with tumor thrombus in the major portal branch. *Hepatogastroenterology.* 2007;54:172–9.
7. Nagano H, Miyamoto A, Wada H, et al. Interferon-alpha and 5-fluorouracil combination therapy after palliative hepatic resection in patients with advanced hepatocellular carcinoma, portal venous tumor thrombus in the major trunk, and multiple nodules. *Cancer.* 2007;110:2493–501.
8. Wada H, Nagano H, Yamamoto H, et al. Combination of interferon-alpha and 5-fluorouracil inhibits endothelial cell growth directly and by regulation of angiogenic factors released by tumor cells. *BMC Cancer.* 2009;9:361.
9. Wada H, Nagano H, Yamamoto H, et al. Combination therapy of interferon-alpha and 5-fluorouracil inhibits tumor angiogenesis in human hepatocellular carcinoma cells by regulating vascular endothelial growth factor and angiopoietins. *Oncol Rep.* 2007;18: 801–9.
10. Ribatti D, Vacca A, Nico B, Sansonno D, Dammacco F. Angiogenesis and anti-angiogenesis in hepatocellular carcinoma. *Cancer Treat Rev.* 2006;32:437–44.
11. Sun HC, Tang ZY. Angiogenesis in hepatocellular carcinoma: the retrospectives and perspectives. *J Cancer Res Clin Oncol.* 2004; 130:307–19.
12. Moon WS, Rhyu KH, Kang MJ, et al. Overexpression of VEGF and angiopoietin 2: a key to high vascularity of hepatocellular carcinoma? *Mod Pathol.* 2003;16:552–7.
13. Yoshiji H, Noguchi R, Kuriyama S, et al. Different cascades in the signaling pathway of two vascular endothelial growth factor (VEGF) receptors for the VEGF-mediated murine hepatocellular carcinoma development. *Oncol Rep.* 2005;13:853–7.
14. Wada H, Nagano H, Yamamoto H, et al. Expression pattern of angiogenic factors and prognosis after hepatic resection in hepatocellular carcinoma: importance of angiopoietin-2 and hypoxia-induced factor-1 alpha. *Liver Int.* 2006;26:414–23.
15. Tanaka S, Arii S. Molecularly targeted therapy for hepatocellular carcinoma. *Cancer Sci.* 2009;100:1–8.
16. Marijon H, Faivre S, Raymond E. Targeted therapies in hepatocellular carcinomas: recent results and future development. *Bull Cancer.* 2009;96:553–61.
17. Liu Y, Poon RT, Li Q, Kok TW, Lau C, Fan ST. Both antiangiogenesis- and angiogenesis-independent effects are responsible for hepatocellular carcinoma growth arrest by tyrosine kinase inhibitor PTK787/ZK222584. *Cancer Res.* 2005;65:3691–9.
18. Eguchi H, Nagano H, Yamamoto H, et al. Augmentation of antitumor activity of 5-fluorouracil by interferon alpha is associated with up-regulation of p27Kip1 in human hepatocellular carcinoma cells. *Clin Cancer Res.* 2000;6:2881–90.
19. Nakamura M, Nagano H, Sakon M, et al. Role of the Fas/FasL pathway in combination therapy with interferon-alpha and fluorouracil against hepatocellular carcinoma in vitro. *J Hepatol.* 2007;46:77–88.
20. Kondo M, Nagano H, Wada H, et al. Combination of IFN-alpha and 5-fluorouracil induces apoptosis through IFN-alpha/beta receptor in human hepatocellular carcinoma cells. *Clin Cancer Res.* 2005;11:1277–86.
21. Yamamoto T, Nagano H, Sakon M, et al. Partial contribution of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/TRAIL receptor pathway to antitumor effects of interferon-alpha/5-fluorouracil against Hepatocellular Carcinoma. *Clin Cancer Res.* 2004;10:7884–95.
22. Noda T, Nagano H, Takemasa I, et al. Activation of Wnt/beta-catenin signalling pathway induces chemoresistance to interferon-alpha/5-fluorouracil combination therapy for hepatocellular carcinoma. *Br J Cancer.* 2009;100:1647–58.
23. Wood JM, Bold G, Buchdunger E, et al. PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. *Cancer Res.* 2000;60:2178–89.
24. Gerber HP, McMurtrey A, Kowalski J, et al. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem.* 1998;273: 30336–43.
25. Nor JE, Christensen J, Liu J, et al. Up-Regulation of Bcl-2 in microvascular endothelial cells enhances intratumoral angiogenesis and accelerates tumor growth. *Cancer Res.* 2001;61:2183–8.
26. Platanias LC. The p38 mitogen-activated protein kinase pathway and its role in interferon signaling. *Pharmacol Ther.* 2003;98: 129–42.
27. Hata T, Yamamoto H, Ngan CY, et al. Role of p21waf1/cip1 in effects of oxaliplatin in colorectal cancer cells. *Mol Cancer Ther.* 2005;4:1585–94.
28. Lee TK, Man K, Poon RT, Lo CM, Ng IO, Fan ST. Disruption of p53-p21/WAF1 cell cycle pathway contributes to progression and worse clinical outcome of hepatocellular carcinoma. *Oncol Rep.* 2004;12:25–31.
29. Martin J, Dufour JF. Tumor suppressor and hepatocellular carcinoma. *World J Gastroenterol.* 2008;14:1720–33.
30. Qin LF, Ng IO, Fan ST, Ng M. p21/WAF1, p53 and PCNA expression and p53 mutation status in hepatocellular carcinoma. *Int J Cancer.* 1998;79:424–8.
31. Hsu IC, Tokiwa T, Bennett W, et al. p53 gene mutation and integrated hepatitis B viral DNA sequences in human liver cancer cell lines. *Carcinogenesis.* 1993;14:987–92.
32. Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE, Givol D. Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res.* 1994;54:3391–5.
33. Nakaji M, Yano Y, Ninomiya T, et al. IFN-alpha prevents the growth of pre-neoplastic lesions and inhibits the development of hepatocellular carcinoma in the rat. *Carcinogenesis.* 2004;25: 389–97.
34. Puisieux A, Galvin K, Troalen F, et al. Retinoblastoma and p53 tumor suppressor genes in human hepatoma cell lines. *FASEB J.* 1993;7:1407–13.
35. Fan G, Ma X, Wong PY, Rodrigues CM, Steer CJ. p53 dephosphorylation and p21(Cip1/Waf1) translocation correlate with caspase-3 activation in TGF-beta1-induced apoptosis of HuH-7 cells. *Apoptosis.* 2004;9:211–21.

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CLINICAL STUDIES

PLOD2 induced under hypoxia is a novel prognostic factor for hepatocellular carcinoma after curative resection

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Abstract

Background: Under hypoxia, tumour cells undergo genetic and adaptive changes that allow their survival. Previously, we reported that high expression of hypoxia-inducible factor (HIF)-1 was a significant predictive factor for recurrence in hepatocellular carcinoma (HCC). Hypoxia also stimulates expression of procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD) genes via the HIF-1 pathway. *Aims:* The aim was to evaluate the relationship between hypoxia stress and expression of PLOD genes in HCC *in vitro* and to identify a new prognostic marker in HCC patients. *Methods:* The PLOD2 expression was assessed under hypoxia in hepatoma cell lines and characterized in 139 HCC samples following hepatic resection using microarray experiments, quantitative RT-PCR and immunohistochemistry. Prognostic factors in HCC patients were assessed using univariate and multivariate analyses. *Results:* The PLOD2 expression was induced under the hypoxia *in vitro*. Disease-free survival in the high PLOD2 expression group of HCC patients was significantly shorter when compared with the low-expression group ($P = 0.002$). In a subset of HCCs, we found that the PLOD2 expression of microarray was correlated with data of quantitative RT-PCR and immunohistochemistry. Of clinicopathological factors, PLOD2 expression was significantly correlated with tumour size ($P = 0.022$) and macroscopic intrahepatic metastasis ($P = 0.049$). In univariate analysis, six prognostic factors (tumour multiplicity, macroscopic intrahepatic metastasis, histological grade, microscopic portal invasion, microscopic intrahepatic metastasis and PLOD2 expression) were significant for disease-free survival. PLOD2 expression was identified as a significant, independent factor of poor prognosis ($P = 0.013$). *Conclusions:* PLOD2 is a potential novel prognostic factor for HCC patients following surgery.

