with HCV infection. Nicola A. Fletcher reported that brain microvascular endothelial cells express all the recognized entry factors for HCV, and brain microvascular endothelial cells actually support infection by HCVpp and HCVcc. This suggests potential disorders of the central nervous system in HCV infection.

Treatment

In the keynote lecture, Masashi Mizokami presented "Genome-wide association study and its application for HCV treatment." He emphasized that the functional relevance of IL-28B single nucleotide polymorphisms should be elucidated to further advance the progress of research on the mechanisms of chronic HCV infection and treatment.

Yasuhiro Asahina presented that genetic variation in IL-28B is associated with gene expression involving innate immunity. Minor alleles of IL-28B, as well as a higher RIG-I/IPS-1 ratio are associated with null viral response. Martin Laggins correlated IL-28B genetic variation with pretreatment levels of IP-10 and HCV RNA throughout therapy. The favorable genetic variation of IL-28B single nucleotide polymorphisms (major allele) was significantly associated with lower baseline IP-10. Masao Honda revealed that hepatic IFN-stimulated genes (ISGs) are associated with genetic variation in IL-28B and the outcome of IFN therapy for chronic hepatitis C using microarray gene expression profiling of the biopsied liver samples. Multivariate logistic regression analysis showed that ISGs, fibrosis stage, and ISDR mutations were strongly associated with viral response. Hepatic ISGs were associated with the IL-28B polymorphism and expression was significantly higher in patients with the minor genotype than in those with the major genotype. Takashi Motomura also analyzed ISG expression using liver transplantation samples. Expression of ISGs in recipients' liver carrying the minor allele of IL-28B was significantly up-regulated when compared with the major allele. Surprisingly, IFN sensitivity for recurrent hepatitis C after liver transplantation is influenced by IL-28B genetic variation not only in recipients, but also in donors.

Drug Development

This session opened with a keynote lecture by Raffaele De Francesco describing the current state of drug development for patients with chronic hepatitis C. Because of the rapid development of NS3/4A, NS5A, and NS5B inhibitors, he finally presented the hopeful message "Will there be an HCV meeting in 2020?".

Lotte Coelmont characterized an NS5A D320E variant showing low-level resistance to DEB025, a cyclophilin (Cyp)-binding molecule. This study suggests that DEB025 presents a high barrier to resistance, and that D320E confers low-level resistance to DEB025 by reducing the need for CypA-dependent isomerization of NS5A. Paul Targett-Adams reported that NS5A inhibitors stimulated redistribution of NS5A from the ER to ring-like structures in the cytoplasm, and disrupted colocalization with NS5B. This study suggests that NS5A inhibitors perturb formation of new replication complexes rather than acting on preformed complexes. Luis M. Schang developed a family of small synthetic rigid amphiphiles with large hydrophilic heads and small, planar and rigid hydrophobic tails, called RAFIs (rigid amphipathic fusion inhibitors), which inhibit the infectivity of enveloped virions including HCV. Emmanuel Thomas screened host genes involving the anti-HCV activity of ribavirin. Among 64 host genes, several candidate genes were identified as host factors involving ribavirin's anti-HCV activity. Interestingly, silencing of the *ITPA* gene increased the anti-HCV activity of ribavirin. Pablo Gastaminza identified a novel family of 1,2-diamines as an anti-HCV reagent from a chemical library. The analysis of ~300 derivatives identified several compounds with enhanced potency and low cytotoxicity.

Vaccines/Epidemiology

HCV therapeutic vaccines are aimed to induce effective T-cell responses. Marianne Mikkelsen reported that vaccination of mice with recombinant adenovirus expressing HCV NS3 fused to the MHC class II chaperon protein invariant chain significantly enhanced NS3 specific $CD8^+$ T-cell responses, and protected mice against NS3-expressing vaccinia virus challenge. This vaccination induced polyfunctional $CD8^+$ memory T cells. Lars Frelin aimed to restore immunologic function through vaccination in a transgenic mouse model with impaired HCV-specific T-cell responses owing to a persistent presence of hepatic HCV NS3/4A antigens. They found that heterologous sequences improved activation and expansion of NS3/4A-specific T cells in a wild-type host, as well as in a tolerant NS3/4A-transgenic mouse model. The authors also suggested an important role for Tregs in the impaired HCV-specific T-cell responses.

Livia M.G. Rossi examined antibody cross-immunoreactivity against different HVR1 variants to identify antigens with a possible application of HCV vaccine development. The authors identified a small set of HVR1 variants that cross-immunoreacted with a large number of HVR1 peptides, thus suggesting their potential use in the development of HCV vaccine candidates.

