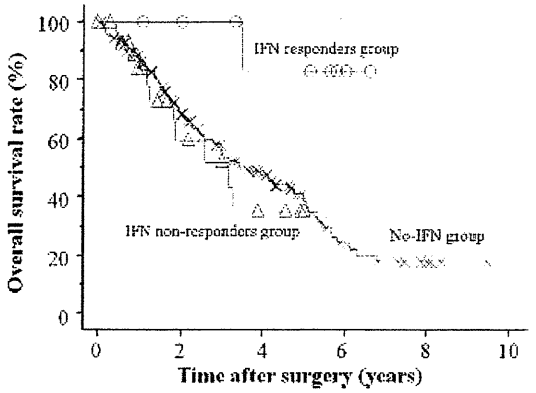

Fig. 5. Overall survival rates calculated from the diagnosis of first HCC recurrence in the responders group, the non-responders group, and the no-IFN group. Open circles: responders (n = 9), open triangles: non-responders (n = 29), crosses: no-IFN group (n = 157). IFN: interferon.

responders group was significantly higher than that of the non-responders group ($P = 0.012$) and that of no-IFN group ($P = 0.011$).

DISCUSSION

The present study demonstrated that a significantly better DFS from the initial hepatectomy in the responders group than the other two groups. This result was similar to that reported previously by Uenishi et al. [16]. Based on the pattern of the DFS curve of the responders group in this study, the recurrence rate appeared to decrease mainly in 2 years later. We have reported that DFS curves for postoperative HCC patients in the early (within 2 years) and late (4 years after surgery) represented both intrahepatic metastasis and multicentric carcinogenesis, respectively [19]. Based on this viewpoint, the decrease in recurrence in the responders group was probably mainly due to the suppression of new multicentric carcinogenesis. A number of investigators have reported that suppression of increased liver inflammation, as assessed by AST and ALT, contributes to inhibition of hepatocarcinogenesis and postoperative intrahepatic recurrence after HCC surgery, which is more likely to originate from multicentric carcinogenesis [20,21]. IFN has been reported also to be effective in eradication of HCV-RNA from the serum and hepatic tissue and prevention of deterioration of liver dysfunction in patients with HCV infection [5–8,10]. It is possible that the suppression of new multicentric carcinogenesis seen in the IFN responders group of this study was due to these effects of IFN therapy. This speculation is supported by the findings of the present study that the levels of aminotransferases and platelet count in the responders group were significantly lower and higher, respectively, than those of the other groups, at the initial hepatectomy and first recurrence, and that the frequency of LC in the responders group was significantly lower than that of the other groups.

On the other hand, IFN has been reported to have anti-tumor effects [22–24]. These anti-tumor effects of IFN had been actually verified also in IFN-alpha/5-fluorouracil combination therapy for advanced HCC in a series of studies by our group [25–32]. Additionally, in a previous report by Uenishi et al. [16], only one patient developed postoperative recurrence about 5 years after the initial surgery among 11 patients of the responders group, and the recurrence pattern of the responders group was also suggestive of the inhibitory effect of IFN on metastasis originating from the primary HCC. Taken together, also in the present study, the decrease of recurrence might be potentially derived from the suppression of intrahepatic metastasis by IFN.

In the present study, overall survival from the initial hepatectomy in the responders group was also significantly better than those of the other two groups. This improvement of overall survival was caused by the aforementioned decrease of HCC recurrence rate in the responders group. In addition, in the responder group, the percentage of patients who underwent selective surgery or percutaneous therapy for the treatment of recurrent HCC was higher than other groups. In general, the treatment for the postoperative HCC recurrence is frequently restricted for the residual liver function, which is one of the reasons for the unfavorable postoperative outcome [3,4]. Considering such restriction of the treatment, the improved liver function by IFN therapy was also speculated to contribute to the better overall survival. Finally, it could be argued that IFN therapy was the main reason for the improvement in both DFS and overall survival rates in the responders group.

To date, several studies examined the impact of IFN therapy after curative loco-regional treatment for HCC [33–37]. For example, in a randomized controlled trial, Ikeda et al. [33] reported that IFN therapy suppressed tumor recurrence after surgery or ethanol injection for HCV-related HCC. Kubo et al. [36] also reported that postoperative IFN therapy significantly decreased recurrence after resection of HCV-related HCC in a randomized controlled trial. That several randomized controlled trials indicated improved posttreamtemt outcome in patients with HCV-related HCC who received postoperative IFN therapy, adds support to our conclusion of the effectiveness of preceding IFN therapy.

Since the present study is retrospective in nature, few details of IFN therapy are unavailable. For example, the duration of HCV-RNA clearance was not clear in several patients treated with IFN. Therefore, in this study, we could not divide patients of the responders group into those with SVR or not. In order to examine more strictly the effectiveness of preceding IFN therapy for surgical outcome, is prospectively designed study is necessary.

CONCLUSIONS

The present study demonstrated the effectiveness of IFN therapy for HCV infection administered before HCC resection as assessed by evaluating the disease-free and overall survival. IFN therapy for HCV might be essential not only for the treatment of HCV infection but also for improvement of prognosis of patients who are susceptible to the development of HCC.

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Research Article

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Circulating *microRNA-21* as a novel biomarker for hepatocellular carcinoma

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Background & Aims: Several groups have reported the significance of circulating microRNA as a biochemical marker of cancer. To our knowledge, however, there are no reports on the significance of circulating microRNA in hepatocellular carcinoma. The aim of this study was to evaluate the significance of plasma *microRNA-21* level as a biochemical marker for hepatocellular carcinoma.

Methods: Plasma *microRNA-21* level was measured by qRT-PCR in 10 patients before and after curative resection of hepatocellular carcinoma. Plasma *microRNA-21* was also compared in other groups of: 126 patients with hepatocellular carcinoma, 30 patients with chronic hepatitis, and 50 healthy volunteers. The power of *microRNA-21* in differentiating hepatocellular carcinoma from chronic hepatitis or from healthy volunteers was compared to that of α-fetoprotein.

Results: In the 10-patient group, plasma *microRNA-21* levels significantly diminished after surgery compared with the pre-operative values ($p = 0.0125$). Plasma *microRNA-21* level in the 126 patients with hepatocellular carcinoma was significantly higher than in patients with chronic hepatitis and healthy volunteers ($p < 0.0001$, $p < 0.0001$, respectively). ROC analysis of plasma *microRNA-21* yielded an AUC of 0.773 with 61.1% sensitivity and 83.3% specificity when differentiating hepatocellular carcinoma from chronic hepatitis, and an AUC of 0.953 with 87.3% sensitivity and 92.0% specificity when differentiating hepatocellular carcinoma from healthy volunteers. Both sets of values were superior to α-fetoprotein and improved for the combination of *microRNA-21* and α-fetoprotein.

Conclusions: Plasma *microRNA-21* level is a promising biochemical marker for hepatocellular carcinoma.

Keywords: Hepatocellular carcinoma; microRNA; *microRNA-21*; Plasma; Biomarker.

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Abbreviations: AFP, α-fetoprotein; AUC, area under the receiver-operator characteristic curve; CH, chronic hepatitis; CT, computed tomography; HCC, hepatocellular carcinoma; HV, healthy volunteer; miRNA, microRNA; MRI, magnetic resonance imaging; PIVKA-II, protein induced by vitamin K absence or antagonists-II; qRT-PCR, quantitative RT-polymerase chain reaction; ROC, receiver-operator characteristic; RT, reverse transcription.

Introduction

MicroRNA (miRNA) is a small noncoding RNA gene product known to post-transcriptionally modulate gene expression by negatively regulating the stability or translational efficiency of its target mRNAs [1,2]. MiRNAs control a wide array of biological processes, such as cell differentiation, proliferation, and apoptosis. Aberrant expressions of miRNAs have been widely reported in human cancers with both up- and down-regulation detected in neoplastic cells compared with their normal counterparts [3,4]. Several recent studies reported that miRNAs are stably detectable in plasma and serum [4–6]. Mitchell *et al.* [5] reported that tumor-associated circulating miRNAs are stably detectable in the plasma of human prostate cancer xenograft mouse models and prostate cancer patients, suggesting that their detection could differentiate cancer-bearing individuals from healthy controls. The finding also raised the possibility that assaying miRNAs in plasma or serum may serve as a novel approach for blood-based detection of human cancers. Actually, since the above study, several investigators have reported the significance of some types of plasma miRNAs as biochemical markers for human cancers [7–13].

Hepatocellular carcinoma (HCC) is a common cancer worldwide, especially in Japan and other East Asian countries, and the third most frequent cause of cancer-related deaths in the world [14]. One of the reasons for the high mortality in HCC is that the tumors are frequently detected at a stage when curative resection is no longer feasible because of intrahepatic and extrahepatic metastases. Today, the diagnosis of HCC relies on the finding of a liver mass in radiology imaging studies including ultrasonography, computed tomography (CT), and/or magnetic resonance imaging (MRI). However, the diagnosis of small lesions is relatively inaccurate [15]. One of the common approaches used for screening HCC in a high risk-population is serum tumor markers such as α-fetoprotein (AFP) and protein induced by vitamin K absence or antagonists-II (PIVKA-II). However, the sensitivity and

Table 1. Clinicopathological characteristics of patients with hepatocellular carcinoma (HCC), patients with chronic hepatitis (CH), and healthy volunteers (HVs).