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and the fourth-ranked cause of cancer-related death in Japan (1). The prognosis for patients with HCC remains unfavourable, with the 5-year survival rate after curative resection reported to be 35–43% (2, 3). Surgery has been established as one of the most effective therapeutic modalities for patients with good liver function; however, frequent recurrence of HCC after curative resection remains a major clinical problem (4). To predict recurrence, metastasis and prognosis of HCC patients after curative resection are significant clinical issues. Recurrence and prognosis closely depend on tumour extent and liver function, and prognostic scoring systems, such as the Cancer of the Liver Italian Program (CLIP) scoring system and the Japan Integrated Staging (JIS) scoring system, have been widely used to evaluate the prognosis of HCC patients (5, 6). However, these systems are not always sufficient for predicting recurrence and prognosis of the individual patient, and identification of novel molecular markers is needed.

Hypoxia regulates the expression of a diverse group of genes that promote tumour growth, and are involved in tissue invasion, angiogenesis, cell proliferation, cell survival and pH balance (7, 8). In tumour cells under hypoxia, the hypoxia-inducible factor (HIF)-1 pathway is activated, leading to upregulation of many hypoxia-response proteins associated with an aggressive tumour phenotype (9, 10). Procollagen-lysine, 2-oxoglutarate 5-dioxygenase (PLOD) genes are

involved in fibrotic processes and tissue remodelling and are also known as lysyl hydroxylases (11). Lysyl hydroxylase catalyses the hydroxylation of lysyl residues as a post-translational event in collagen biosynthesis. Three isoforms of the enzyme have been characterized so far: PLOD1, PLOD2 and PLOD3 (12). Furthermore, recent work has shown that hypoxia stimulates expression of PLOD via the HIF-1 pathway (13, 14). Among these PLOD genes, PLOD2 contributes to cancer prognosis and angiogenesis. Several authors have reported that PLOD2 expression might provide prognostic information about malignant tumours such as glioblastoma (15), and PLOD2 expression is a useful biomarker for the effects of anti-angiogenic treatment for malignancy (16).

We previously reported a relationship between HIF-1 expression and angiogenic and clinicopathological factors or prognosis, in which the high nuclear expression of HIF-1 was a significant predictive factor for recurrence after curative resection in HCC patients (17). Furthermore, we identified several potential prognostic factors and therapeutic targets by culturing of colorectal cancer cells under hypoxic conditions *in vitro* (18). In these experiments, PLOD2 expression was induced in the hypoxic region of liver metastases. We thus hypothesized that PLOD2 expression is regulated by hypoxia stress in hepatoma cell lines as well as in colorectal cancer cells and could be a novel prognostic factor in HCC patients after surgery.

Materials and methods

Cell lines

The human hepatoma cell lines PLC/PRF/5, HuH7 and HepG2 were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan). They were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 units/ml penicillinand 100 μg/ml streptomycin at 37°C in a humidified incubator with 5% CO_2 in air. For hypoxia treatment, cells were maintained in a humidified incubator with 0.1% O_2, 5% CO_2 and 94% N_2.

Drugs and reagents

We used the following antibodies for the western blot: monoclonal mouse antihuman PLOD2 antibody (R&D Systems, Minneapolis, MN, USA) and polyclonal rabbit antihuman β-actin (Sigma, St Louis, MO, USA). We also used the rabbit polyclonal antihuman PLOD2 antibody (Proteintech Group, Inc., Chicago, IL, USA) for immunohistochemistry.

Patients and specimens

A total of 139 HCC patients who underwent a hepatectomy at Osaka University Hospital and at its related hospitals were enrolled in this study. All aspects of our study protocol were approved by the ethics committee of the Graduate School of Medicine, Osaka University. Informed consent was obtained from all patients to use their surgical specimens and clinicopathological data for research purposes. Histological classification was based on the Edmondson grading system, and clinical stage was based on the staging system of the Liver Cancer Study Group of Japan (19). For reference for the microarray experiments, a mixture of RNA from normal liver specimens of seven patients with liver metastasis from intestinal carcinomas was obtained. All cases for reference had no infections of hepatitis B or hepatitis C virus, and all had liver function values within normal limits. All tissues were snap-frozen in liquid nitrogen and stored at −80°C. Other samples were fixed in 10% buffered formalin, embedded in paraffin and stained with haematoxylin–eosin for study of the pathological features of HCC in accordance with the classification proposed by the Liver Cancer Study Group of Japan.

Reverse transcription polymerase chain reaction analysis

Complementary DNA (cDNA) was generated from 1 μg RNA with avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA), as described previously (20). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays were performed using the Light Cycler and detection system (Roche Diagnostics, Mannheim, Germany), as described previously (21). Gene expression was measured in duplicate. PCR conditions for PLOD1, PLOD2 and PLOD3 were as follows: one cycle of denaturing at 95° C for 10 min, followed by 40 cycles of 95°C for 15 s, 60° C for 15 s and 72°C for 35 s and final extension at 72°C for 10 min. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified quantitatively at the same time to verify the integrity of the RNA. The primer sequences were as follows: PLOD1 forward primer, 5′-GCAGCAGGATGTGTTCATGT-3′; PLOD1 reverse primer, 5′- GGGCTTTGGTGTAGTT-CTGG-3′; PLOD2 forward primer, 5′-GCGTTCTCTT-CGTCCTCATC-3′; PLOD2 reverse primer, 5′-GTGTG AGTCTCCCAGGATGC-3′; PLOD3 forward primer, 5′-GGTACGAGGACCAGTGGCT-3′; PLOD3 reverse primer, 5′-GAAGGTGGATGAGTCGTGGT-3′; GAPDH forward primer, 5′-CAACTACATGGTTTACATGTTC-3′; and GAPDH reverse primer, 5′-GCCAGTGGACTC-CACGAC-3′.

Western blot analysis

The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and collected with a rubber scraper. After centrifugation, the cell pellets were resuspended in RIPA buffer [25 mM Tris (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP-40,

0.2% SDS, 1 mM phenylmethylsulfonyl fluoride and 500 KIE/ml proteinase inhibitor (Bayer, LeverKusen, Germany)] with phosphate inhibitor (Sigma). The extracts were clarified at 15 000*g* for 10 min at 4°C, and the supernatant fraction was collected. Western blot analysis was performed as described previously (22).

Microarray experiments

The microarray results were evaluated according to previously described methods (23). Briefly, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the instructions supplied by the manufacturer. The integrity of RNA was assessed by Agilent 2100 Bioanalyzer and RNA 6000 LabChip kits (Yokokawa Analytical Systems, Tokyo, Japan). Only high-quality RNA was used for analysis. Seven RNA extractions from different normal liver tissue were mixed as the control reference. Next, 2 μg of total RNA was used to synthesize double-stranded cDNA that contained a promoter for T7 RNA polymerase. Amplified antisense RNA was synthesized by *in vitro* transcription of the cDNA templates using the Amino Allyl MessageAmp aRNA kit (Ambion, Austin, TX, USA). The reference and test samples were labelled with Cy3 and Cy5, mixed and hybridized on a microarray covering 30 336 human probes (AceGene Human 30K; DNA Chip Research Inc. and Hitachi Software Engineering Co., Yokohama, Japan). The microarrays were scanned using ScanArray Lite, and signal values were calculated using DNASIS array software (Hitachi Software Engineering Co.). The local background was subtracted from each spot, and the ratio of the intensity of fluorescence from the Cy5 channel to the intensity of fluorescence from the Cy3 channel was calculated for each spot. The ratio of expression level of each gene was converted to a logarithmic scale (base 2), and the data matrix was normalized.