Conclusion

HCV2010 in Yokohama was successful and contributed to the progress of research in the field. HCV infection remains one of the most serious worldwide health problems. The goals of this symposium were to

increase the scientific understanding of this virus and gain insights applicable to future efforts to control its infection. From this point of view, we gained further fundamental understanding about HCV at the meeting. The discovery of IL-28B as a new host factor involved in HCV treatment and pathogenesis had a major impact on HCV research. New treatment advances have been made in recent years and will continue in the near future. We would like to conclude that this meeting was successful in providing opportunities for exchanging up-to-date information and international collaboration. The next meeting will take place in Seattle, Washington, from September 8–12, 2011 (http://www.hcv2011.org/).

Reprint requests

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Conflicts of interest

The authors disclose no conflicts.

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 (x10⁴/μl)*	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.

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.

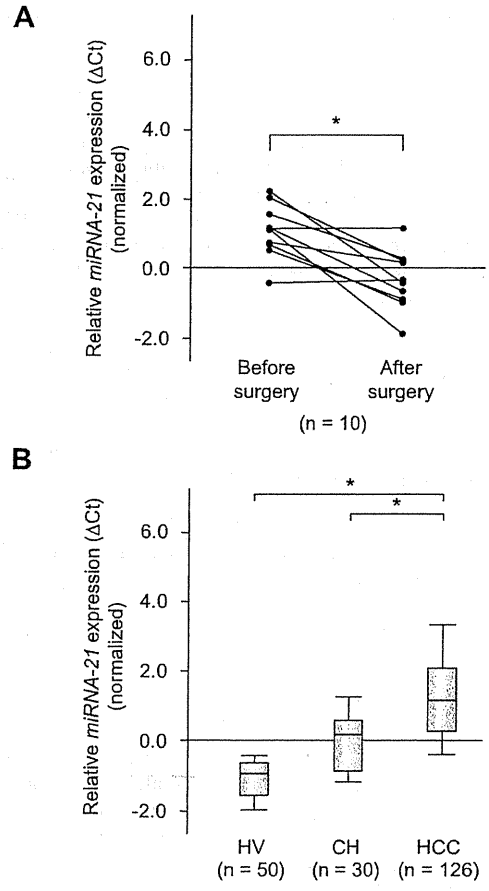

Fig. 1. Plasma *miRNA-21* levels (ΔCt). (A) Plasma *miRNA-21* levels (ΔCt) before and after curative resection in patients with HCC (n = 10). *p <0.05. (B) Plasma *miRNA-21* levels (ΔCt) in patients with HCC (n = 126), CH (n = 30), and HVs (n = 50) measured by qRT-PCR *p <0.05. In this box-and-whisker 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 (p <0.0001, r = 0.403). The ROC curve analysis indicated that AFP was useful in differentiating HCC from CH with AUC of

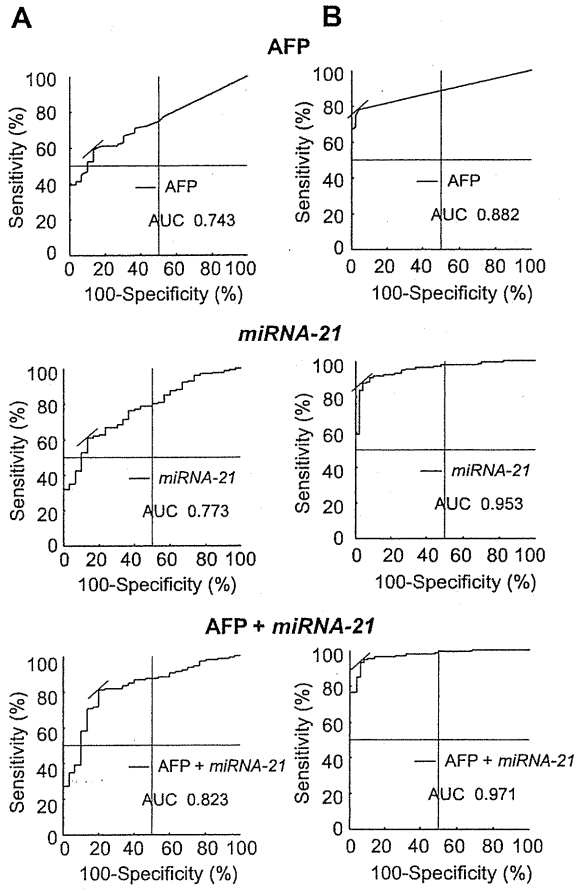

Fig. 2. The diagnostic power of AFP, *miRNA-21*, and the combination of AFP and *miRNA-21* for HCC (n = 126) against CH (n = 30) and HVs (n = 50). (A) Power of AFP, plasma *miRNA-21*, and the combination of AFP and plasma *miRNA-21* in differentiating HCC patients from CH patients. Optimal cutoff values, 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.