	HCC patients		CH patients	HVs	*p* value	
	(n = 10)‡	(n = 126)	(n = 30)	(n = 50)	(HCC *vs.* CH)	(HCC *vs.* HVs)
Clinical factors						
Gender (male/female)	9/1	99/27	20/10	37/13	0.1683	0.5140
Age (years)*	66 ± 9	63 ± 10	62 ± 8	62 ± 8	0.4062	0.6935
Viral status (B-C-/B+C-/B-C+/B+C+)†	1/3/6/0	14/25/84/3	0/4/26/0		0.1129	
AST (IU/L)*	39 ± 19	38 ± 20	56 ± 28		0.0002	
ALT (IU/L)*	39 ± 20	41 ± 25	57 ± 36		0.0048	
Platelet count ($x10^4/\mu l$)*	14.8 ± 5.1	16.1 ± 6.0	15.1 ± 5.8		0.4334	
Prothrombin time (%)*	82 ± 13	76 ± 12	74 ± 11		0.4400	
Albumin (g/dl)*	3.9 ± 0.2	3.9 ± 0.3	3.9 ± 0.4		0.9697	
Total bilirubin (mg/dl)*	0.7 ± 0.3	0.7 ± 0.3	0.7 ± 0.3		0.8564	
Child-Pugh classification (A/B)	8/2	112/14	25/5		0.3693	
Liver cirrhosis (-/+)	6/4	67/59	30/0		<0.0001	
Tumor-related factors						
AFP (ng/ml)*	431 ± 424	8715 ± 46,095	13 ± 16	5 ± 1	0.3039	0.1840
PIVKA-II (mAU/ml)*	736 ± 785	8061 ± 26,319				
Tumor number (single/multiple)	6/4	75/51				
Maximum tumor size (cm)*	3.5 ± 1.8	4.9 ± 3.3				
Vascular invasion (-/+)	8/2	95/31				
TNM staging (I/II/IIIA)	6/2/2	67/16/43				
CLIP scoring (0/1/2/3-)	2/4/3/1	52/37/23/14				
JIS scorings (0/1/2/3-)	1/4/4/1	11/62/27/26				
BCLC staging (A/B/C)	5/3/2	58/37/31				
Edmondson-Steiner grade (I, II/III, IV/unknown)	5/5/0	42/76/8				

*Data are mean ± SD.
†Negative HBs-Ag, positive HBs-Ag, negative anti-HCV Ab, and positive anti-HCV Ab were defined as B–, B+, C–, and C+, respectively.
‡Patients with blood samples before and after surgical resection.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; NL, normal liver; LC, liver cirrhosis; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence; HBs-Ag, hepatitis B surface antigen; anti-HCV Ab, anti-hepatic C virus antibody.

Cancer

specificity of high serum AFP and PIVKA-II levels for HCC were reported to range from 39–64% and 76–91%, and 41–77% and 72–98%, respectively, suggesting that elevated serum AFP and PIVKA-II levels have insufficient sensitivity and specificity [16–18]. Accordingly, to identify novel biochemical markers for early detection of HCC is desirable.

To our knowledge, there are no reports on the significance of circulating miRNAs in HCC. In this study, we focused on *miRNA-21*, which is one of the first miRNAs detected abundantly in certain human cancers [4,19–21]. *miRNA-21* targets tumor suppressor genes, such as PDCD4, PTEN, and matrix metalloproteinase inhibitors, such as TIMP3 and RECK. Furthermore, *miRNA-21* increased cell proliferation and suppressed apoptosis in a cancer xenograft model, further defining *miRNA-21* as an oncogenic miRNA [22–25]. Overexpression of *miRNA-21* is reported in many types of cancers [26–29]. Also in HCC, it is previously reported that the expression was significantly increased in cancer tissues and cell lines, and that *miRNA-21* contributed to the malignant potential such as cell proliferation, migration, and invasion by reducing the aforementioned targets [30,31]. In other studies, *miRNA-21* was reported to be secreted by cells and detected in plasma [5,32]. It was also confirmed that plasma *miRNA-21* was a useful biomarker for some types of cancer [5,7,9,13]. Thus, we postulated that plasma *miRNA-21* expression could be a novel biochemical marker for HCC. In the present study, we evaluated the usefulness of plasma *miRNA-21* as a biochemical marker for HCC by comparing the expression in patients with HCC and control patients. In addition, we also examined the prognostic significance of plasma *miRNA-21* and investigated the correlation between *miRNA-21* expression in tumoral tissue and its plasma levels.

Materials and methods

Patients and samples

From 10 patients with HCC who had consecutively undergone curative hepatic resection at the Department of Surgery, Osaka University Hospital between January 2010 and February 2010, pre-operative and post-operative plasma samples were collected for the measurement of *miRNA-21*. In the present study, curative resection was defined as complete removal of all macroscopically evident tumors. Post-operative plasma samples were obtained 10–30 days after surgery under the confirmation of no obvious recurrence by ultrasonography, CT, and/or MRI. The clinicopathological features of the 10 patients are shown in Table 1. Plasma

samples, tumoral tissues, and non-tumoral tissues were also obtained from 126 consecutive patients with HCC who had undergone curative hepatic resection and were followed after surgery for 43.4 ± 25.5 months (mean ± SD) at the Department of Surgery, Osaka University Hospital between January 2001 and December 2005. The clinicopathological backgrounds of the 126 patients are also shown in Table 1. Plasma samples were collected before hepatic resection, and the tumoral tissue and non-tumoral tissue were collected from the resected specimens just after the resection. HCC was confirmed histologically in the entire group of 136 patients. For enrollment in the study, the following inclusion criteria were adopted: a good performance status (ECOG level <2), adequate bone marrow function (platelet count >8.0 × 10^4/μl), normal renal function (serum creatinine level <1.5 mg/dl), and adequate liver function (total bilirubin of <1.5 mg/dl, serum transaminases <150 IU/L) [33]. Patients with concomitant neoplasms and serious inflammatory diseases were excluded from the study. For comparison, plasma samples were also collected from age- and gender-matched control patients including 30 patients with chronic hepatitis (CH) and 50 healthy volunteers (HVs). In the 30 patients with CH, laboratory tests and ultrasonographic findings, CT and/or MRI were performed for the diagnosis of CH. In several cases with the possibility of liver cirrhosis among them, liver biopsy was additionally performed for histological assessment, and the diagnosis of CH was based on the histological assessment. Furthermore, patients with CH were confirmed to be free of HCC. In HVs, tumor markers including carcinoembryonic antigen, carbohydrate antigen 19–9, carbohydrate antigen, squamous cell carcinoma-related antigen, prostate specific antigen (in males), and carbohydrate antigen 15–3 (in females) were confirmed to be within normal ranges. They were also confirmed to be free of malignant disease for more than 2 years. The clinicopathological backgrounds of these control patients are also shown in Table 1.

The aim of the study was explained in details to all patients, and each provided written informed consent before enrollment in the study. The study protocol was approved by the Human Ethics Review Committee of Osaka University Hospital.

RNA extraction

Total RNA was isolated from tissue samples by TRIzol agent (Invitrogen, Carlsbad, CA), and the quality of the RNA was assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Total RNA was isolated from plasma samples using mirVana PARIS kit (Ambion Inc., Austin, TX) according to the instructions provided by the manufacturer.

Real-time quantitative reverse transcription-polymerase chain reaction for miRNA expression

Reverse transcription (RT) reaction and real-time quantitative RT-polymerase chain reaction (qRT-PCR) were performed using Taqman human miRNA assay kit (Applied Biosystems, Foster City, CA) according to the instruction supplied by the manufacturer. The expression of the target miRNA in the tumoral tissue and the non-tumoral tissue was normalized relative to the expression of RNU48, which was used as an internal control. On the other hand, there is no established endogenous plasma miRNA control for normalization of plasma miRNA levels [34]. Therefore, in the present study, the expression of the target miRNAs in the plasma was normalized relative to the expression of *miRNA-16*, which was confirmed to exist abundantly and stably in the plasma, as an internal control in previous reports [5,8,10,12]. Data were analyzed according to the comparative Ct method ($2^{-\Delta Ct}$) [35].

Statistical analysis

The HCC staging was performed according to the UICC/AJCC TNM staging system (sixth edition), CLIP scoring system, JIS scoring system, and BCLC staging system [36–39]. Data were expressed as mean ± SD. Differences between groups were assessed by the χ^2-test, Fisher's exact test, or the Mann–Whitney *U* test. Statistical analysis of paired samples was performed using Wilcoxon's signed-rank test. Time-to-recurrence was calculated according to the Kaplan–Meier method and compared using the log-rank test. The Pearson's correlation coefficient was used to calculate correlations. The diagnostic value for differentiating between HCC patients and the control was assessed by calculating the area under the receiver–operator characteristic (ROC) curve (AUC). Validation of the ROC results was performed by the leave-one-out cross-validation method as described by Simon *et al.* [40]. In the validation, first, by using the subset of all but one sample, we built a ROC model, and defined the cut-off in such a way that the sum of sensitivity and specificity was maximum. Then, using the cut-off value, the model is used to predict the left-out recorded samples. When this process was repeated for each sample, the prediction was obtained for every record in the data set using a model that was blind to the predicted observation. The calculated AUC was compared by using the jackknife method [41,42]. All statistical analyses were performed using StatView (version 5.0; SAS Institute Inc., Cary, NC). A *p* value <0.05 denoted the presence of a statistically significant difference.