Immunohistochemical staining

Immunohistochemical staining of the PLOD2 protein was performed as previously described (23). Briefly, formalin-fixed, paraffin-embedded sections were deparaffinized and then treated with an antigen retrieval procedure and incubated in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase. The sections were incubated with normal protein block serum solution. Then, the sections were incubated with anti-PLOD2 antibody at a dilution of 1:100. After washing in PBS, the sections were incubated with a biotin-conjugated secondary antibody and with peroxidase-conjugated streptavidin. The peroxidase reaction was then developed with 0.02% 3, 30-diaminobenzidine tetrachloride solution with 0.03% hydrogen peroxide. Finally, the sections were counterstained with Meyer's haematoxylin. The sections of pancreas cancer were stained with the same way for positive control. For negative controls, sections were incubated with PBS instead of the primary antibody.

Statistical analysis

Clinicopathological characteristics were compared using chi-square tests, and continuous variables were compared using Student's *t*-tests. Survival curves were computed using the Kaplan–Meier method, and differences between disease-survival curves were compared using the log-rank test. Univariate analysis was conducted incorporating 16 prognostic factors including age, gender, hepatitis B virus infection, hepatitis C virus infection, cirrhosis, alpha-foetoprotein, protein-induced by vitamin K absence or antagonist II (known as PIVKA-II), tumour size, tumour multiplicity, macroscopic portal invasion, macroscopic intrahepatic metastasis, stage, histological grade, microscopic portal invasion, microscopic intrahepatic metastasis and PLOD2 expression. To evaluate the risk associated with the prognostic variables, a Cox model with the determination of the hazard ratio was applied employing six factors found to be significant in univariate analysis; a 95% confidence interval was adopted. All statistical analyses were calculated with SPSS software (version 11.0.1 J; SPSS Inc., Chicago, IL, USA), and a *P*-value <0.05 was considered statistically significant.

Results

Expression of PLOD genes under the hypoxic condition

Among the PLOD genes, PLOD1 and PLOD3 expression did not change under hypoxic conditions based on qRT-PCR analysis in hepatoma cell lines (Fig. 1a and c). Only PLOD2 expression gradually increased under hypoxia in HuH7 and HepG2 cell lines after 6 h of exposure, but not in PLC/PRF/5 cells (Fig. 1b). In western blotting analysis, PLOD2 expression was induced in HuH7 and HepG2 cell lines under hypoxic conditions of 24 and 72 h, whereas PLC/PRF/5 cell lines did not exhibit an increase in PLOD2 expression (Fig. 1d).

Disease-free survival curve analysis according to PLOD gene expression

We examined the correlation between the expression level of PLOD genes and disease-free survival curves of 139 HCC patients with hepatic resection. The 139 patients were divided into two groups [high-expression group ($n = 70$) or low-expression group ($n = 69$)] based on median expression levels from the microarray data for each gene in Fig. 2a–c. Figure 2b shows that the disease-free survival rate in the high-expression group for the PLOD2 gene was significantly shorter than that of the low-expression group (log-rank test: $P = 0.002$). The 24- and 60-month disease-free

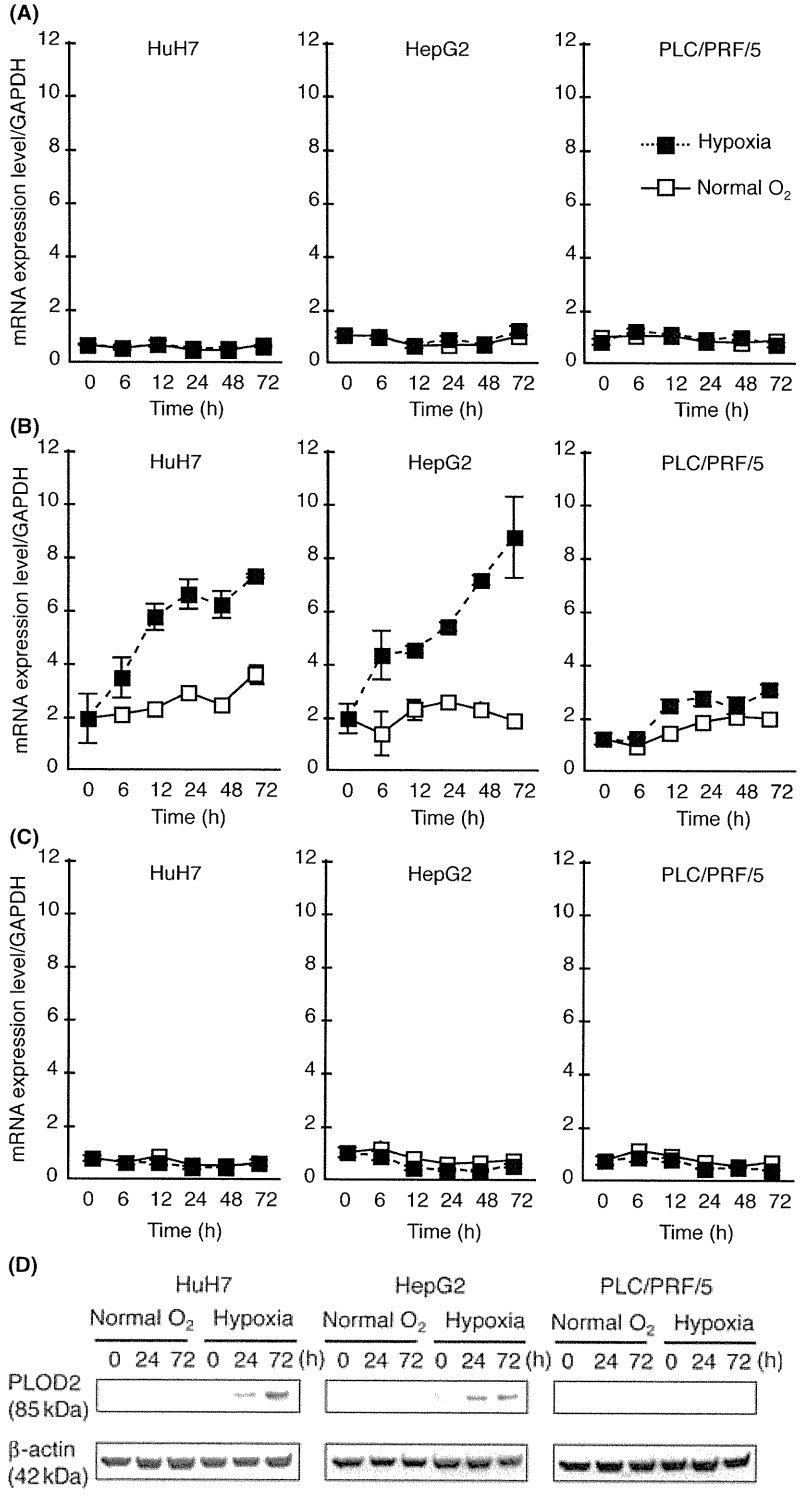

Fig. 1. *In vitro* assay for expression of PLOD genes (PLOD1, PLOD2 and PLOD3) under hypoxic conditions in hepatoma cell lines. (a–c) Comparison of PLOD mRNA expression in HuH7, HepG2 and PLC/PRF/5 cells. (a) PLOD1 expression. (b) PLOD2 expression. (c) PLOD3 expression. (d) Expression of PLOD2 protein in hepatoma cell lines as measured by western blotting at different time points of 0, 24 and 72 h. Expression of PLOD2 protein was increased under hypoxic conditions in HuH7 and HepG2 cells.