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).

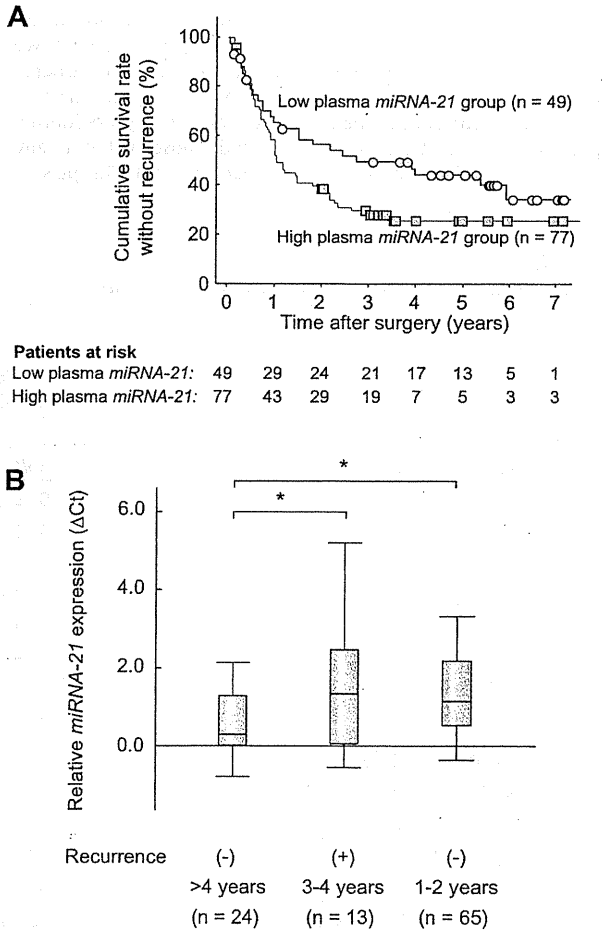

Fig. 3. Correlation between plasma *miRNA-21* expression level and post-operative tumor recurrence. (A) Cumulative survival rate without recurrence after curative surgery for HCC according to plasma *miRNA-21* expression levels. Patients the low plasma *miRNA-21* expression (n = 49) (solid line) (open 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.

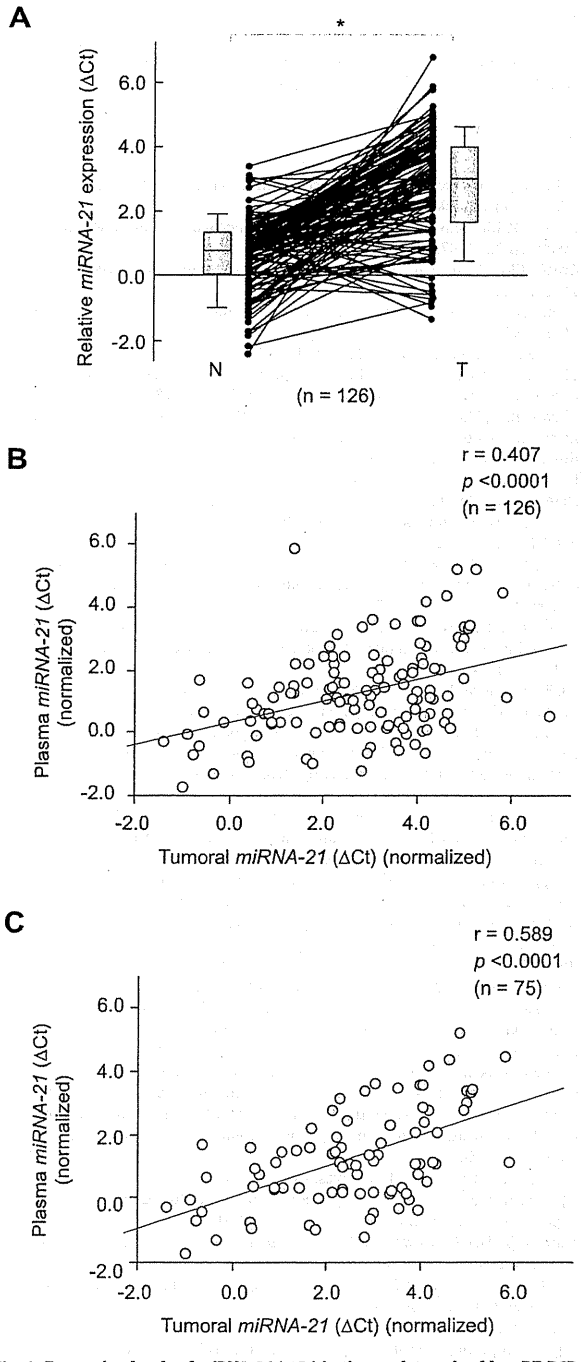

Fig. 4. Expression levels of *miRNA-21* (*Δ*Ct) in tissues determined by qRT-PCR. (A) Tumoral (T) and non-tumoral (N) tissues of patients with HCC (n = 126). $*p < 0.05$. In this box-and-whisker plot, the lines within the boxes represent median values; the upper and lower lines of the boxes represent 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 (*Δ*Ct) in the tumoral tissue and plasma *miRNA-21* levels (*Δ*Ct). 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.

