Association between ephrin-A1 mRNA expression and poor prognosis after hepatectomy to treat hepatocellular carcinoma

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Abstract. Hypoxia regulates the expression of genes that promote tumor growth, angiogenesis and invasion. We previously studied hypoxic tumor cells *in vitro* and from hepatic metastases of colorectal cancer and determined several potential prognostic factors for hepatocellular carcinoma (HCC). In this study, we evaluated the prognostic impact of the expression of ephrin-A1 (EFNA1) and its receptor, EPHA2, in patients with HCC after curative resection. Samples from a total of 139 HCC patients were analyzed by either microarray alone (n=86) or by microarray and quantitative PCR (n=53). There was no correlation between *EFNA1* expression and clinicopathological factors. *EPHA2* expression was not significantly correlated with any clinicopathological factors, except for microscopic portal invasion. *EFNA1* was an independent prognostic factor for HCC (p=0.0277). These findings suggest that *EFNA1* expression may be a useful marker for predicting high risk of recurrence in patients who have undergone curative resection for HCC.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies and the fifth leading cause of cancer-related death worldwide. Despite recent advances in diagnostic technology and new therapeutic modalities for HCC, the prognosis for patients with advanced-stage HCC is still poor (1). Thus, it is crucial to find novel cancer-related genes that may serve as diagnostic markers and molecular targets in HCC therapy, especially after curative treatment.

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Abbreviations: RT-PCR, reverse transcription PCR

Key words: hepatocellular carcinoma, ephrin-A1, hypoxia, prognosis

Hypoxia is a central feature of solid tumors, and it regulates the expression of a diverse group of genes that promote tumor growth, invasion, angiogenesis and cell survival (2-5). In tumor cells under hypoxic conditions, the hypoxia-inducible factor-1 (HIF-1) pathway is activated and leads to upregulation of many hypoxia-response genes, which are associated with an aggressive tumor phenotype (5-7). We previously reported that these hypoxia-related genes include several angiogenic factors that play important roles in cancer biology (3,8-10). The anti-VEGF antibody bevacizumab is used clinically for treatment of several human cancers (11), and the multi-tyrosine kinase inhibitor sorafenib was shown to have survival benefits for patients with advanced HCC in two phase III clinical trials (12,13). These findings support the use of hypoxia-induced genes as clinically relevant therapeutic targets.

Ephrin-A1 (EFNA1) is known as an angiogenesis factor and is induced through an HIF-1-dependent pathway (14,15). EFNA1 was originally isolated as a secreted protein in conditioned media from cultures of human umbilical vein endothelial cells treated with tumor necrosis factor-α (16,17). Binding of EFNA1 ligand to its receptor EPHA2 promotes autophosphorylation, which triggers downstream signals that regulate cell growth and migration. EFNA1 expression has been observed in tumor cells and in endothelial cells and has been shown to induce endothelial cell migration (18), capillary assembly *in vitro* and corneal angiogenesis *in vivo* (19). EFNA1 and EPHA2 expression is associated with carcinogenesis, angiogenesis (18,20-22), and tumorigenesis in various types of cancer (23-28).

We previously reported that HIF1A expression is correlated with tumor angiogenesis in HCC and that high nuclear expression of HIF-1 is a significant predictive factor for recurrence after curative resection in HCC patients (9). Previously, we detected several potential prognostic factors and therapeutic targets in hypoxic tumor cells from hepatic metastases of CRC *in vivo* (8). Of the 3,000 genes ranked in the microarray data, the top 30 were identified as hypoxia-inducible genes. Among these hypoxia-inducible genes, Jumonji domain containing 1A (*JMJD1A*, also known as *KDM3A*) and procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (*PLOD2*) were novel prognostic factors of HCC (3,10). In these experiments, *EFNA1* expression was highly induced in hypoxic regions of liver metastases. Thus, we hypothesized that *EFNA1* expression

may be a novel prognostic factor in patients with HCC. In the present study, we examined the correlation between *EFNA1* expression and prognosis in HCC patients and analyzed the biological significance of *EFNA1* expression in human HCC.

Materials and methods

Cell culture. The human hepatoma cell lines PLC/PRF/5, HuH7, and HpeG2 were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan), and the Hep3B cell line was obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C in a humidified incubator with 5% CO_2. For hypoxic conditions, cells were maintained in a continuously monitored atmosphere of 1% O_2, 5% CO_2, and 94% N_2 in a multigas incubator (model 9200; Wakenyaku Company, Kyoto, Japan).

Patients and clinical sample collection. A total of 139 HCC patients who underwent hepatectomy at Osaka University Hospital and its associated 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. All patients provided written informed consent to use their surgical specimens and clinicopathological data for research purposes. Clinical staging was based on the TNM classification of the Union for International Cancer Control (UICC), and histological grading was based on World Health Organization classification.

Immediately after surgical resection, a tissue sample was collected from the fresh specimens and stored in RNA Stabilization Reagent (RNA Later; Ambion, Inc., Austin, TX, USA) at -80˚C until RNA extraction.