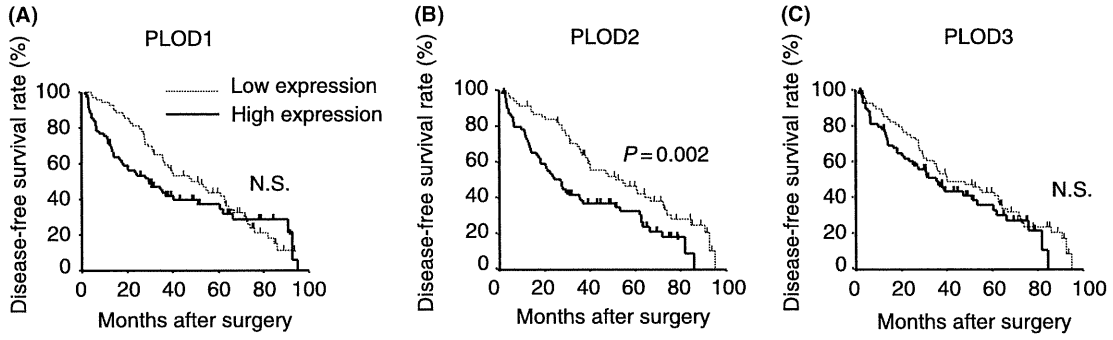

Fig. 2. Disease-free survival curves calculated using the Kaplan–Meier method for 139 hepatocellular carcinoma cases according to PLOD gene expression. (a–c) The 139 patient samples were divided into two groups (high expression; n = 70 or low expression; n = 69) based on the median expression values of each gene (PLOD1, PLOD2 and PLOD3). Differences in disease-free survival curves were estimated using the log-rank test. (a) PLOD1 expression. (b) PLOD2 expression. (c) PLOD3 expression.

survival rates in the PLOD2 high-expression group were 52 and 33%, respectively, and those in the PLOD2 low-expression group were 84 and 47% respectively. There were no significant differences in disease-free survival curves between the high-expression and low-expression groups for either PLOD1 or PLOD3 genes in Fig. 2a and c.

PLOD2 expression by qRT-PCR and correlation with microarray data

We next examined the correlation between the expression data from microarray and qRT-PCR analysis of PLOD2 to validate the microarray data. qRT-PCR analysis was performed on 23 HCC tissue samples, randomly selected from among the 139 HCC tissues. Individual mRNA levels were normalized to GAPDH and expressed relative to those in a mixture of seven normal livers. In 23 samples, qRT-PCR data for PLOD2 were significantly correlated with the results obtained from the microarray data (Fig. 3). The Pearson correlation coefficients (P-value) for PLOD2 were 0.794 ($P < 0.001$).

PLOD2 expression by immunohistochemistry and correlation with microarray data

We examined the protein expression of HCC tissue samples using immunohistochemistry and investigated the correlation between the PLOD2 positivity on tissue samples and the microarray expression to validate the PLOD2 expression of post-transcriptional level. Immunohistochemistry staining was performed on 26 HCC tissue samples. Of 26 cases, 15 cases (57.7%) showed the positive PLOD2 expression on cytoplasm (Fig. 4a). In 26 samples, the PLOD2 positive cases significantly showed the higher PLOD2 expression in microarray than the PLOD2 negative cases (Fig. 4b) and the PLOD2 expression in protein level was well correlated with the mRNA expression in microarray ($P < 0.05$).

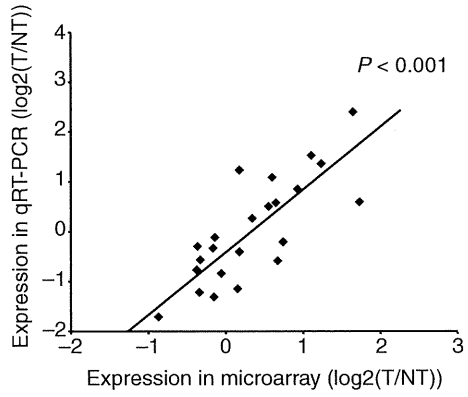

Fig. 3. Correlation of PLOD2 expression between the microarray data and quantitative reverse transcription polymerase chain reaction (qRT-PCR). qRT-PCR data were significantly correlated with the microarray data in 23 hepatocellular carcinoma samples surgically resected. The Pearson correlation coefficients (P-value) for PLOD2 were 0.794 ($P < 0.001$).

Correlations between PLOD2 expression and clinicopathological findings

Table 1 summarizes the correlation between the clinicopathological characteristics of the HCC patients and PLOD2 expression. PLOD2 expression was significantly correlated with tumour size ($P = 0.022$) and macroscopic intrahepatic metastasis ($P = 0.049$). There was no significant association between PLOD2 expression and other characteristics.

Univariate and multivariate analysis of disease-free survival according to PLOD2 expression

Table 2 shows the clinical and pathological features and results of univariate analysis used to identify the significant prognostic factors for disease-free survival for all 139 patients. In univariate analysis, six significant prognostic factors (tumour multiplicity,

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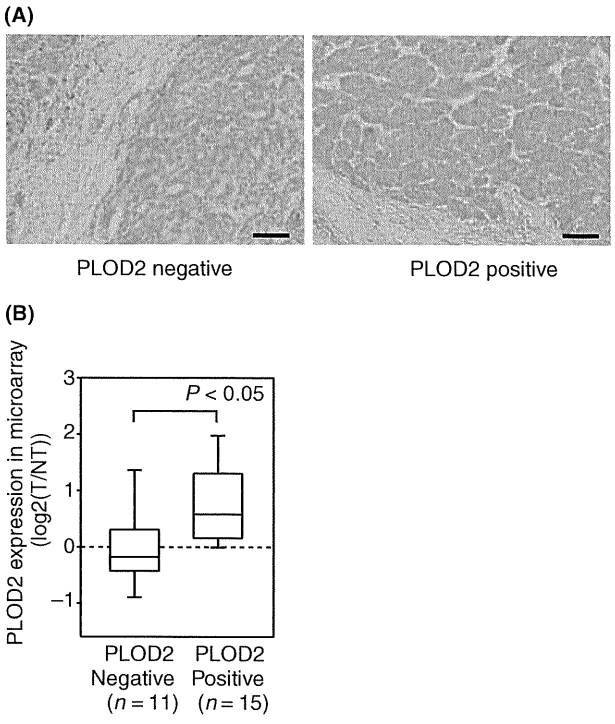

Fig. 4. PLOD2 expression by immunohistochemistry. (a) The representative cases of PLOD2-negative hepatocellular carcinoma (HCC) and PLOD2-positive HCC (bar: 100 μm). (b) The PLOD2 positive cases significantly showed the higher PLOD2 expression in microarray than the PLOD2 negative cases ($P < 0.05$).

macroscopic intrahepatic metastasis, histological grade, microscopic portal invasion, microscopic intrahepatic metastasis and PLOD2 expression) were the significant prognostic factors for disease-free survival. Furthermore, PLOD2 expression was identified as a significant and independent factor of poor prognosis by a Cox proportional hazards model using these six factors ($P = 0.013$).

Discussion

Hepatocellular carcinoma is generally known as a hypervascular tumour, but it is hypovascular in its early stage, and the rapid proliferation of tumour cells continuously induces local hypoxia in the advanced stages. Angiogenesis, the formation of new blood vessels, is an essential process in carcinogenesis in HCC, and several angiogenic factors play important roles. We previously reported that the expression of vascular endothelial growth factor (VEGF) and angiopoietin-2 correlated with microvessel density, and we demonstrated strong expression of VEGF in hypervascular HCC. We also showed that a high nuclear expression of HIF-1 was a significant predictive factor for recurrence after curative resection in HCC patients (17). HIF-1 is a key transcription factor induced by hypoxia.

Table 1. Clinicopathological characteristics and PLOD2 expression

Characteristics	Low expression (n = 69)	High expression (n = 70)	P-value
Age (year)			0.178
<65	33	30	
≥65	36	40	
Gender			0.556
Male	53	60	
Female	16	10	
HBV infection			0.928
Present	32	33	
Absent	37	37	
HCV infection			0.946
Present	42	43	
Absent	27	27	
Cirrhosis			0.806
Present	31	30	
Absent	38	40	
AFP (ng/ml)			0.869
<400	55	55	
≥400	14	15	
PIVKA-II (mAU/ml)			0.764
<40	22	24	
≥40	47	46	
Tumour size (cm)			0.022
<2	17	7	
≥2	52	63	
Tumour multiplicity			0.276
Single	53	48	
Multiple	16	22	
Macroscopic portal invasion			0.595
Present	4	11	
Absent	65	59	
Macroscopic intrahepatic metastasis			0.049
Present	11	21	
Absent	58	49	
Stage			0.087
I–II	56	48	
III–IV	13	22	
Histological grade (Edmondson)			0.924
I–II	39	39	
III–IV	30	31	
Microscopic portal invasion			0.129
Present	21	30	
Absent	48	40	
Microscopic intrahepatic metastasis			0.087
Present	13	22	
Absent	56	48	

HBV, hepatitis B virus; HCV, hepatitis C virus; AFP, alpha-foetoprotein; PIVKA-II, protein-induced by vitamin K absence or antagonist II.