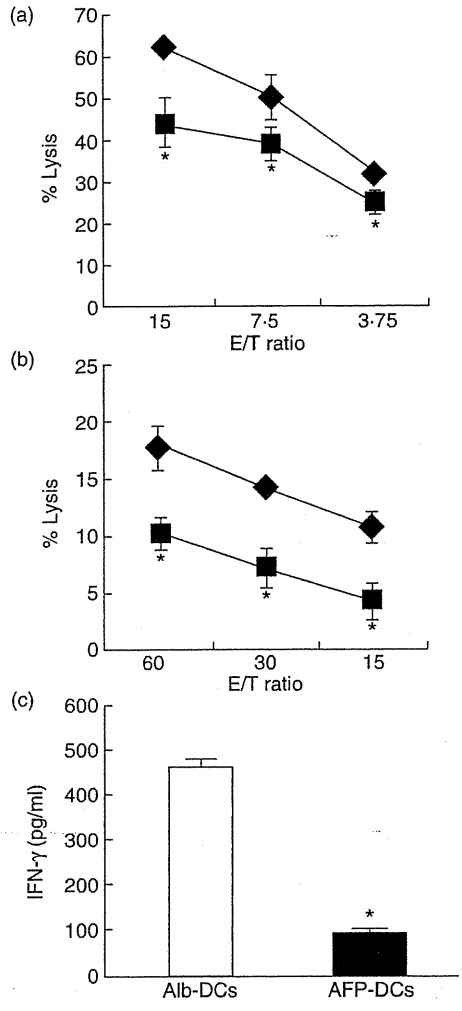

Fig. 1. The cytolytic activity and interferon (IFN)-γ production of natural killer (NK) cells co-cultured with α-fetoprotein-dendritic cells (AFP-DCs) were impaired. (a,b) NK cells were isolated from peripheral blood mononuclear cells (PBMCs) by magnetic cell sorting using CD56 MicroBeads according to 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. * $P < 0{\cdot}05$.

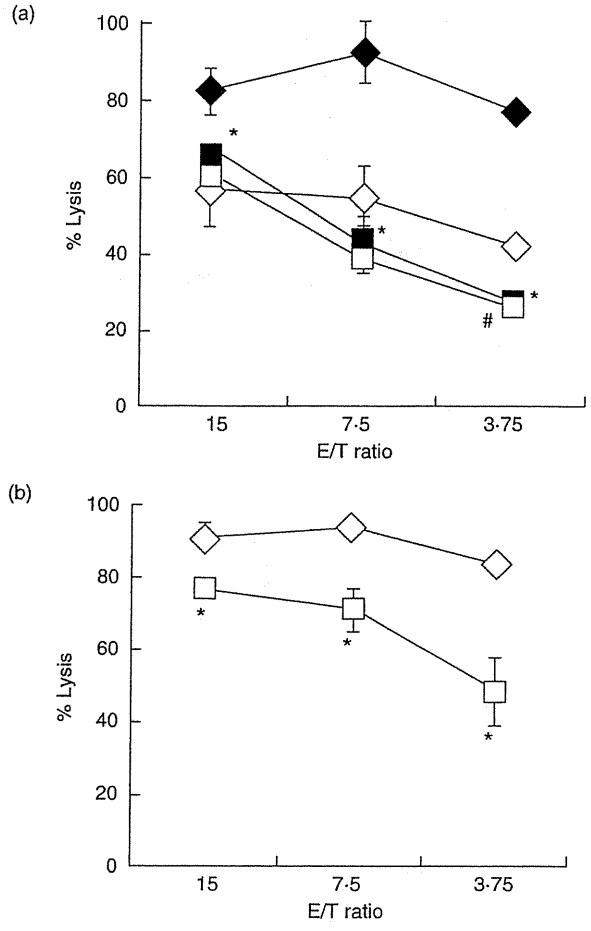

Fig. 2. α-Fetoprotein (AFP) did not directly affect the cytolytic activity of natural killer (NK) cells and soluble factor from dendritic cells (DCs) played a role in the inhibition of NK activity. (a) NK cells were cultured with AFP (25 μg/ml, □, AFP-NK cells) or albumin (Alb) (25 μg/ml, ◇, Alb-NK cells) 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