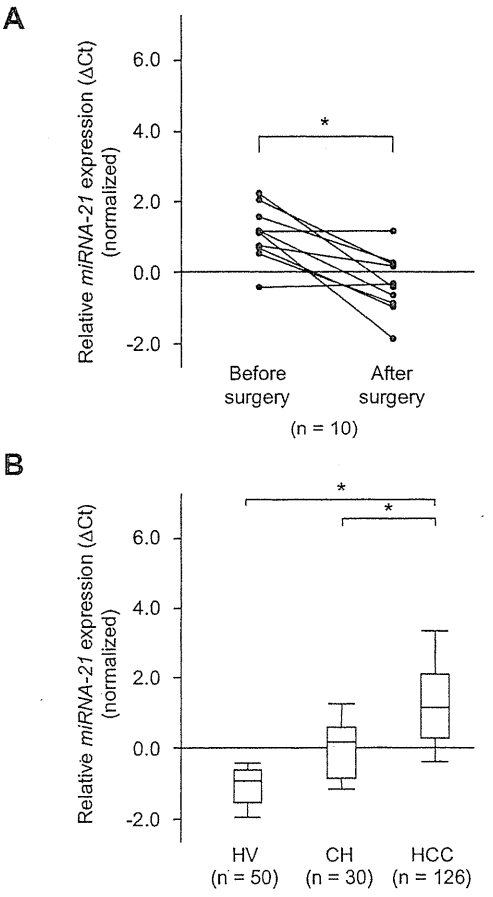

Fig. 1. Plasma *miRNA-21* levels (ΔCt). (A) Plasma *miRNA-21* levels (ΔCt) before and after curative resection in patients with HCC (n = 10). *p <0.05. (B) Plasma *miRNA-21* levels (ΔCt) in patients with HCC (n = 126), CH (n = 30), and HVs (n = 50) measured by qRT-PCR *p <0.05. In this box-and-whisker plot, the lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively.

Results

Plasma miRNA-21 levels before and after surgery

In the 10 patients with curative resection, *miRNA-21* expression level in plasma samples was measured before (baseline) and after surgery by qRT-PCR (Fig. 1A). Plasma *miRNA-21* expression level was significantly lower after surgery than at baseline ($p = 0.0125$). Because the results suggested that plasma *miRNA-21* was derived from tumoral tissue, we measured its plasma *miRNA-21* level and its significance as a biomarker and a prognostic factor for HCC in another group of 126 patients whose long-term prognostic data and samples including tumoral tissue, non-tumoral tissue, and plasma were available.

Plasma miRNA-21 expression is a potential biochemical marker for HCC

Plasma *miRNA-21* expression level was examined by qRT-PCR in the 126 patients with HCC and control subjects (30 patients with CH and 50 HVs). The plasma *miRNA-21* expression in patients with HCC was significantly higher than in patients with CH and HVs (p <0.0001, p <0.0001, respectively) (Fig. 1B). Next, we examined the correlation between plasma *miRNA-21* levels and TNM staging, and the results showed no significant differences in plasma *miRNA-21* levels among patients with stage I, II, and IIIA (Supplementary Fig. 1A). In addition, the plasma *miRNA-21* levels were not also significantly different in subgroups divided on the basis of CLIP scoring system, JIS scoring system, and BCLC staging system (Supplementary Fig. 1A).

On the other hand, the incidence of liver cirrhosis was different between patients with HCC and those with CH (p <0.0001). To examine whether plasma *miRNA-21* expression is influenced by cirrhosis, we compared plasma *miRNA-21* levels between cirrhotic (n = 59) patients with HCC and the remaining non-cirrhotic patients with HCC (n = 67). The results showed that plasma *miRNA-21* expression was similar in the two groups, suggesting that cirrhosis does not influence plasma *miRNA-21* expression level (Supplementary Fig. 1B). Next, we compared the extent of liver fibrosis such as the liver function test evaluated by Child-Pugh classification and platelet count between the non-cirrhotic patients with HCC and the chronic hepatitis patients. By this comparison, we found no significant differences in the extent of liver fibrosis among the two groups [Platelet: non-cirrhotic patients with HCC; 16.4 ± 5.7 ($\times 10^4/\mu l$), chronic hepatitis patients; 15.1 ± 5.8 ($\times 10^4/\mu l$), p = 0.3001)] (Child-Pugh classification: non-cirrhotic patients with HCC; A in 61 patients and B in 6 patients, chronic hepatitis patients; A in 25 patients and B in 5 patients, p = 0.4447). Furthermore, to examine whether the viral status influences plasma *miRNA-21* expression level, we compared the plasma *miRNA-21* level in the HCC and CH groups based on the viral status. The result showed that the viral status had no influence on *miRNA-21* level in both groups (Supplementary Fig. 1B).

Differentiating power of AFP, miRNA-21, and combination of AFP and miRNA-21

We evaluated the differentiating power of plasma *miRNA-21* expression in patients with HCC and the control by comparison with that of AFP. Prior to the comparison, it was found that plasma *miRNA-21* levels correlated weakly with those of AFP

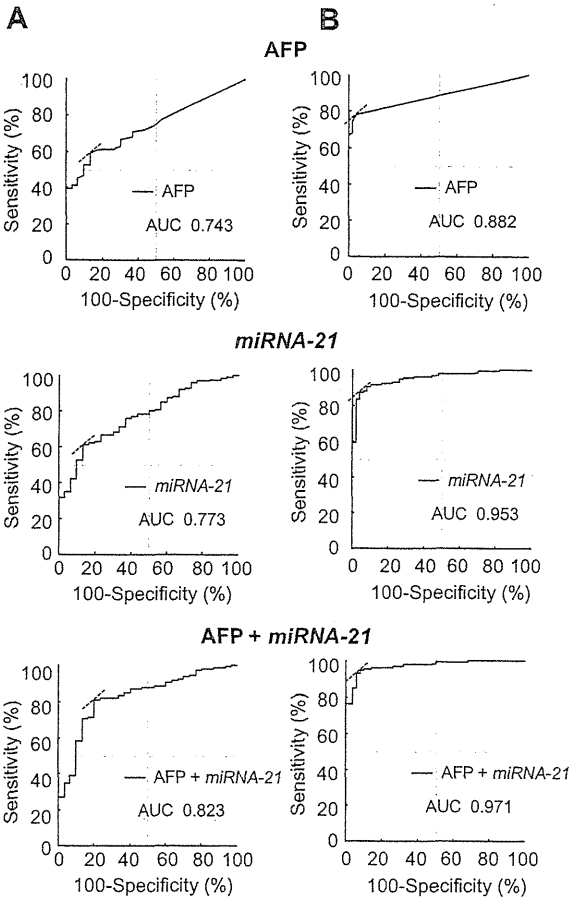

Fig. 2. The diagnostic power of AFP, *miRNA-21*, and the combination of AFP and *miRNA-21* for HCC (n = 126) against CH (n = 30) and HVs (n = 50). (A) Power of AFP, plasma *miRNA-21*, and the combination of AFP and plasma *miRNA-21* in differentiating HCC patients from CH patients. Optimal cutoff values, where the sum of sensitivity and specificity was maximum, were 19.0 ng/ml for AFP and 0.754 for plasma *miRNA-21*. (B) Power of AFP, plasma *miRNA-21*, and the combination of AFP and plasma *miRNA-21* in differentiating HCC patients from HVs. The optimal cutoff values were 6.0 ng/ml for AFP and −0.108 for plasma *miRNA-21*. The power of plasma *miRNA-21* was superior to that of AFP and the combination of the two enhanced the power of AFP in differentiating HCC from the control.

(p <0.0001, r = 0.403). The ROC curve analysis indicated that AFP was useful in differentiating HCC from CH with AUC of

Table 2. Differentiating power of AFP, *miRNA-21*, and the combination of AFP and *miRNA-21*.

	AUC	95% CI	Sensitivity (%)	Specificity (%)	Accuracy (%)
HCC patients *vs.* CH patients					
AFP	0.743	0.662-0.824	59.5	83.3	64.7
miRNA-21	0.773	0.690-0.856	61.1	83.3	65.4
AFP + *miRNA-21*	0.823	0.744-0.902	81.0	76.7	80.1
HCC patients *vs.* HVs					
AFP	0.882	0.834-0.931	77.8	96.0	83.0
miRNA-21	0.953	0.924-0.983	87.3	92.0	88.6
AFP + *miRNA-21*	0.971	0.949-0.992	92.9	90.0	92.0

Abbreviations as in Table 1. *miRNA-21*; *microRNA-21*.

0.743 (Fig. 2A, left panel). At a cutoff value of 19.0 ng/ml for AFP expression level, the optimal sensitivity and specificity were 58.7% and 86.7%, respectively. Similar analysis indicated the AUC for plasma *miRNA-21* was 0.773 (Fig. 2A, middle panel), which was significantly superior to AFP ($p < 0.0001$). At the cutoff value of 0.754 for plasma *miRNA-21* expression level (ΔCt), the optimal sensitivity and specificity were 61.1% and 86.7%, respectively. Next, the differentiation power of the combination of plasma *miRNA-21* with AFP was analyzed by fixing the cut-off value of AFP. The combination of plasma *miRNA-21* with AFP improved the differentiation power between HCC and CH, with an increase in AUC of 0.823 and 81.0% sensitivity and 80.0% specificity (Fig. 2A, right panel). Next, we validated these results by using the leave-one-out cross-validation method. The results indicated that the sensitivity, specificity, and accuracy of AFP were 59.5%, 83.3%, and 64.7%, respectively, while those of plasma *miRNA-21* were 61.1%, 83.3%, and 65.4%, respectively. Furthermore, the sensitivity, specificity, and accuracy for the combination of plasma *miRNA-21* with AFP, as obtained by the cross-validation, were 81.0%, 76.7%, and 80.1%, respectively (Table 2).