In the absence of oxygen, it binds to hypoxia-response elements, activating the expression of numerous hypoxia-response genes, such as VEGF, glucose transporter-1 and erythropoietin (9).

Table 2. Univariate and multivariate analysis in disease-free survival

Characteristics	*n*	50% DFS (m)	*P*-value	Hazard ratio	95% CI	*P*-value
Age (year)			0.298			
<65	63	39.1				
≥65	76	38.1				
Gender			0.364			
Male	113	35.6				
Female	26	62.7				
HBV infection			0.633			
Present	65	35.6				
Absent	74	40.2				
HCV infection			0.286			
Present	85	47.2				
Absent	54	29.1				
Cirrhosis			0.256			
Present	61	39.8				
Absent	78	38.1				
AFP (ng/ml)			0.102			
<400	110	40.2				
≥400	29	35.4				
PIVKA-II (mAU/ml)			0.425			
<40	46	39.8				
≥40	93	35.6				
Tumour size (cm)			0.609			
<2	24	55.5				
≥2	115	36.9				
Tumour multiplicity			0.007	0.992	0.391-2.518	0.987
Single	101	47.2				
Multiple	38	27.7				
Macroscopic portal invasion			0.204			
Present	15	20.9				
Absent	124	39.8				
Macroscopic intrahepatic metastasis			0.001	1.778	0.536-5.898	0.347
Present	32	20.9				
Absent	107	48.1				
Stage			0.252			
I–II	104	39.8				
III–IV	35	36.9				
Histological grade (Edmondson)			0.049	1.292	0.849-1.964	0.232
I–II	78	55.5				
III–IV	61	35.4				
Microscopic portal invasion			0.004	1.338	0.855-2.093	0.203
Present	51	27.0				
Absent	88	54.6				
Microscopic intrahepatic metastasis			0.001	1.199	0.541-2.659	0.654
Present	35	21.4				
Absent	104	51.1				
PLOD2 expression			0.002	1.688	1.115-2.557	0.013
Low expression	69	54.6				
High expression	70	27.0				

AFP, alpha-foetoprotein; CI, confidential interval; DFS, disease-free survival; HBV, hepatitis B virus; HCV, hepatitis C virus; PIVKA-II, protein-induced by vitamin K absence or antagonist II.

The PLOD genes are responsible for the catalytic conversion of lysine into hydroxylysine. The specificity of PLOD1 is probably directed towards lysine residues in the helical domain of the collagen molecule (24). PLOD2 is involved in the hydroxylation of telopeptide lysine residues (25). PLOD3 differs from the other two lysyl hydroxylases in that it also displays glucosyl and galactosyl transferase activity and contributes to the glycosylation of hydroxylysine residues in the collagen molecule (26). Hofbauer *et al.* (13) reported that oxygen tension regulates the expression of PLOD1 and PLOD2 *in vitro*. The change in expression of PLOD genes under stimulation by hypoxia differed, however, based on the cell type and species. In our study, only

PLOD2 expression in human hepatoma cell lines was stimulated by hypoxia. In other malignant tumours, further studies will be needed.

We also investigated the transcriptional level of PLOD2 mRNA in HCC tumour tissues from 139 patients. Our clinicopathological analysis demonstrated that high expression of PLOD2 was related to the tumour size and macroscopic intrahepatic metastasis. In addition, the disease-free survival curves showed that high PLOD2 expression was associated with a significant poor disease-free survival rate. The mechanism of regulation of PLOD2 by oxygen tension during hypoxia suggests coordination by HIF-1 (13).

Few reports have addressed the relationship between PLOD2 and malignant tumours. Chang *et al.* (27) proposed PLOD2 as one of the fibroblast core serum response genes associated with cancer progression. Dong *et al.* (15) analysed the prognosis for patients with glioblastoma and demonstrated that PLOD2 median expression values could significantly dichotomize patient survival. Arao *et al.* (16) analysed gene expression in a metastatic gastric cancer model treated by ZD6474, an agent inhibiting both VEGFR-2 and epidermal growth factor receptor tyrosine kinase; they found that PLOD2 expression was upregulated 2.4-fold by angiogenesis inhibitor treatment. These reports indicate that PLOD2 is located downstream from HIF-1, and that PLOD2 expression is associated with angiogenesis in malignant tumours. Although the mechanism by which PLOD2 influences the clinicopathological features and prognosis in HCC patients is unclear, our results highlight the importance of PLOD2 in HCC and its significance for further biological investigation in hypoxia and HCC.

PLOD2 functions as a telepeptide lysyl hydroxylase, and the hydroxylation of lysine residues in collagen is thought to lead to the formation and stabilization of hydroxylysine-derived cross-links in fibrosis and accumulation of excessive collagen (11, 28). In this study, we identified no significant correlation between PLOD2 expression and the presence of cirrhosis. In previous reports, lysyl hydroxylase and many other collagen processing enzymes such as lysyl oxidase, prolyl hydroxylase, collagen galactosyltransferase and collagen glucosyltransferase contributed to hepatic fibrosis in mice and rat fibrosis models (29, 30). However, the patients enrolled in this study were ones who underwent surgical resection, and their liver functions were relatively preserved. As a result of the limitations, we could not find the relation between PLOD2 expression and the cirrhosis. Further experiments are needed for analysis of cirrhosis and collagen processing enzymes.

In summary, we demonstrated increased PLOD2 expression under hypoxia in hepatoma cell lines. Furthermore, we showed that the disease-free survival rate of the high PLOD2 expression group was significantly lower than that of the low-expression group. PLOD2 expression may be a novel prognostic factor in HCC patients.

References

1. Farmer DG, Rosove MH, Shaked A, Busuttil RW. Current treatment modalities for hepatocellular carcinoma. *Ann Surg* 1994; **219**: 236–47.
2. Lai EC, Fan ST, Lo CM, Chu KM, Liu CL, Wong J. Hepatic resection for hepatocellular carcinoma. An audit of 343 patients. *Ann Surg* 1995; **221**: 291–8.
3. Ercolani G, Grazi GL, Ravaioli M, *et al.* Liver resection for hepatocellular carcinoma on cirrhosis: univariate and multivariate analysis of risk factors for intrahepatic recurrence. *Ann Surg* 2003; **237**: 536–43.
4. Sasaki Y, Yamada T, Tanaka H, *et al.* Risk of recurrence in a long-term follow-up after surgery in 417 patients with hepatitis B- or hepatitis C-related hepatocellular carcinoma. *Ann Surg* 2006; **244**: 771–80.
5. A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients: the Cancer of the Liver Italian Program (CLIP) investigators. *Hepatology* 1998; **28**: 751–5.
6. Kudo M, Chung H, Osaki Y. Prognostic staging system for hepatocellular carcinoma (CLIP score): its value and limitations, and a proposal for a new staging system, the Japan Integrated Staging Score (JIS score). *J Gastroenterol* 2003; **38**: 207–15.
7. Maxwell PH, Ratcliffe PJ. Oxygen sensors and angiogenesis. *Semin Cell Dev Biol* 2002; **13**: 29–37.
8. Pugh CW, Ratcliffe PJ. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med* 2003; **9**: 677–84.
9. Harris AL. Hypoxia – a key regulatory factor in tumour growth. *Nat Rev Cancer* 2002; **2**: 38–47.
10. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003; **3**: 721–32.
11. van der Slot AJ, Zuurmond AM, Bardoel AF, *et al.* Identification of PLOD2 as telopeptide lysyl hydroxylase, an important enzyme in fibrosis. *J Biol Chem* 2003; **278**: 40967–72.
12. Ruotsalainen H, Vanhatupa S, Tampio M, Sipila L, Valtavaara M, Myllyla R. Complete genomic structure of mouse lysyl hydroxylase 2 and lysyl hydroxylase 3/collagen glucosyltransferase. *Matrix Biol* 2001; **20**: 137–46.
13. Hofbauer KH, Gess B, Lohaus C, Meyer HE, Katschinski D, Kurtz A. Oxygen tension regulates the expression of a group of procollagen hydroxylases. *Eur J Biochem* 2003; **270**: 4515–22.
14. Evens AM, David KA, Helenowski I, *et al.* Multicenter analysis of 80 solid organ transplantation recipients with post-transplantation lymphoproliferative disease: outcomes and prognostic factors in the modern era. *J Clin Oncol* 2010; **28**: 1038–46.
15. Dong S, Nutt CL, Betensky RA, *et al.* Histology-based expression profiling yields novel prognostic markers in human glioblastoma. *J Neuropathol Exp Neurol* 2005; **64**: 948–55.
16. Arao T, Yanagihara K, Takigahira M, *et al.* ZD6474 inhibits tumor growth and intraperitoneal dissemination in a highly metastatic orthotopic gastric cancer model. *Int J Cancer* 2006; **118**: 483–9.