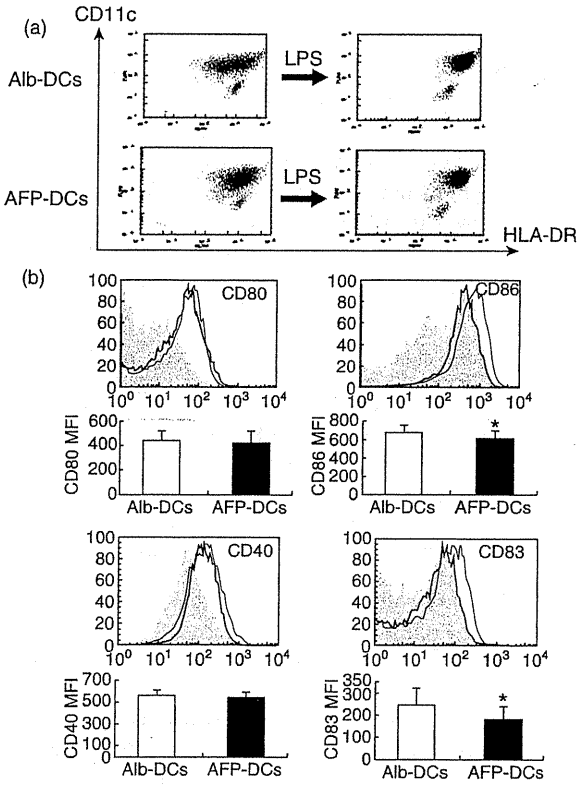

Fig. 3. The maturation of α-fetoprotein-dendritic cells (AFP-DCs) was inhibited more than that of Alb-DCs. Monocyte-derived DCs were generated from eight healthy volunteers. DCs were cultured for 7 days in RPMI-1640 with AFP (25 μg/ml) or albumin (Alb) (25 μg/ml). On day 6, lipopolysaccharide (LPS) was 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

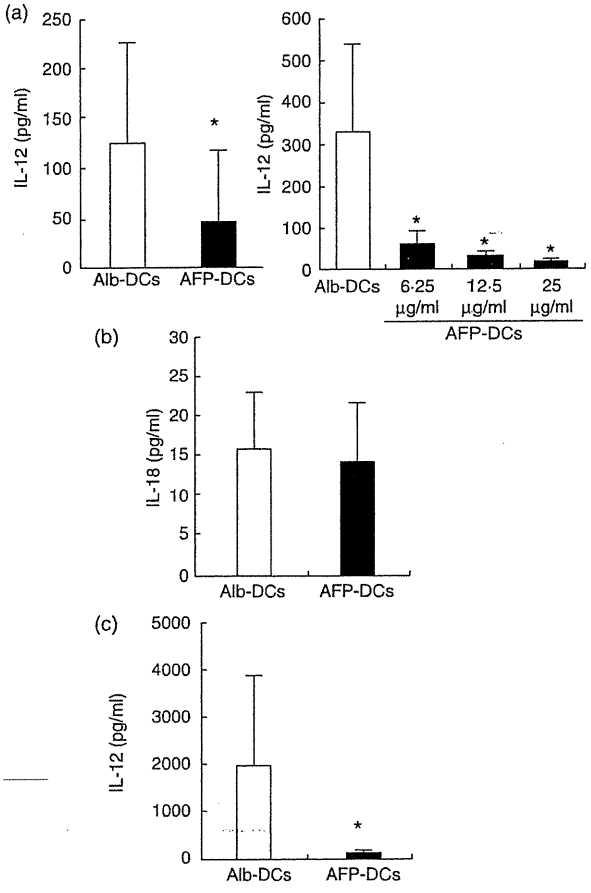

Fig. 4. The production of interleukin (IL)-12p70 from α-fetoprotein-dendritic cells (AFP-DCs) was lower than that from Alb-DCs, but that of interleukin (IL)-18 was not. We cultured DCs for 7 days in RPMI-1640 with AFP (25 μg/ml, 12·5 μg/ml, 6·25 μg/ml) or albumin (Alb) (25 μg/ml). On day 6, we added 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