The significance of plasma *miRNA-21* expression in differentiating HCC patients from HVs was also examined. The ROC curve analysis showed that AUC for AFP was 0.882 (Fig. 2B, left panel). At the cutoff value of 6.0 ng/ml for plasma AFP, the optimal sensitivity and specificity were 77.8% and 96.0%, respectively. Similar analysis for *miRNA-21* showed AUC of 0.953 (Fig. 2B, central panel), which was also significantly superior to AFP ($p < 0.0001$). At the cutoff value of −0.108 for plasma *miRNA-21* expression level (ΔCt), the optimal sensitivity and specificity were 87.3% and 96.0%, respectively. The combination of plasma *miRNA-21* with AFP also enhanced the differentiating power between HCC patients and HVs with an increase in AUC to 0.971 with 92.9% sensitivity and 94.0% specificity (Fig. 2B, right panel). Validation of these results indicated that the sensitivity, specificity, and accuracy of AFP were 77.8%, 96.0%, and 83.0%, respectively, while those of plasma *miRNA-21* were 87.3%, 92.0%, and 88.6%, respectively. The validation also indicated that the sensitivity, specificity, and accuracy for the combination of plasma *miRNA-21* with AFP were 92.9%, 90.0%, and 92.0%, respectively (Table 2).

Furthermore, to confirm the usefulness of plasma *miRNA-21* in screening for HCC, we compared the sensitivity of plasma *miRNA-21* with that of AFP only in 20 patients with small HCC (<2.0 cm). In differentiating HCC patients from CH patients, the sensitivity of plasma *miRNA-21* and that of AFP were 55.0% and 55.0%, respectively, and that of the combination of plasma *miRNA-21* and AFP was 75.0%, which was superior to plasma *miRNA-21* or AFP alone. ROC curve and AUC are shown in Supplementary Fig. 2A. Also, a similar tendency in differentiating HCC patients from HVs was obtained (sensitivity of plasma *miRNA-21*, AFP, combination; 80.0%, 75.0%, 90.0%, respectively). ROC curve and AUC are shown in Supplementary Fig. 2B.

Plasma miRNA-21 level correlates with prognosis of patients with HCC

Next, patients were divided into two groups; the tumoral *miRNA-21* high expression group (n = 34), representing patients with an *miRNA-21* level more than the optimal cutoff level in differentiation between tumoral tissue and non-tumoral tissue, and the tumoral *miRNA-21* low expression group representing the remaining 92 patients. Univariate analyses showed significant relationships between tumoral *miRNA-21* expression and each of the following factors: AFP, PIVKA-II, number of tumors, maximum tumor size, vascular invasion, Edmondson-Steiner grade, and integrative prognostic staging/scoring systems. Patients with high tumoral *miRNA-21* expression had a significant shorter time-to-recurrence compared to those with low tumoral *miRNA-21* expression (Supplementary Fig. 3). The multivariate analysis identified tumoral *miRNA-21* expression as an independent significant factor for recurrence ($p = 0.0206$, Supplementary Table 1).

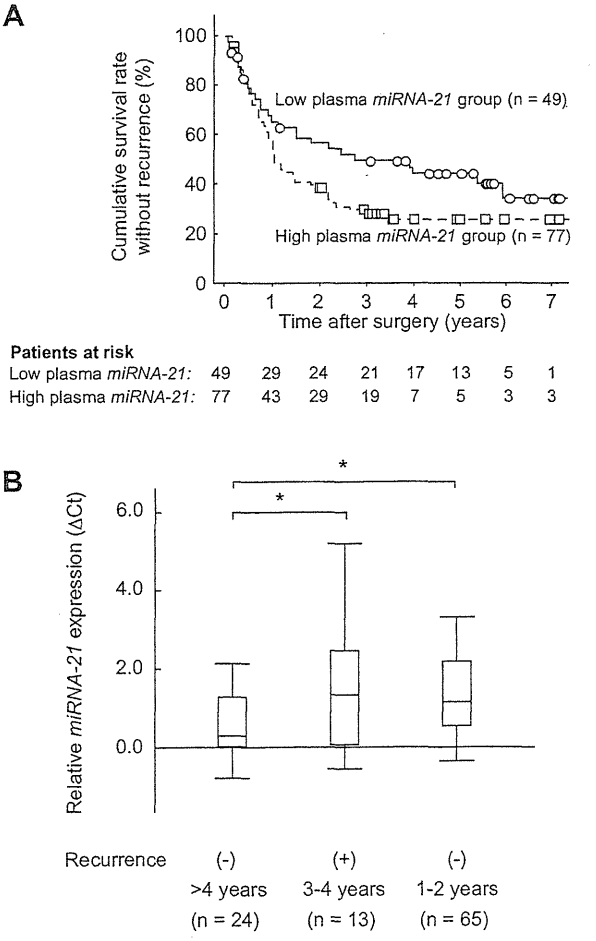

Fig. 3. Correlation between plasma *miRNA-21* expression level and post-operative tumor recurrence. (A) Cumulative survival rate without recurrence after curative surgery for HCC according to plasma *miRNA-21* expression levels. Patients the low plasma *miRNA-21* expression (n = 49) (solid line) (open circles; censored) tended to have a longer time-to-recurrence compared to those with high plasma *miRNA-21* expression (n = 77) (dotted line) (closed squares; censored) ($p = 0.0722$). (B) Plasma *miRNA-21* levels (ΔCt) in patients with recurrence within 2 post-operative years (n = 65), those with recurrence in the next 2 post-operative years (during post-operative 2–4 years) (n = 13), and those without any recurrence during 4 post-operative years (n = 24). *$p < 0.05$. In this box-and-whisker plot, the lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively.

Next, the prognostic value of high plasma *miRNA-21* levels was also examined by dividing the patients into two groups; the high plasma *miRNA-21* group (n = 49), representing patients with plasma *miRNA-21* levels above the aforementioned optimal cutoff level in differentiating HCC from CH, and the low plasma *miRNA-21* group, representing the remaining 77 patients. The clinicopathological backgrounds of the two groups are listed in Table 3. The proportion of patients with undifferentiated tumors was significantly higher in the high plasma *miRNA-21* group than the low plasma *miRNA-21* group ($p = 0.0338$). On the other hand, no significant differences were observed in other tumor-related factors between the two groups. Patients with low plasma *miRNA-21* expression tended to have a longer time-to-recurrence compared to those with high plasma *miRNA-21* expression, albeit statistically insignificant ($p = 0.0722$, Fig. 3A). Furthermore, we also compared plasma *miRNA-21* levels among the three groups; patients with recurrence within 2 post-operative years (n = 65), those with recurrence in the next 2 post-operative years (during post-operative 2–4 years) (n = 13), and those without any recurrence during 4 post-operative years (n = 24). The plasma *miRNA-21* level was significantly lower in patients without any recurrence than in those with recurrence within 2 post-operative years and those with recurrence in the next 2 post-operative years ($p = 0.0125$, $p = 0.0483$, respectively).

Plasma miRNA-21 level correlates significantly with that in tumoral tissue

In the group of 126 patients, *miRNA-21* expression level was significantly higher in tumoral tissue than in non-tumoral tissue with a median fold increase in tumoral tissue of 2.5 (ΔCt) ($p < 0.0001$, Fig. 4A). This over-expression of *miRNA-21* in tumoral tissue was in agreement with previous results [30,31]. Further analysis showed that plasma *miRNA-21* levels correlated significantly with *miRNA-21* expression levels in tumoral tissue ($p < 0.0001$), though the correlation coefficient was not high ($r = 0.407$, Fig. 4B). A similar analysis using data of only 75 patients with solitary HCC without vascular invasion showed a more significant correlation between the two parameters with a higher correlation coefficient ($p < 0.0001$, $r = 0.589$, Fig. 4C).