17. Wada H, Nagano H, Yamamoto H, *et al.* Expression pattern of angiogenic factors and prognosis after hepatic resection in hepatocellular carcinoma: importance of angiopoietin-2 and hypoxia-induced factor-1 alpha. *Liver Int* 2006; **26**: 414–23.
18. Uemura M, Yamamoto H, Takemasa I, *et al.* Jumonji domain containing 1A is a novel prognostic marker for colorectal cancer: in vivo identification from hypoxic tumor cells. *Clin Cancer Res* 2010; **16**: 4636–46.
19. Liver Cancer Study Group of Japan. *The General Rules for the Clinical and Pathological Study of Primary Liver Cancer*. Tokyo: Kanehara, 1992.
20. Damdinsuren B, Nagano H, Kondo M, *et al.* TGF-beta1-induced cell growth arrest and partial differentiation is related to the suppression of Id1 in human hepatoma cells. *Oncol Rep* 2006; **15**: 401–8.
21. Ogawa M, Yamamoto H, Nagano H, *et al.* Hepatic expression of ANG2 RNA in metastatic colorectal cancer. *Hepatology* 2004; **39**: 528–39.
22. Kondo M, Nagano H, Wada H, *et al.* Combination of IFN-alpha and 5-fluorouracil induces apoptosis through IFN-alpha/beta receptor in human hepatocellular carcinoma cells. *Clin Cancer Res* 2005; **11**: 1277–86.
23. Noda T, Nagano H, Takemasa I, *et al.* Activation of Wnt/beta-catenin signalling pathway induces chemoresistance to interferon-alpha/5-fluorouracil combination therapy for hepatocellular carcinoma. *Br J Cancer* 2009; **100**: 1647–58.
24. Pasquali M, Still MJ, Vales T, *et al.* Abnormal formation of collagen cross-links in skin fibroblasts cultured from patients with Ehlers-Danlos syndrome type VI. *Proc Assoc Am Physicians* 1997; **109**: 33–41.
25. Uzawa K, Grzesik WJ, Nishiura T, *et al.* Differential expression of human lysyl hydroxylase genes, lysine hydroxylation, and cross-linking of type I collagen during osteoblastic differentiation in vitro. *J Bone Miner Res* 1999; **14**: 1272–80.
26. Wang C, Risteli M, Heikkinen J, Hussa AK, Uitto L, Myllyla R. Identification of amino acids important for the catalytic activity of the collagen glucosyltransferase associated with the multifunctional lysyl hydroxylase 3 (LH3). *J Biol Chem* 2002; **277**: 18568–73.
27. Chang HY, Sneddon JB, Alizadeh AA, *et al.* Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. *PLoS Biol* 2004; **2**: E7.
28. Prockop DJ, Kivirikko KI. Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem* 1995; **64**: 403–34.
29. Siegel RC, Chen KH, Greenspan JS, Aguiar JM. Biochemical and immunochemical study of lysyl oxidase in experimental hepatic fibrosis in the rat. *Proc Natl Acad Sci USA* 1978; **75**: 2945–9.
30. Carter EA, McCarron MJ, Alpert E, Isselbacher KJ. Lysyl oxidase and collagenase in experimental acute and chronic liver injury. *Gastroenterology* 1982; **82**: 526–34.

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/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.

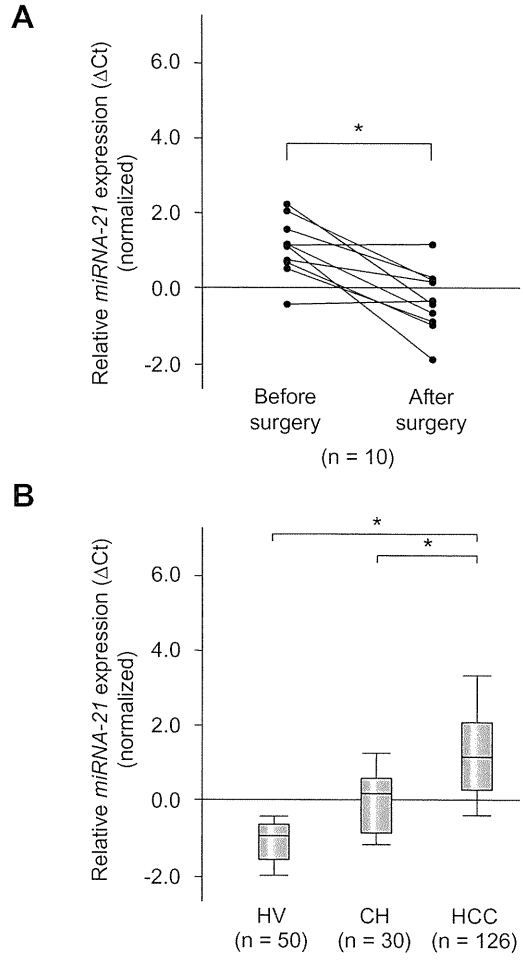

Fig. 1. Plasma *miRNA-21* levels (*ΔCt*). (A) Plasma *miRNA-21* levels (*ΔCt*) before and after curative resection in patients with HCC (n = 10). *p <0.05. (B) Plasma *miRNA-21* levels (*ΔCt*) in patients with HCC (n = 126), CH (n = 30), and HVs (n = 50) measured by qRT-PCR *p <0.05. In this box-and-whisker 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

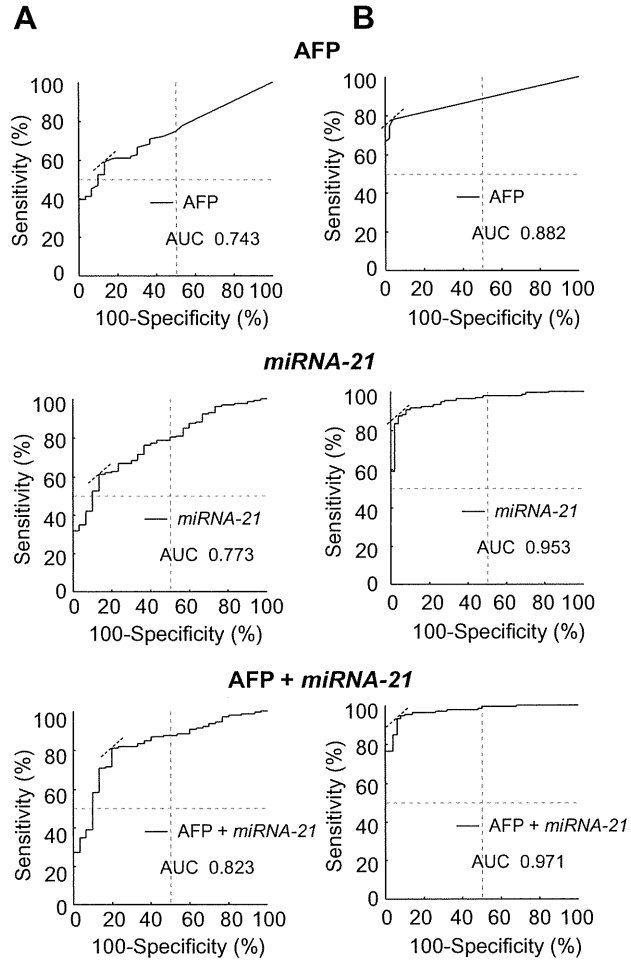

Fig. 2. The diagnostic power of AFP, *miRNA-21*, and the combination of AFP and *miRNA-21* for HCC (n = 126) against CH (n = 30) and HVs (n = 50). (A) Power of AFP, plasma *miRNA-21*, and the combination of AFP and plasma *miRNA-21* in differentiating HCC patients from CH patients. Optimal cutoff values,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