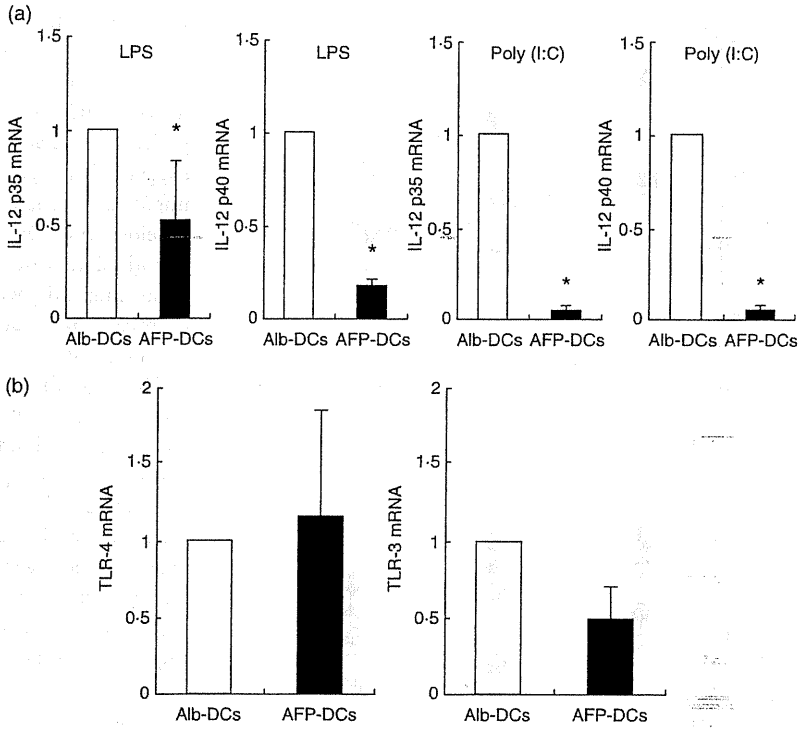

Fig. 5. The mRNAs of interleukin (IL)-12p35 and p40 from α-fetoprotein-dendritic cells (AFP-DCs) were lower than that from albumin (Alb)-DCs, but the mRNA of Toll-like receptor (TLR)-4 and TLRL-3 were not. We cultured DCs for 7 days in RPMI-1640 with AFP (25 μg/ml) or Alb (25 μg/ml). On day 6, we added lipopolysaccharide (LPS) (10 μg/ml) or Poly(I:C) (10 μg/ml) to induce DC maturation. Twenty-four hours later, total RNA was isolated from LPS or Poly(I:C)-treated AFP-DCs or Alb-DCs and was subjected to real-time polymerase chain reaction (PCR) to detect mRNA of IL-12 (a: IL-12p35 mRNA, IL-12p40 mRNA) or mRNA of TLRs (b, TLR-4 mRNA or TLR-3 mRNA). Similar results were obtained from three independent experiments. We analysed statistically the mRNA levels of IL-12p35, IL-12p40, TLR-4 and TLR-3 between AFP-DCs and Alb-DCs. $^*P < 0{\cdot}05$.

cells co-cultured with AFP-DCs to the levels of that with Alb-DCs (Fig. 6b). These results demonstrated that NK activity was impaired in the co-culture with AFP-DCs possibly because of less IL-12 production from AFP-DCs.

Discussion

A variety of tumour-derived soluble factors have been reported to contribute to the emerging of complex local and regional immunosuppressive networks [15]. Recent study has demonstrated that innate immune system via NKG2D signals, expressed on NK cells, might play a critical role in tumour surveillance [16]. This led us to try to identify the immunosuppressive factors in innate immunity to develop a new strategy for cancer prevention. Elevation of serum AFP in cirrhosis patients is believed to be a high risk factor for HCC development [17]. AFP has already been reported to have immune regulatory function in T cells and B cells [9–11]. In this study, we hypothesized that AFP elevation might affect the immune-surveillance of innate immunity in HCC patients. We used a concentration of AFP (6·25–25 μg/ml) that is in a range similar to that detected in the sera of cirrhosis or HCC patients. Our data show that AFP inhibited DC maturation and IL-12 production from DCs which might impair NK activity. This suggested that elevated AFP might affect HCC development by inhibiting NK activity in HCC patients.