Table 3. Clinicopathological characteristics of patients categorized according to the plasma *miRNA-21* expression status.

	plasma *miRNA-21* level		*p* value
	Low group [≤0.754 (ΔCt)] (n = 49)	High group [>0.754 (ΔCt)] (n = 77)	
Clinical factors			
Gender (male/female)	39/10	60/17	0.8238
Age (years)*	62 ± 11	63 ± 9	0.6184
Viral status (B-C-/B+C-/B-C+/B+C+)†	5/13/30/1	9/12/54/2	0.5197
AST (IU/L)*	37 ± 22	40 ± 19	0.5756
ALT (IU/L)*	39 ± 27	42 ± 24	0.5399
Platelet count (x10⁴/μl)*	16.4 ± 6.8	15.8 ± 5.5	0.6002
Prothrombin time (%)*	74 ± 9	76 ± 14	0.2325
Albumin (g/dl)*	3.9 ± 0.3	3.9 ± 0.4	0.6920
Total bilirubin (mg/dl)*	0.7 ± 0.2	0.7 ± 0.3	0.2450
Child-Pugh classification (A/B)	42/7	70/7	0.3657
Tumor-related factors			
AFP (ng/ml)*	606 ± 1839	13,875 ± 58,508	0.1156
PIVKA-II (mAU/ml)*	5511 ± 18,433	9684 ± 30,292	0.3878
Tumor number (single/multiple)	20/29	31/46	0.9505
Maximum tumor size (cm)*	4.6 ± 3.0	5.2 ± 4.0	0.2012
Vascular invasion (-/+)	39/10	56/21	0.3831
TNM staging (I/II/IIIA)	29/5/15	38/11/28	0.5413
CLIP scoring (0/1/2/3-)	21/15/9/4	31/22/14/10	0.8687
JIS scorings (0/1/2/3-)	6/25/7/11	5/37/20/15	0.3582
BCLC staging (A/B/C)	21/18/10	37/19/21	0.3276
Edmondson-Steiner grade (I, II/III, IV/unknown)	21/23/5	21/53/3	0.0338

*Data are mean ± SD.
†Negative HBs-Ag, positive HBs-Ag, negative anti-HCV Ab, and positive anti-HCV Ab were defined as B–, B+, C–, and C+, respectively. Abbreviations as in Tables 1 and 2.

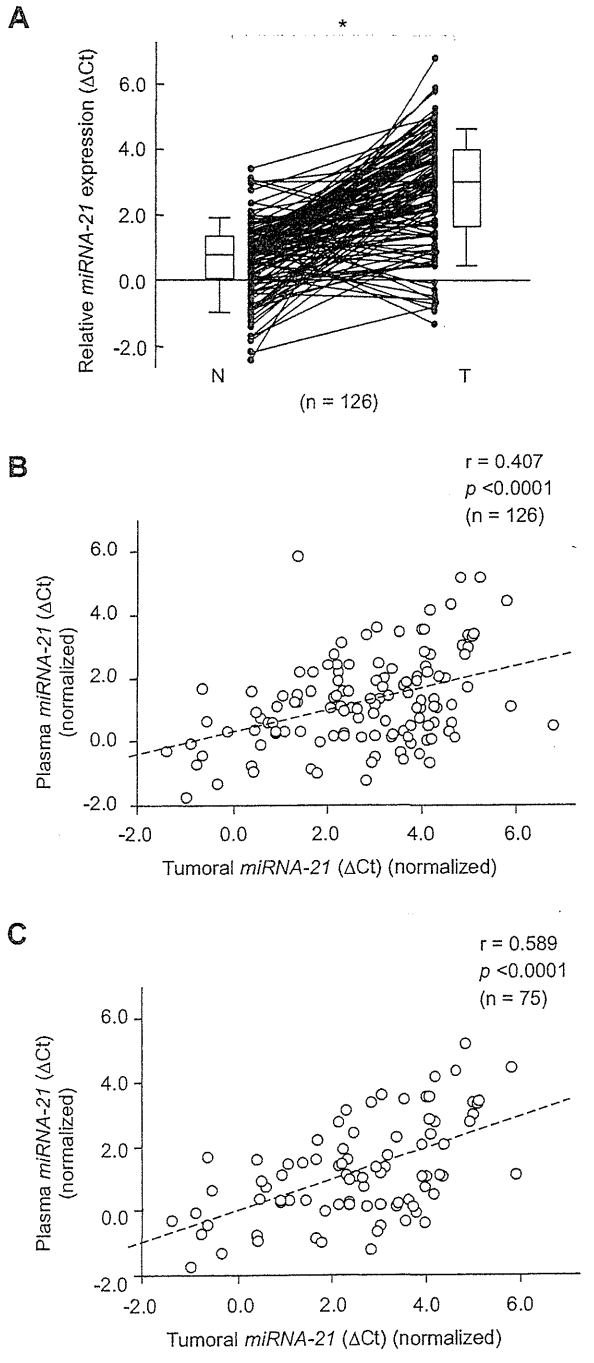

Fig. 4. Expression levels of *miRNA-21* (ΔCt) in tissues determined by qRT-PCR. (A) Tumoral (T) and non-tumoral (N) tissues of patients with HCC (n = 126). $*p < 0.05$. In this box-and-whisker plot, the lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (B and C) Correlation between *miRNA-21* expression level (ΔCt) in the tumoral tissue and plasma *miRNA-21* levels (ΔCt). Note the weak correlation in the 126 patients ($p < 0.0001$, r = 0.407) (B), and the enhanced correlation in the 75 patients with solitary HCC without vascular invasion ($p < 0.0001$, r = 0.589) (C).

Discussion

The present study demonstrated that plasma *miRNA-21* levels were significantly reduced in the post-operative plasma samples compared to the pre-operative samples, and that the levels in patients with HCC were significantly higher than in patients with CH and HVs. ROC analyses for the diagnostic power of plasma *miRNA-21* yielded an AUC of 0.773 with 61.1% sensitivity and 83.3% specificity in differentiating patients with HCC from those with CH, and AUC of 0.953 with 87.3% sensitivity and 92.0% specificity in differentiating patients with HCC from HVs. These results suggest that plasma *miRNA-21* is a valuable biochemical marker of HCC. Furthermore, the superiority of the differentiating power of a single measurement of plasma *miRNA-21* compared with AFP was statistically confirmed, and the differentiating power of the combination of plasma *miRNA-21* and AFP was significantly stronger than AFP alone, suggesting that measurement of both plasma *miRNA-21* and AFP has a better differentiating power than plasma *miRNA-21* and AFP alone. Furthermore, plasma *miRNA-21* level was significantly elevated even in HCC patients with early tumor stage. While the exact reason for this observation is not clear, it may reflect a larger increase in plasma *miRNA-21* at cancer initiation than during cancer progression. Whatever the reason, considering that high plasma *miRNA-21* levels were identified even in patients with early tumor stage and that the differentiating power of plasma *miRNA-21* was significantly superior to that of AFP, we suggest that plasma *miRNA-21* is a useful diagnostic marker for HCC. To our knowledge, this is the first report to evaluate the diagnostic value of a specific plasma miRNA as a biochemical marker for HCC. At the same time, we must keep in mind that, as even patients with advanced HCC were included in the present study, the enrollment of the patients was not designed for the examination of diagnostic markers, suggesting the possibility that the aforementioned sensitivity and specificity might be over-estimated. In addition, we did not examine plasma *miRNA-21* level in cirrhotic patients who also have possibility for developing HCC, in the present study. We should investigate whether the *miRNA-21* measurement is useful in differentiating HCC patients from cirrhotic patients in the future.

Unfortunately, the plasma *miRNA-21* level had a low specificity as a biomarker of HCC. The expression level of *miRNA-21* was reported in several studies in various normal tissues, though the expression level was lower than in tumoral tissues [31,43]. In the present study, *miRNA-21* expression was also detected in non-tumoral liver tissues. In addition to its expression in non-tumoral tissues, high plasma *miRNA-21* levels were reported in other types of cancers such as lymphoma, glioblastoma, ovarian cancer, and pancreatic cancer, which is conceivable, considering that *miRNA-21* is one of the miRNAs over-expressed in many types of cancers [7,9,13]. Thus, while the measurement of plasma *miRNA-21* level can be useful for HCC detection, high plasma *miRNA-21* levels should not mean presence of HCC. To overcome this limitation, the combination of plasma *miRNA-21* and other tumor markers with certain specificity to HCC, such as AFP, might be useful.

In the present study, the correlation between plasma and tumoral tissue *miRNA-21* levels was investigated in patients with HCC. To date, several studies have investigated the correlation between plasma miRNAs and tumoral miRNAs. Skog *et al.* [13] reported a poor overall correlation between miRNAs

expression levels in glioblastoma based on microarray analysis, but found significant correlations for several miRNAs between tumoral tissue and plasma. Moreover, similar results of significant correlation for several miRNAs were also reported in ovarian cancer and lung cancer [44,45]. These reports also identified a significant correlation between *miRNA-21* levels in plasma and tumor cells. Consistent with the above reports, the present study demonstrated that plasma *miRNA-21* levels correlated significantly with *miRNA-21* expression levels in tumoral tissues, though the correlation coefficient was relatively low. At present, the high plasma miRNA levels in cancer are considered to be due to excessive secretion by primary cancer cells [5,13,34,46]. The above studies showing significant correlation and the present study seems to support this speculation. Admittedly, however, some patients in the present study showed discrepancy between the *miRNA-21* expression level in tumoral tissue and plasma *miRNA-21* levels. Although the reason for this discrepancy is not clear at present, one possible explanation is the heterogeneity of the tumor. In the present study, the aforementioned modest correlation improved when data from patients with solitary HCC without vascular invasion were analyzed, which may support the speculation. Another possible explanation may be the aforementioned *miRNA-21* expression in various normal tissues. Future studies are needed to shed light on this discrepancy.

We also examined the correlation between *miRNA-21* expression level and tumor progression and prognosis. The results showed that both tumoral and plasma *miRNA-21* expression levels correlated significantly with tumor progression and prognosis, but there were no significant differences in the analysis of time-to-recurrence. This shortfall in the significance might be possibly related to the abovementioned modest correlation between *miRNA-21* expression levels in tumoral tissue and plasma.