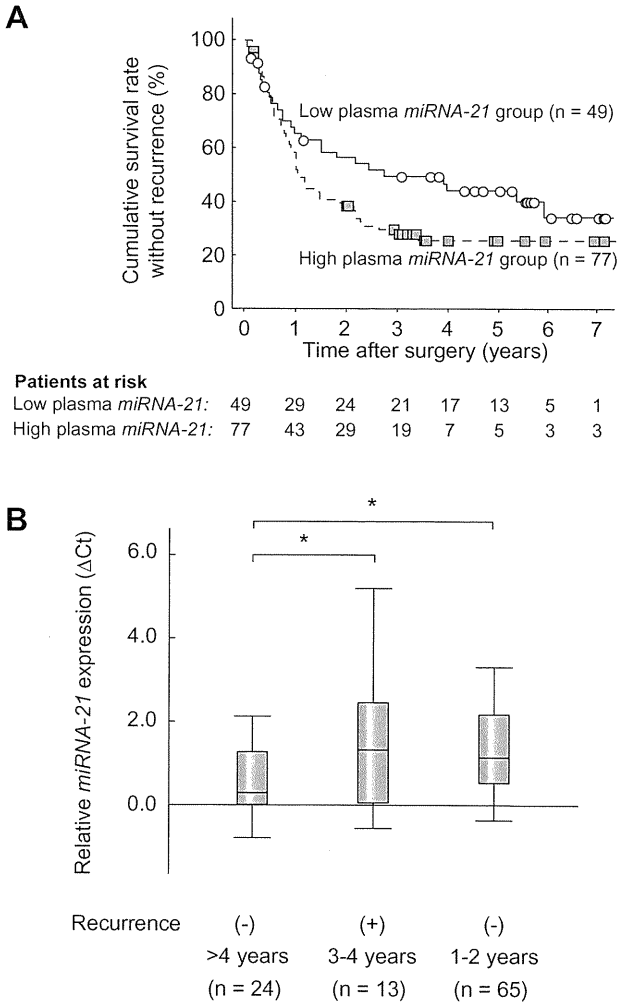

Fig. 3. Correlation between plasma *miRNA-21* expression level and post-operative tumor recurrence. (A) Cumulative survival rate without recurrence after curative surgery for HCC according to plasma *miRNA-21* expression levels. Patients the low plasma *miRNA-21* expression (n = 49) (solid line) (open 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 (x10^4/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.

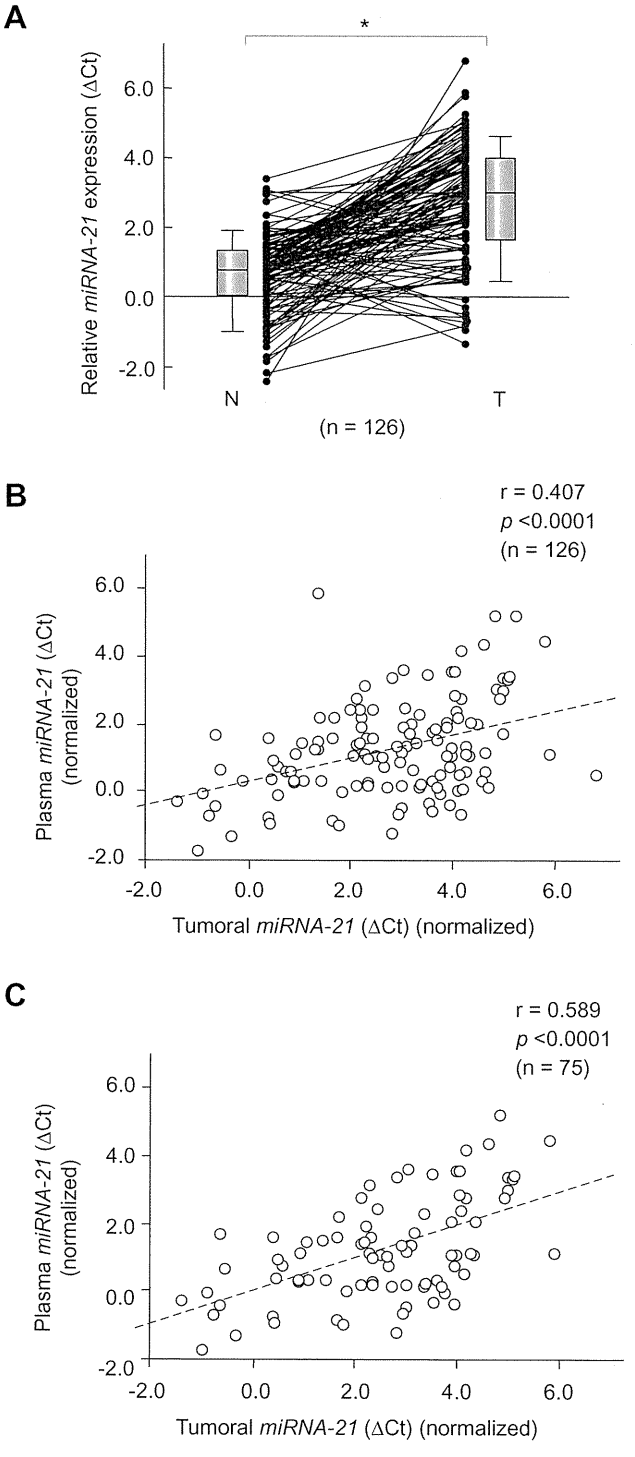

Fig. 4. Expression levels of *miRNA-21* (ΔCt) in tissues determined by qRT-PCR. (A) Tumoral (T) and non-tumoral (N) tissues of patients with HCC (n = 126). $*p < 0.05$. In this box-and-whisker plot, the lines within the boxes represent median values; the upper and lower lines of the boxes represent the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

References

[1] Calin GA, Croce CM. MicroRNA-cancer connection: the beginning of a new tale. Cancer Res 2006;66:7390–7394.
[2] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–297.
[3] Croce CM, Calin GA. MiRNAs, cancer, and stem cell division. Cell 2005;122:6–7.
[4] Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006;6:857–866.
[5] Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci USA 2008;105:10513–10518.
[6] Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, et al. Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 2008;18:997–1006.
[7] Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol 2008;141:672–675.
[8] Wong TS, Liu XB, Wong BY, Ng RW, Yuen AP, Wei WI. Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue. Clin Cancer Res 2008;14:2588–2592.
[9] Tsujiura M, Ichikawa D, Komatsu S, Shiozaki A, Takeshita H, Kosuga T, et al. Circulating microRNAs in plasma of patients with gastric cancers. Br J Cancer 2010;102:1174–1179.
[10] Ng EK, Chong WW, Jin H, Lam EK, Shin VY, Yu J, et al. Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening. Gut 2009;58:1375–1381.
[11] Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X. Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer. Int J Cancer 2010;127:118–126.
[12] Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Newell J, Kerin MJ. Circulating microRNAs as novel minimally invasive biomarkers for breast cancer. Ann Surg 2010;251:499–505.
[13] Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol 2008;10:1470–1476.
[14] Bosch FX, Ribes J, Diaz M, Cleries R. Primary liver cancer: worldwide incidence and trends. Gastroenterology 2004;127 (Suppl 1):S5–S16.
[15] Murakami T, Kim T, Oi H, Nakamura H, Igarashi H, Matsushita M, et al. Detectability of hypervascular hepatocellular carcinoma by arterial phase images of MR and spiral CT. Acta Radiol 1995;36:372–376.
[16] Okuda H, Nakanishi T, Takatsu K, Saito A, Hayashi N, Takasaki K, et al. Serum levels of des-gamma-carboxy prothrombin measured using the revised enzyme immunoassay kit with increased sensitivity in relation to clinicopathologic features of solitary hepatocellular carcinoma. Cancer 2000;88:544–549.
[17] Oka H, Tamori A, Kuroki T, Kobayashi K, Yamamoto S. Prospective study of alpha-fetoprotein in cirrhotic patients monitored for development of hepatocellular carcinoma. Hepatology 1994;19:61–66.
[18] Marrero JA, Lok AS. Newer markers for hepatocellular carcinoma. Gastroenterology 2004;127 (Suppl. 1):S113–119.
[19] Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY. MiR-21-mediated tumor growth. Oncogene 2007;26:2799–2803.
[20] Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834–838.
[21] Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res 2005;65:7065–7070.
[22] Asangani IA, Rasheed SA, Nikolova DA, Leupold JH, Colburn NH, Post S, et al. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 2008;27:2128–2136.