The cytolytic activities of NK cells co-cultured with AFP-DCs against K562, NK-sensitive cells as well as Huh7 hepatoma cells were lower than those co-cultured with Alb-DCs. These results suggested that the presence of AFP-stimulated DCs could alter NK cytotoxicity. We have demonstrated previously that the expression of MICA/B on DCs, NK-activating molecules, plays a critical role in the pathogenesis of chronic hepatitis and HCC [14,18]. In this study, we examined these molecules on AFP-DCs and Alb-DCs. However, the expression of MICA/B on AFP-DCs were similar to those on Alb-DCs (Yamamoto *et al.* unpublished data), which suggested that the soluble factor from DCs was more important in the impairment of NK cytotoxicity. In NK activation by DCs, both direct contact with these cells and soluble factors such as IL-12 from activated DCs contribute to NK activation [19]. We demonstrated that the cytolytic activities of NK cells co-cultured with AFP-DCs were also lower than those with Alb-DCs in using Transwell culture, suggesting that the soluble factor contributed to the impairment of NK cytotoxicity. To examine the involvement of IL-12 from DCs in the activation of NK cells, we co-cultured NK cells with AFP-DCs or Alb-DCs with or without the presence of neutralizing antibody for IL-12. The cytolytic activity of NK cells co-cultured with Alb-DCs decreased to the level of that with AFP-DCs on addition of anti-IL-12 neutralizing antibody. Moreover, adding IL-12 to the co-culture of AFP-DCs and NK cells resulted in enhancement of the cytolytic activity of NK cells to the levels of Alb-DCs and NK cells. Taken together, these data demonstrated that IL-12 derived from AFP-DCs plays essential roles in the impairment of NK cytotoxicity, which is consistent

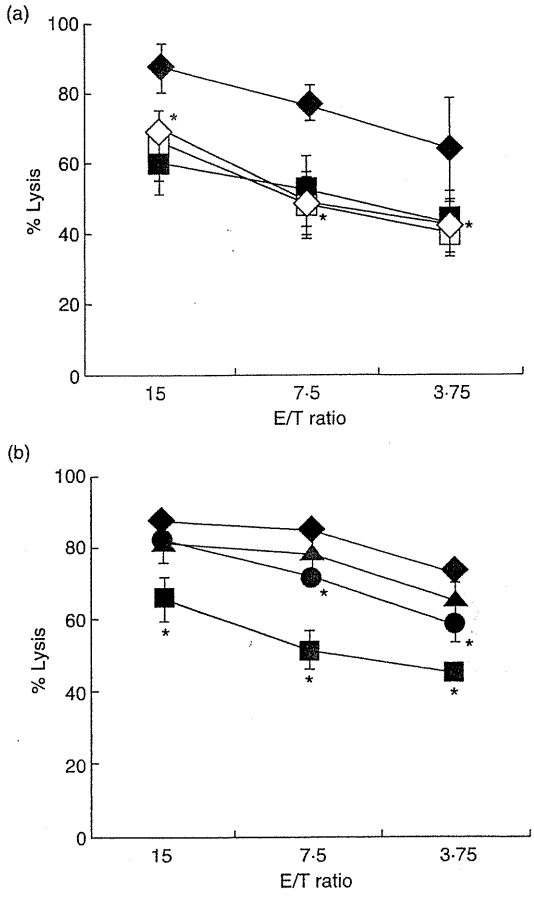

Fig. 6. Interleukin (IL)-12 derived from dendritic cells (DCs) played a critical role in natural killer (NK) cell activation. (a) Enriched NK cells were co-cultured with α-fetoprotein (AFP)-DCs or albumin (Alb)-DCs for 24 h with or without the presence of the neutralizing antibody of IL-12. The cytolytic activity of NK cells against K562 cells were evaluated by ^{51}Cr-releasing assay. The cytolytic activity against K562 cells of NK cells co-cultured with AFP-DCs (■) or Alb-DCs (◆) without neutralizing antibody of IL-12 or AFP-DCs (□) or Alb-DCs (◇) with neutralizing antibody of IL-12. We analysed statistically between the antibody-adding and not-adding groups in both AFP-DC and Alb-DC cultures, respectively. *$P < 0{\cdot}05$ *versus* the cytolytic activity of NK cells cultured with Alb-DCs. Significant difference was observed between the antibody-adding group and not-adding group in the cytolytic activity of NK cells cultured with Alb-DCs. In contrast, no significant difference was observed between the groups in the cytolytic activity of NK cells cultured with AFP-DCs. (b) Enriched NK cells were co-cultured with AFP-DCs or Alb-DCs for 24 h with or without recombinant IL-12p70 protein (150 pg/ml, 300 pg/ml). NK cells were harvested and subjected to examine the cytolytic activity against K562 cells by ^{51}Cr releasing assay. The cytolytic activity co-cultured with AFP-DCs without IL-12 (■) or with IL-12 (150 pg/ml, ●; 300 pg/ml, ▲) or Alb-DCs (◆). *$P < 0{\cdot}05$ *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.

with the results of the production of IL-12 from AFP-DCs and Alb-DCs. Serum AFP is often high in patients with cirrhosis without HCC [8]. Oka *et al.* reported that the incidence of HCC development is significantly high in cirrhosis patients who had elevated serum levels of AFP [17], which suggests that high production of AFP in cirrhosis patients might also impair innate immunity, leading to HCC development. Our results might offer support for the hypothesis that elevation of AFP in cirrhosis patients impairs innate immunity which plays an essential role in the deletion of micro HCC, and thus results in promotion of HCC development.