Considered together, the present results and those of previous studies suggest that plasma miRNAs, reflecting those in tumoral tissue, are potentially suitable biochemical markers of cancer, when they are used clinically with special attention to their specificity. However, today, the mechanism involved in the secretion of miRNA from cancer cells into plasma remains to be unanswered. Further studies are needed to determine the exact time during cancer progression at which circulating miRNAs become detectable in the bloodstream and whether such time point is similar or different between tumoral tissues and non-tumoral tissues and among each tumoral tissue, as described by Cortez *et al.* [34]. Clinical application of plasma miRNAs for cancer detection is not feasible until these issues are resolved.

In summary, plasma *miRNA-21* expression, which was significantly associated with *miRNA-21* expression in tumoral tissue, is a promising biochemical marker of HCC.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2011.04.026.

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α-Fetoprotein impairs activation of natural killer cells by inhibiting the function of dendritic cells

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Summary

α-Fetoprotein (AFP) is a tumour-associated antigen in hepatocellular carcinoma (HCC). The biological properties of AFP have been identified in its regulatory effects on immune responses of T cells and B cells. However, AFP effects on natural killer (NK) cells are still unclear. In this study, we examined the immunoregulation of AFP on NK activity. The cytolytic activity against K562 cells and Huh7 cells of NK cells co-cultured with AFP-treated dendritic cells (DCs) (AFP-DCs) was lower than that with albumin-treated DCs (Alb-DCs). Direct addition of AFP to NK cells did not alter the cytolytic activity of NK cells. Adding AFP inhibited the interleukin (IL)-12 production of DCs after stimulation with lipopolysaccharide (LPS) [Toll-like receptor (TLR)-4 ligand], or Poly(I:C) (TLR-3 ligand), but not IL-18 production. The mRNAs of IL-12p35 and IL-12p40 were significantly inhibited in AFP-DCs compared with Alb-DCs, but those of TLR-4 or TLR-3 were not. Transwell experiments revealed that soluble factors derived from DCs played roles in inhibition of the ability of activating NK cells by AFP-DCs. Adding the neutralizing antibody of IL-12 to NK cells co-cultured with Alb-DCs resulted in a decrease of cytolytic activity to the levels of NK cells co-cultured with AFP-DCs. Adding IL-12 to NK cells co-cultured with AFP-DCs resulted in an increase of cytolytic activity to the levels of NK cells co-cultured with Alb-DCs. These demonstrated that the impairment of IL-12 production from AFP-DCs resulted in inhibition of the ability of the activation of NK cells by DCs, and thus suggests a role of AFP in HCC development.

Keywords: α-fetoprotein, dendritic cells, hepatocellular carcinoma, NK cells

Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer deaths worldwide. Chronic liver disease caused by hepatitis virus infection and non-alcoholic steatohepatitis leads to a predisposition for HCC, with liver cirrhosis, in particular, being considered a premalignant condition [1,2]. With regard to treatment, surgical resection or percutaneous techniques such as ethanol injection and radiofrequency ablation are considered to be choices for the curable treatment of localized HCC, whereas transarterial chemo-embolization is a well-established technique for more advanced HCC [3]. Recently the Sorafenib Hepatocellular carcinoma Assessment Randomized Protocol (SHARP) trial has demonstrated that sorafenib, a multi-targeting kinase molecule that inhibits receptor tyrosine kinases [vascular endothelial growth factor receptor (VEFGR)-2, VEGFR-3, Flt ligand (Flt)-3, platelet-derived growth factor receptor beta (PDGFR) and fibroblast growth factor receptors (FGFR)-1] as well as Raf serine–threonine kinase in the signal transduction, is effective for prolonging median survival and time-to-progression in patients with advanced HCC [4]. The liver contains a large compartment of innate immune cells [natural killer (NK) cells and NK T cells] and acquired immune cells (T cells) [5,6]. However, what remain unclear are the details of the activation of these immune cells in the process of HCC development. If the mechanism of tumour surveillance by immune cells in HCC development can be elucidated, this could lead to the establishment of new strategies for HCC treatment.

α-Fetoprotein (AFP), a glycoprotein of molecular mass 68–72 kDa, is a tumour-associated antigen in HCC and a target for immunotherapy [7]. Measurement of serum levels of AFP is important for the diagnosis of HCC and

monitoring of treatment [8]. Recently, several biological properties of AFP have been identified in its regulatory effects on immune responses [9–13]. AFP induces the suppression of cytotoxic T lymphocytes (CTLs) activity and antibody responses of B lymphocytes [9–11]. Alisa *et al.* demonstrated that AFP may contain specific epitopes which activate the expansion of inducible transforming growth factor (TGF)-β producing regulatory T cells, leading to evasion of tumour control [12]. Antigen-presenting cells (APCs) of HCC patients with high levels of AFP are dysfunctional, and AFP impairs dendritic cell (DC) function and induces their apoptosis [13]. However, the biological role of AFP on innate immune responses still remains unclear.

In this study, we investigated the immunoregulation of NK activity and DC function by AFP. We demonstrate that AFP impairs NK activity via inhibition of interleukin (IL)-12 production from DCs. The present study sheds light on previously unrecognized immunological effects of AFP on NK cells, and thus suggests a role of AFP in HCC development.

Materials and methods

Cell culture

Cell culture was maintained in a medium (RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 ug/ml streptomycin and 10 mM L-glutamine: all reagents from GIBCO/Life Technologies, Grand Island, NY, USA) in a humidified incubator at 5% CO_2 and 37°C. Purified human cord blood AFP [purity, > 98%; sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)] and purified human serum albumin (Alb) (purity, > 97%; SDS-PAGE) were obtained from HyTest (Turku, Finland) and Sigma-Aldrich (St Louis, MO, USA), respectively.

Generation of monocyte-derived DCs from peripheral blood monocytes (PBMCs)

Monocyte-derived DCs were generated from PBMCs of healthy volunteers. PBMCs, isolated by Ficoll Hypaque density centrifugation, were washed twice in phosphate-buffered saline (PBS) and resuspended in AIM-V medium for 60 min. Non-adherent cells were removed by gentle washing, and adherent cells were cultured in DC medium (RPMI-1640 supplemented with 10% fetal calf serum) containing human granulocyte–macrophage colony-stimulating factor (GM-CSF) (50 pg/ml; PeproTech, Rocky Hill, NJ, USA) and human IL-4 (50 pg/ml; PeproTech) with either AFP (25 μg/ml) or Alb (25 μg/ml). On day 6, immature DCs were harvested. DC maturation was induced by the addition of lipopolysaccharide (LPS) (10 μg/ml; Sigma-Aldrich) or Poly(I:C) (10 μg/ml; InvivoGen, San Diego, CA, USA) to immature DCs for 24 h.

Analysis of DC surface markers

For phenotypic analysis of DCs, allophycocyanin (APC)-, peridinin chlorophyll protein complex (PerCP)- or phycoerythrin (PE)-labelled monoclonal antibodies (mAbs) [anti-human CD11c, CD40, CD80, CD83, CD86, human leucocyte antigen D-related (HLA-DR) relevant isotype controls; BD Pharmingen, San Diego, CA, USA], according to the manufacturer's instructions. Flow cytometric analysis was performed using a fluorescence activated cell sorter (FACS)Calibur (Becton Dickinson, San Jose, CA, USA) flow cytometer. We defined DCs with $CD11c^+$ $HLA\text{-}DR^+$ cells by flow cytometry and evaluated the expression of these antigen-presenting related molecules. Data were analysed using FlowJo software (Tree Star, Ashland, OR, USA) and reported as the mean fluorescence intensity (MFI).

Measurements of cytokine production of DCs

IL-12p70, IL-15, IL-18 and interferon (IFN)-γ of the DC culture were measured by a single solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) using paired specific mAbs and recombinant cytokine standards, according to the manufacturer's instructions (IL-12p70, IL-15 and IFN-γ from BD Pharmingen, IL-18 from MBL, Woburn, MA, USA).

Real time reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was isolated using an RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan), and was reverse-transcribed using the high-capacity RNA-to-cDNA Master Mix (Invitrogen, Carlsbad, CA, USA). Random hexamers were added as primers. The mRNA levels were evaluated using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Ready-to-use assays (Applied Biosystems) were used for the quantification of Toll-like receptor (TLR)-3, TLR-4, IL-12p35, IL-12p40 and β-actin, according to the manufacturer's instructions. The thermal cycling conditions for all genes were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. β-Actin mRNA from each sample was quantified as an endogenous control of internal RNA.

DC/NK cell co-culture

NK cells were isolated from PBMCs by magnetic cell sorting using CD56 MicroBeads according to the manufacturer's instructions (Miltenyi Biotech, Auburn, CA, USA). More than 95% of the cells were $CD56^+CD3^-$ lymphocytes. Enriched NK cells were co-cultured with AFP (25 μg/ml,

AFP-DCs) or Alb (25 μg/ml, Alb-DCs) pretreated DCs for 24 h. The cytolytic activity of NK cells co-cultured with AFP-DCs or Alb-DCs against target cells (K562, NK sensitive cells, or Huh7, human HCC cells) was assessed by 4-h ^{51}Cr-releasing assay with or without the presence of neutralizing antibody of IL-12 (BD Pharmingen) or recombinant IL-12p70 protein (PeproTech), as described previously [14]. In some experiments, a Transwell insert was also used to prevent direct contact of NK cells and DCs in co-culture systems, as described previously [14].

Statistical analysis

The statistical significance of differences between the two groups was determined by applying the Mann–Whitney *U*-test. We defined statistical significance as $P < 0{\cdot}05$.