RNA extraction and real-time quantitative RT-PCR analysis. Total RNA was extracted by a single-step method with TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD, USA) at Osaka University. Complementary DNA (cDNA) was generated by using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI, USA), as described previously (3). Real-time monitoring of PCR reactions was performed with the LightCycler system (Roche Applied Science, Indianapolis, IN, USA) for quantification of mRNA expression, as described previously (29). The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard. The sequences of the GAPDH primers were as follows: sense primer, 5'-CAACTACATGGTTTACATGTTC-3' and antisense primer, 5'-GCCAGTGGACTCCACGAC-3'. *EFNA1* primer sets were designed to flank one intron and were tested to ensure amplification of only cDNA to avoid amplification of possible contaminating genomic DNA. The sequences of these PCR primers were as follows: *EFNA1* sense primer, 5'-TGCC GTCCGGACGAGACAGGC-3' and antisense primer, 5'-CTG GAGCCAGGACCGGGACTG-3'.

Microarray experiment. Microarray results were evaluated in accordance with previously described methods (30). Briefly, total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the instructions supplied by the manufacturer. The integrity of RNA was assessed with 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 samples were mixed and used 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 labeled 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 Company, Yokohama, Japan). The microarrays were scanned using ScanArray Lite, and signal values were calculated using DNASIS array software (Hitachi Software Engineering Company). 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 levels of each gene was converted to a logarithmic scale (base 2), and the data matrix was normalized.

Statistical analysis. For clinicopathological analyses, study samples were divided into high- and low-expression groups based on the median *EFNA1* mRNA expression levels in tumor tissue. All statistical analyses were carried out using the StatView J-5.0 program (Abacus Concepts, Inc., Berkeley, CA), USA. The post-operative period was measured from the date of surgery to the date of the last follow-up or death. Differences were estimated using Fisher's exact probability test. Survival curves were calculated by the Kaplan-Meier method and compared statistically using the log-rank test. To estimate relative risk (RR) and 95% confidence intervals (95% CI), univariate and multivariate analyses were performed using the Cox proportional hazards regression model. Data are reported as mean ± standard deviation. Mean values were compared using the Mann-Whitney test. A probability value of <0.05 was deemed to be statistically significant.

Results

Expression of EFNA1 under hypoxic conditions. First, we evaluated expression of *EFNA1* under hypoxic conditions. *EFNA1* was expressed in all four hepatoma cell lines and gradually increased under hypoxia in HuH7, HepG2 and Hep3B cell lines, but not in PLC/PRF/5 cells (Fig. 1). This result suggests that hypoxic conditions are associated with increased *EFNA1* expression in HCC.

Patient profiles. Next, we evaluated the expression of *EFNA1* in clinical samples by using microarray analysis. The patients selected for microarray analysis included 113 (81.3%) men and 26 (18.7%) women. Twenty-six patients had hepatitis B virus infection, and 85 patients were positive for hepatitis C virus antibody. A total of 102 patients had a single tumor in the liver, and 65 patients had a tumor <3 cm in diameter. Macroscopic vascular invasion was seen in 15 patients. With regard to TNM