[23] Selaru FM, Olaru AV, Kan T, David S, Cheng Y, Mori Y, et al. MicroRNA-21 is overexpressed in human cholangiocarcinoma and regulates programmed cell death 4 and tissue inhibitor of metalloproteinase 3. Hepatology 2009;49:1595–1601.
[24] Bourguignon LY, Spevak CC, Wong G, Xia W, Gilad E. Hyaluronan-CD44 interaction with protein kinase C(epsilon) promotes oncogenic signaling by the stem cell marker Nanog and the Production of microRNA-21, leading to down-regulation of the tumor suppressor protein PDCD4, anti-apoptosis, and chemotherapy resistance in breast tumor cells. J Biol Chem 2009;284:26533–26546.
[25] Meng F, Henson R, Lang M, Wehbe H, Maheshwari S, Mendell JT, et al. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology 2006;130:2113–2129.
[26] Sempere LF, Christensen M, Silahtaroglu A, Bak M, Heath CV, Schwartz G, et al. Altered MicroRNA expression confined to specific epithelial cell subpopulations in breast cancer. Cancer Res 2007;67:11612–11620.
[27] Lee EJ, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL, et al. Expression profiling identifies microRNA signature in pancreatic cancer. Int J Cancer 2007;120:1046–1054.
[28] Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA 2006;103:2257–2261.
[29] Chan JA, Krichevsky AM, Kosik KS. MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells. Cancer Res 2005;65:6029–6033.
[30] Meng F, Henson R, Wehbe-Janek H, Ghoshal K, Jacob ST, Patel T. MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. Gastroenterology 2007;133:647–658.
[31] Liu C, Yu J, Yu S, Lavker RM, Cai L, Liu W, et al. MicroRNA-21 acts as an oncomir through multiple targets in human hepatocellular carcinoma. J Hepatol 2010;53:98–107.
[32] Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem 2010;285:17442–17452.
[33] Oken MM, Creech RH, Tormey DC, Horton J, Davis TE, McFadden ET, et al. Toxicity and response criteria of the eastern cooperative oncology group. Am J Clin Oncol 1982;5:649–655.
[34] Cortez MA, Calin GA. MicroRNA identification in plasma and serum: a new tool to diagnose and monitor diseases. Expert Opin Biol Ther 2009;9:703–711.
[35] Schmittgen TD, Jiang J, Liu Q, Yang L. A high-throughput method to monitor the expression of microRNA precursors. Nucleic Acids Res 2004;32:e43.
[36] Sobin LHWC. International union against cancer (UICC). 6th ed. New York: Wiley; 2002.
[37] A new prognostic system for hepatocellular carcinoma: a retrospective study of 435 patients: the Cancer of the Liver Italian Program (CLIP) investigators. Hepatology 1998;28:751–755.
[38] Kudo M, Chung H, Osaki Y. Prognostic staging system for hepatocellular carcinoma (CLIP score): its value and limitations, and a proposal for a new staging system, the Japan Integrated Staging Score (JIS score). J Gastroenterol 2003;38:207–215.
[39] Llovet JM, Bru C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. Semin Liver Dis 1999;19:329–338.
[40] Simon R, Radmacher MD, Dobbin K, McShane LM. Pitfalls in the use of DNA microarray data for diagnostic and prognostic classification. J Natl Cancer Inst 2003;95:14–18.
[41] Hanley JA, Hajian-Tilaki KO. Sampling variability of nonparametric estimates of the areas under receiver operating characteristic curves: an update. Acad Radiol 1997;4:49–58.
[42] Dorfman DD, Berbaum KS, Metz CE. Receiver operating characteristic rating analysis. Generalization to the population of readers and patients with the jackknife method. Invest Radiol 1992;27:723–731.
[43] Marquez RT, Wendlandt E, Galle CS, Keck K, McCaffrey AP. MicroRNA-21 is upregulated during the proliferative phase of liver regeneration, targets Pellino-1, and inhibits NF-kappaB signaling. Am J Physiol Gastrointest Liver Physiol 2010;298:G535–G541.
[44] Rabinowits G, Gercel-Taylor C, Day JM, Taylor DD, Kloecker GH. Exosomal microRNA: a diagnostic marker for lung cancer. Clin Lung Cancer 2009;10:42–46.
[45] Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. Gynecol Oncol 2008;110:13–21.
[46] Pegtel DM, Cosmopoulos K, Thorley-Lawson DA, van Eijndhoven MA, Hopmans ES, Lindenberg JL, et al. Functional delivery of viral miRNAs via exosomes. Proc Natl Acad Sci USA 2010;107:6328–6333.

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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Role of the Hypoxia-Related Gene, JMJD1A, in Hepatocellular Carcinoma: Clinical Impact on Recurrence after Hepatic Resection

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ABSTRACT

Background and Aims. Intratumoral hypoxia affects every major aspect of cancer biology, but the relationship between hypoxia-induced genes and hepatocellular carcinoma has not been fully investigated. From a previously ranked microarray of hypoxia-inducible genes related to hepatocellular carcinoma, we focused on a histone H3 lysine 9 demethylase, known as Jumonji domain containing 1A. One function of this demethylase is to amplify hypoxia-inducible gene expression. We hypothesized that the demethylase would be a significant marker of hepatocellular carcinoma.

Methods. We examined Jumonji domain containing 1A expression in 110 hepatocellular carcinoma samples with quantitative real-time polymerase chain reaction and immunohistochemistry. We performed a small interfering RNA suppression analysis to determine the biological roles of the demethylase in proliferation, invasion, and the expression of epithelial–mesenchymal transition-related genes.

Results. The level of Jumonji domain containing 1A in cancer tissues was higher than in normal tissues ($P < 0.0001$). Protein expression was significantly related to gene expression ($P < 0.0001$). Samples with high Jumonji domain containing 1A expression ($n = 47$) had higher recurrence rates ($P = 0.0006$) than those with low expression. Multivariate Cox regression analysis revealed that Jumonji domain containing 1A expression was an independent predictor of recurrence ($P = 0.0016$), but was not significantly associated with any clinicopathological characteristics. Moreover, suppression of Jumonji domain containing 1A expression in hepatocellular carcinoma cell lines under hypoxic conditions reduced cell growth inhibition, reduced invasion ability, and arrested epithelial–mesenchymal transitions.

Conclusion. Jumonji domain containing 1A is a useful prognostic marker and may ameliorate malignant transformation in hepatocellular carcinoma.

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world. HCC causes 500,000 deaths globally each year, and its incidence is increasing worldwide, due to the dissemination of hepatitis B and C virus infections.[1] Advances in surgical techniques and perioperative care have greatly improved the outcome of hepatic resection for HCC.[2–4] Nonetheless, long-term survival after hepatectomy remains unsatisfactory, due to the high incidence of recurrence or metastasis. For these recurrent HCCs, inducing hypoxia by intercepting the hepatic arterial blood flow, i.e., transarterial chemoembolization (TACE), has achieved a pronounced therapeutic effect.[1,5] Although overall survival was prolonged after HCC treatment, success was transient in most patients. In some cases, high incidence of intrahepatic and/or distant metastases was observed after hepatic artery occlusion;[6–9] this suggested that hypoxia-inducible genes may play a role in increasing malignant potency in HCC.

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