Although the expression of antigen-presenting related molecules on AFP-DCs was not altered, the maturation of AFP-DCs was inhibited compared with Alb-DCs. This is consistent with a previous report [13] suggesting that the presence of AFP impairs DC maturation. DCs have been implicated in the activation of NK cells [19]. However, activated NK cells have been shown to recognize and lyse DCs *in vitro* and *in vivo*, but maturation of DCs results in resistance to NK lysis [19]. In HCC patients, it has been shown that impairment of DCs is associated with increased tumour progression [20] and that the levels of activated DCs are significantly low in HCC tissues [21]. High levels of AFP produced by HCC tissues may impair DC maturation, which would enhance HCC progression by removing DCs from HCC tumour areas.

IL-12 exhibits a number of immunologically important activities, including the ability to enhance NK cell and CTL functionality, to polarize $CD4^+$ T cell responses by preferentially supporting the T helper type 1/cytotoxic T cell (Th1/Tc1)-type and to suppress Th2-type immunity [22]. We have demonstrated that the production of IL-12 protein from LPS-stimulated or Poly(I:C)-stimulated AFP-DCs was impaired significantly compared with that from Alb-DCs, which might affect immunosuppression in AFP-elevated patients. The expression of mRNA of both IL-12p35 and IL-12p40 were also inhibited significantly in AFP-DCs compared with Alb-DCs but not those of TLR-4, LPS receptor and TLR-3, Poly(I:C) receptor. The production of IL-12 is regulated strictly by positive and negative regulatory mechanisms and differential expression of TLRs affect the IL-12 production from DCs [23]. Our data revealed that adding AFP resulted in inhibition of IL-12 production at the transcriptional level, not by decreased expression of TLRs. Although the regulation of transcription of IL-12p40 and IL-12p35 has been elucidated in various studies [23,24], the detailed mechanism of inhibition of IL-12 transcription by AFP remains unclear. Further study is needed to clarify the detailed mechanism of inhibition of IL-12 by AFP. Taken together, IL-12 might play a mainly essential role in the impairment of NK activity by AFP. To evaluate the possibility of involvement of other immunosuppressive cytokines inhibiting NK activity, we examined the IL-6 and IL-10 levels in the supernatants of the co-cultures of NK cells and

AFP-DCs/Alb-DCs by specific ELISAs. IL-6 levels in the supernatants of AFP-DCs were similar to those of Alb-DCs, and IL-10 levels in the supernatants of AFP-DCs were significantly lower than those of Alb-DCs (M. Yamamoto, unpublished data). These results suggest that the addition of AFP might impair the ability of cytokine production of DCs.

In a previous report, Um *et al.* demonstrated that AFP impairs the function of dendritic cells and induces their apoptosis [13]. In their report, they used the commercially available human cord blood AFP. Thus, we used human cord blood AFP because this is the only commercially available AFP. The carbohydrates of AFP are heterogeneous, which is reflected by differences in the binding of individual AFP molecules to lectins. Therefore, we also added the supernatants of Huh7 cells, AFP-producing HCC cells or control medium on the DCs and evaluated IL-12 production after LPS stimulation by specific ELISA. The supernatants of Huh7 cells contained AFP (1·76 μg/ml) and control medium contained no AFP. The IL-12 production of DCs co-cultured with the supernatants of Huh7 cells was significantly lower than that with control medium (M. Yamamoto, unpublished data). These results were consistent with the results using human cord blood AFP. Although we cannot deny the possibility that unknown factors, except AFP, in the supernatants of Huh7 cell might affect the IL-12 production of DCs, these results suggest that another type of AFP might also have immunoregulatory ability on DCs.

In this study, we demonstrate that AFP might down-regulate IL-12 production from DCs which inhibit NK activity. Zhang *et al.* demonstrated that IL-12 improves the cytotoxicity of NK cells via up-regulated expression of NKG2D on NK cells [25]. We have demonstrated previously that NKG2D expression on NK cells was down-regulated in the progression of chronic liver disease, including HCC [18], which suggested that NK activities were impaired in HCC patients. The expression of NKG2D on NK cells in HCC patients with high serum AFP was significantly lower than those in HCC patients with low serum AFP (M. Yamamoto, unpublished data). This might be the result of long-term continuous suppression of NK cells by elevated AFP.

In conclusion, this study demonstrates that AFP impair the DC ability of activation of NK cells. These findings might provide new insight into understanding the mechanisms underlying the suppression of innate immune responses in chronic liver disease patients with high serum AFP levels.

Acknowledgements

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Disclosure

The authors have no conflicts of interest.

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