Results

NK activity co-cultured with AFP-DCs was lower than that with Alb-DCs

We investigated the activity of NK cells co-cultured with AFP-DCs or Alb-DCs. NK cells from the same healthy volunteers were co-cultured with AFP-DCs or Alb-DCs for 24 h, and we evaluated the cytolytic activity of NK cells co-cultured with DCs against K562 cells as target cells with the ^{51}Cr-releasing assay. The cytotoxicity of NK cells co-cultured with AFP-DCs against K562 cells was significantly lower than those with Alb-DCs (Fig. 1a). Similarly, the cytotoxicity of NK cells co-cultured with AFP-DCs against Huh7 cells was significantly lower than that with Alb-DCs (Fig. 1b). We also evaluated the IFN-γ production from NK cells co-cultured with AFP-DCs or Alb-DCs by specific ELISA. IFN-γ production from NK cells co-cultured with AFP-DCs was significantly lower than that from NK cells co-cultured with Alb-DCs (Fig. 1c). These results demonstrated that NK activity co-cultured with AFP-DCS was lower than that with Alb-DCs. Next, NK cells were cultured with AFP (AFP-NK cells) or Alb (Alb-NK cells) for 24 h, and we evaluated the cytolytic activity of AFP-NK and Alb-NK against K562 cells with the ^{51}Cr-releasing assay. The cytotoxicity of AFP-NK cells was almost similar to that of Alb-NK cells, and the presence of DCs could enhance the cytotoxicity of NK cells (Fig. 2a). These results suggested that AFP does not directly impair NK cell function and that DCs play a critical role in activating NK cells. To examine whether this attenuation of NK cells was caused by the cytokine from DCs or by direct contact with DCs, NK cells were co-cultured with AFP-DCs or Alb-DCs in Transwell culture for 24 h. The cytotoxicity of NK cells co-cultured with AFP-DCs was lower than that with Alb-DCs, which was similar to the results without Transwell membrane (Fig. 2b). These results suggested that soluble factors derived from DCs played a role in activating NK cells.

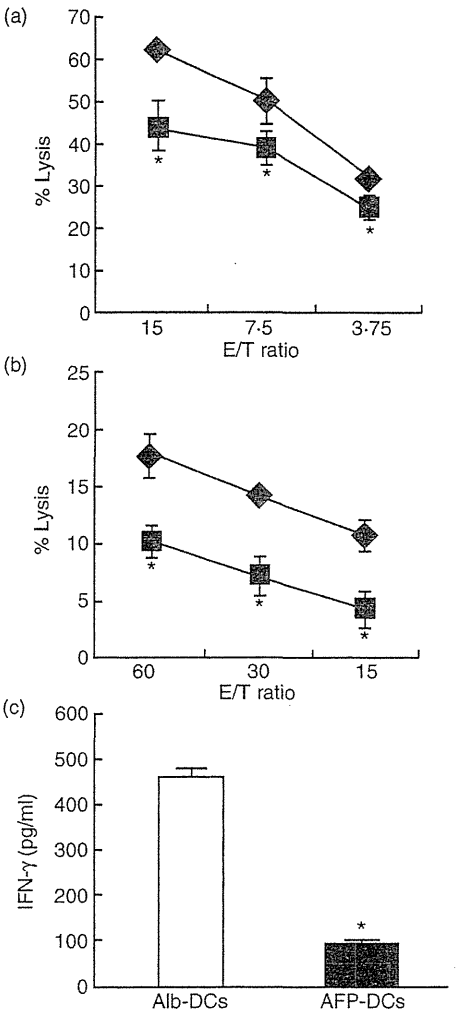

Fig. 1. The cytolytic activity and interferon (IFN)-γ production of natural killer (NK) cells co-cultured with α-fetoprotein-dendritic cells (AFP-DCs) were impaired. (a,b) NK cells were isolated from peripheral blood mononuclear cells (PBMCs) by magnetic cell sorting using CD56 MicroBeads according to the manufacturer's instructions. Enriched NK cells were co-cultured with AFP (25 μg/ml, AFP-DCs) or albumin (Alb) (25 μg/ml, Alb-DCs) pretreated DCs for 24 h. The cytolytic activities of NK cells co-cultured with AFP-DCs (■) or Alb-DCs (◆) against K562 cells (a) or Huh7 cells (b) were evaluated by ^{51}Cr-releasing assay. * $P < 0{\cdot}05$ *versus* the cytolytic activity of NK cells cultured with Alb-DCs. (c) NK cells were isolated from PBMCs by magnetic cell sorting using CD56 MicroBeads, according to the manufacturer's instructions. Enriched NK cells were co-cultured with AFP (25 μg/ml, AFP-DCs) or Alb (25 μg/ml, Alb-DCs) pretreated DCs for 24 h. The IFN-γ productions from NK cells were analysed by specific enzyme-linked immunosorbent assay (ELISA). To evaluate the IFN-γ production from NK cells, we also evaluated the IFN-γ production from AFP-DCs or Alb-DCs cultured without NK cells, and these values were subtracted from all experimental determinations to determine specific IFN-γ productions of NK cells (results in pg/ml; mean ± standard deviation of triplicate samples). We analysed statistically the production of IFN-γ between AFP-DCs and Alb-DCs. * $P < 0{\cdot}05$.

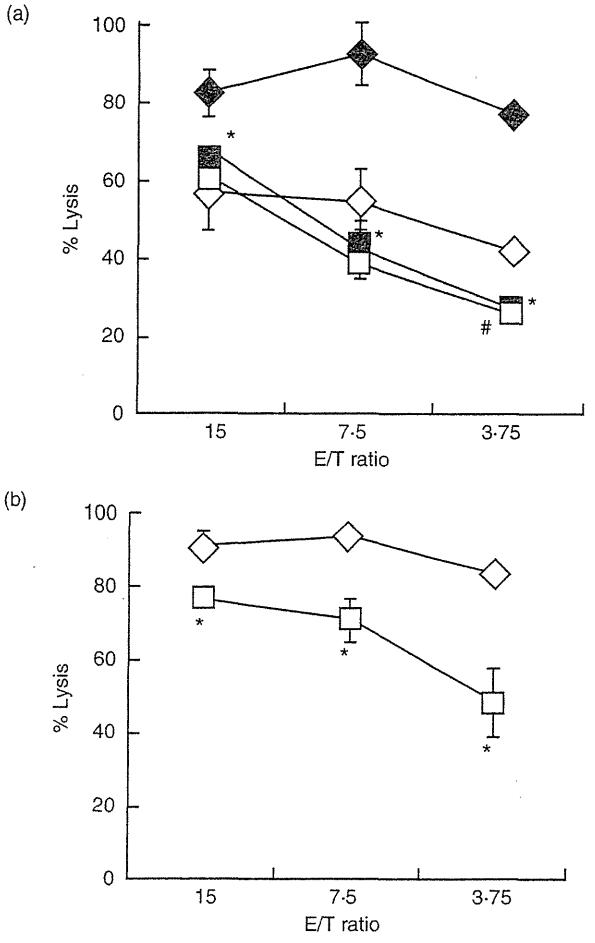

Fig. 2. α-Fetoprotein (AFP) did not directly affect the cytolytic activity of natural killer (NK) cells and soluble factor from dendritic cells (DCs) played a role in the inhibition of NK activity. (a) NK cells were cultured with AFP (25 μg/ml, □, AFP-NK cells) or albumin (Alb) (25 μg/ml, ◇, Alb-NK cells) or cultured with AFP-DCs (▩) or Alb-DCs (◆) for 24 h. We evaluated the cytolytic activity of AFP-NK cells and Alb-NK cells or NK cells stimulated by AFP-DCs or Alb-DCs using K562 cells as target cells by ^{51}Cr-releasing assay. We analysed statistically between the cytolytic activity of NK cells co-cultured with AFP-DCs (▩) and Alb-DCs (◆) or between that of AFP-NK cells (□) and Alb-NK cells (◇). $*P < 0{\cdot}05$ *versus* the cytolytic activity of NK cells cultured with Alb-DCs, $\#P < 0{\cdot}05$ *versus* the cytolytic activity of Alb-NK cells. (b) Enriched NK cells were co-cultured with AFP-DCs (□) or Alb-DCs (◇) for 24 h in the presence of 0·4 μm of inserting membrane (Transwell). NK cells were harvested and subjected to examine the cytolytic activity against K562 cells by ^{51}Cr-releasing assay. $*P < 0{\cdot}05$ *versus* the cytolytic activity of NK cells cultured with Alb-DCs. Representative results are shown. Similar results were obtained from three independent experiments in all experiments.

Maturation of AFP-treated DCs was impaired

We next examined the function of AFP-DCs. We obtained DCs from eight healthy volunteers and cultured the DCs for 7 days in RPMI-1640 with AFP (AFP-DCs) or Alb (Alb-DCs). On day 6, we added LPS to induce DC maturation. We identified DCs with CD11c$^+$/HLA-DR$^+$ cells by flow cytometry. As shown in Fig. 3a, adding LPS, the TLR-4 ligand, resulted in increasing the expression of HLA-DR in both AFP-DCs and Alb-DCs. The numbers of harvested AFP-DCs or Alb-DCs were $(1{\cdot}64 \pm 0{\cdot}62) \times 10^6$ and $(1{\cdot}77 \pm 0{\cdot}73) \times 10^6$, respectively, with no significant difference being observed between the two groups. We evaluated the expression of the antigen-presenting related molecules on AFP-DCs and Alb-DCs. The expression of CD80, CD86, CD40 and CD83 increased on both AFP-DCs and Alb-DCs after addition of LPS. The expression of these

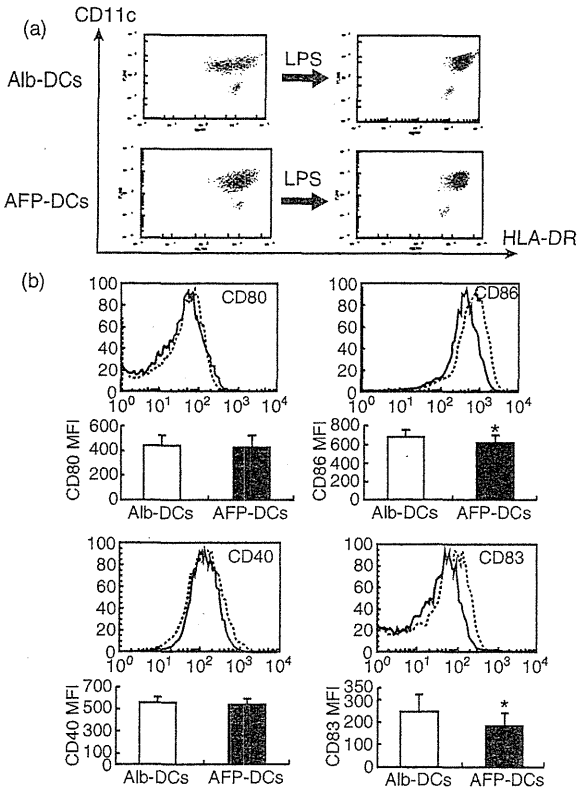

Fig. 3. The maturation of α-fetoprotein-dendritic cells (AFP-DCs) was inhibited more than that of Alb-DCs. Monocyte-derived DCs were generated from eight healthy volunteers. DCs were cultured for 7 days in RPMI-1640 with AFP (25 μg/ml) or albumin (Alb) (25 μg/ml). On day 6, lipopolysaccharide (LPS) was added to induce DC maturation. (a) We defined DCs with CD11c$^+$ and human leucocyte antigen D-related (HLA-DR$^+$) cells by flow cytometry. (b) We evaluated the expression of CD80, CD86, CD40 and CD83 on AFP-DCs (black line) and Alb-DCs (dotted line). Grey histogram indicates control immunoglobulin (Ig)G staining. The expression of each molecule on AFP-DCs and Alb-DCs from seven healthy volunteers was evaluated by the mean fluorescence intensity (MFI) ± standard deviation. All experiments were performed three times independently and representative results (upper panels) as well as the statistical analysis (lower panels) are shown as the MFI of the staining cells. $*P < 0{\cdot}05$.

molecules was not significantly different between immature (day 6) AFP-DCs and immature (day 6) Alb-DCs (data not shown). The expression of CD83 and CD86 on LPS-treated mature AFP-DCs was inhibited significantly compared with those on LPS-treated mature Alb-DCs, although the expression of CD80 and CD40 was not (Fig. 3b), suggesting that maturation of AFP-DCs was impaired. We also examined the expression of antigen-presenting related molecules on AFP-DCs or Alb-DCs which were matured by Poly(I:C), the TLR-3 ligand. On day 6 of the DC culture, we added Poly(I:C) (10 μg/ml) to immature-DC. The results of Poly(I:C)-matured AFP-DCs was similar to those of LPS-matured AFP-DCs (data not shown).

IL-12 production from AFP-DCs was impaired

We examined IL-12, IL-15 and IL-18 production in the supernatant of LPS (TLR-4 ligand)-treated DC culture by specific ELISA. IL-12 was not detected in the supernatants of the non-treated immature AFP-DCs and Alb-DCs (data not shown). The production of IL-12 from mature AFP-DCs was significantly lower than that from mature Alb-DCs (Fig. 4a). When mature DCs were generated under various AFP concentrations (25 μg/ml, 12·5 μg/ml or 6·25 μg/ml), the production of IL-12 from DCs decreased in a dose-dependent manner (Fig. 4a). IL-15 was not detected from the supernatants of both LPS-treated AFP-DCs and Alb-DCs (data not shown), and IL-18 was detected equally in the supernatants of both LPS-treated mature AFP-DCs and Alb-DCs (Fig. 4b). We also examined IL-12 production of AFP-DCs or Alb-DCs which were matured by Poly(I:C). The IL-12 production of mature AFP-DCs was significantly lower than that of Alb-DCs (Fig. 4c), which is consistent with the results of LPS-treated DCs.

mRNA of IL-12 in AFP-DCs was lower than that in Alb-DCs

The bioactive form of IL-12 is a 75 kDa heterodimer (IL-12p70) comprised of independently regulated disulphide-linked 40 kDa (p40) and 35 kDa (p35) subunits. Next, we examined the expression of mRNA of IL-12p35 and IL-12p40 by real-time PCR. Both IL-12p35-mRNA and IL-12p40 mRNA of AFP-DCs were significantly lower than those of Alb-DCs with both LPS and Poly(I:C) stimulation (Fig. 5a). We examined the expression of mRNA of TLR-3 and TLR-4 in the mature DCs. The expression of TLR-3-mRNA and TLR-4-mRNA of AFP-DCs were similar to those of Alb-DCs (Fig. 5b). These results suggested that AFP might cause inhibition downstream of the TLR-3 or TLR-4 signalling pathway, resulting in inhibition of translation of the IL-12 gene at the mRNA level.

IL-12 from DCs played critical roles in NK activation

To examine the involvement of IL-12 in the activated NK cells, NK cells were co-cultured with AFP-DCs or Alb-DCs with or

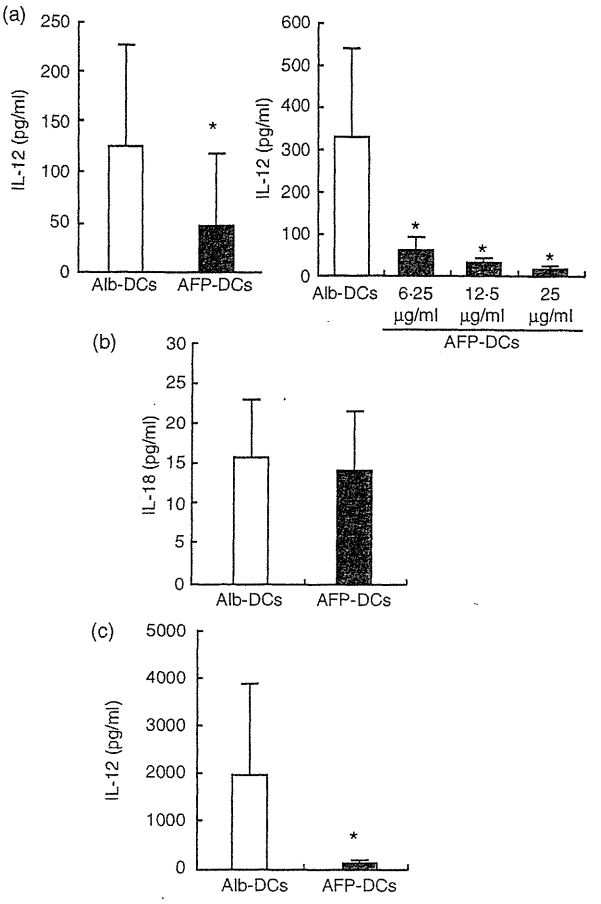

Fig. 4. The production of interleukin (IL)-12p70 from α-fetoprotein-dendritic cells (AFP-DCs) was lower than that from Alb-DCs, but that of interleukin (IL)-18 was not. We cultured DCs for 7 days in RPMI-1640 with AFP (25 μg/ml, 12·5 μg/ml, 6·25 μg/ml) or albumin (Alb) (25 μg/ml). On day 6, we added lipopolysaccharide (LPS) (10 μg/ml, a,b) or Poly(I:C) (10 μg/ml, c) to induce DC maturation. Twenty-four hours later, IL-12p70 (a,c) or IL-18 (b) production from LPS- or Poly(I:C)-treated DCs was measured by specific enzyme-linked immunosorbent assay (ELISA) (results in pg/ml; mean ± standard deviation of triplicate samples). (a, left panel; b,c) We analysed statistically the production of both cytokines between AFP-DCs and Alb-DCs. $^*P < 0·05$. (a, right panel) We analysed statistically the production of IL-12p70 between Alb-DCs and AFP (6·25 μg/ml)-DCs, AFP (12·5 μg/ml)-DCs or AFP(25 μg/ml)-DCs. $^*P < 0·05$ *versus* IL-12p70 production of Alb-DCs.

without the presence of neutralizing antibody for IL-12. The cytolytic activity of NK cells co-cultured with Alb-DCs was significantly higher than that with adding anti-IL-12 neutralizing antibody, but the cytolytic activity of NK cells co-culture with AFP-DCs did not decrease significantly on addition of anti-IL-12 neutralizing antibody (Fig. 6a). Next, NK cells were co-cultured with AFP-DCs or Alb-DCs, and IL-12 was added to the NK cell/AFP-DC co-cultures. Adding IL-12 resulted in significant enhancement of the cytotoxicity of NK