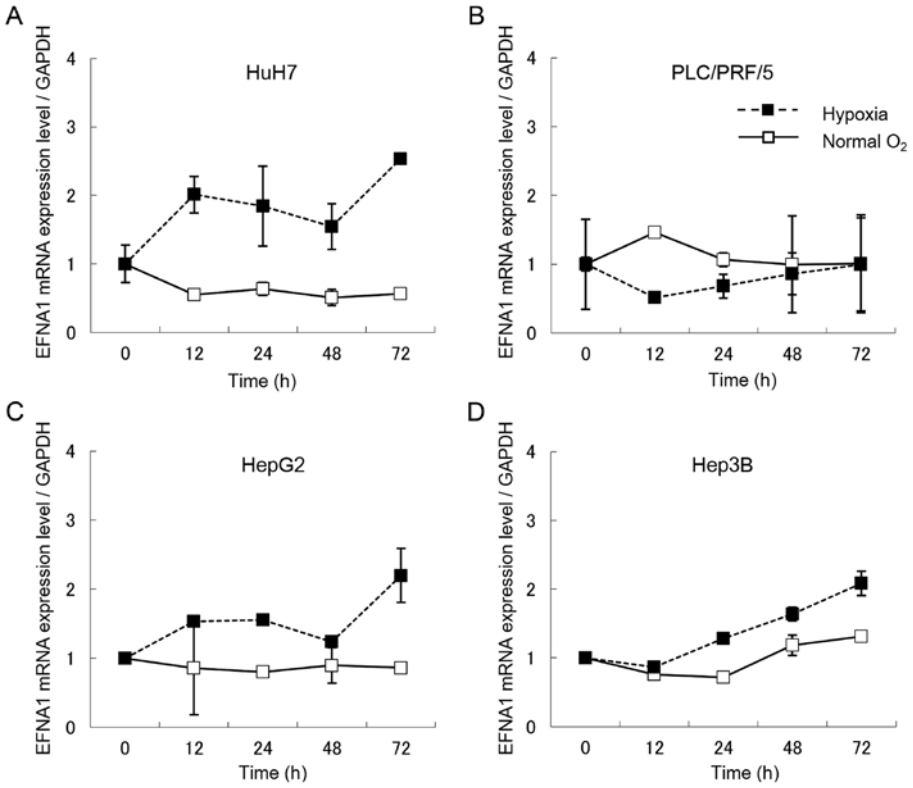

Figure 1. *In vitro* assay to measure *EFNA1* expression under hypoxic conditions in hepatoma cell lines. Comparison of *EFNA1* mRNA expression under hypoxic and normal conditions in (A) HuH7, (B) PLC/PRF/5, (C) HepG2 and (D) Hep3B cell lines.

staging, 96 patients (69.1%) were stage I, 31 patients (22.3%) were stage II, and 12 patients (8.6%) were stage III. The characteristics of the 139 patients are summarized in Table I.

Microarray analysis of EFNA1 mRNA expression. We examined the correlation between expression levels of *EFNA1* and *EPHA2* and the clinicopathological factors of the 139 HCC patients who had undergone hepatic resection. The 139 patients were divided into two groups, a high-expression group (n=70) and a low-expression group (n=69), based on median expression levels from the microarray data for each gene in Table II. There was no correlation between *EFNA1* expression and clinicopathological factors including tumor size, vascular invasion and number of tumors. *EPHA2* expression was not significantly correlated with any clinicopathological factors, except for microscopic portal invasion. Tumors with high expression of *EPHA2* had a tendency to have microscopic vascular invasion, although this result was not statistically significant (p=0.0786) (Tables I and II).

Correlation between EFNA1 and EPHA2 expression levels. We next evaluated the correlation between *EFNA1* and *EphA2* expression levels using microarray data. We found that *EFNA1* expression levels were significantly correlated with those of *EPHA2* (Fig. 2).

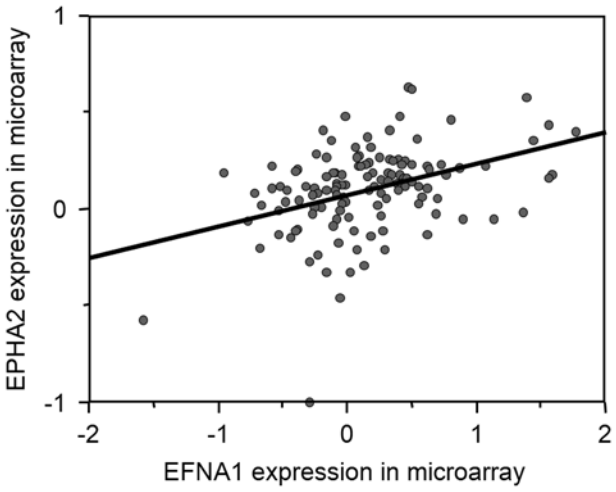

Figure 2. Correlation between *EFNA1* and *EPHA2* expression based on microarray data. The Pearson correlation coefficient was 0.455.

EFNA1 expression measured by quantitative RT-PCR correlated with microarray data. We next examined the correlation between expression data from the microarray and quantitative RT-PCR (qRT-PCR) analysis of *EFNA1* to validate the microarray data. qRT-PCR analysis was performed on 53 HCC tissue samples that were randomly selected from among the 139 HCC tissue specimens. Individual mRNA levels were normalized to GAPDH. In the 53 samples, qRT-PCR data for *EFNA1* were significantly correlated with the results obtained from the microarray data (Fig. 3).

Survival analysis stratified by EFNA1 and EPHA2 mRNA expression. Kaplan-Meier survival curves demonstrated that

Table I. Association between clinicopathological factors and EFNA1 expression.

Characteristics	Low expression (n=69)	High expression (n=70)	p-value
Age (years)			0.9999
<65	31	32	
≥65	38	38	
Gender			0.1271
Male	60	53	
Female	9	17	
HBV infection			0.5150
Present	11	15	
Absent	58	55	
HCV infection			0.9999
Present	42	43	
Absent	27	27	
Child-Pugh grade			0.0761
A	53	62	
B	16	8	
Cirrhosis			0.4955
Absent	41	37	
Present	28	33	
α-fetoprotein (ng/ml)			0.1519
<100	42	51	
≥100	27	19	
PIVKA-II (mAU/ml)			0.2829
<40	26	20	
≥40	43	50	
Tumor size (cm)			0.9999
<3	32	33	
≥3	37	37	
Tumor multiplicity			0.2500
Single	54	48	
Multiple	15	22	
Macroscopic portal invasion			0.1829
Absent	59	65	
Present	10	5	
Stage (TNM)			0.7055
I/II	64	63	
IIIA/IIIB	5	7	
Histological grade			0.2796
Well/moderately	42	40	
Poorly	27	30	
Microscopic portal vein invasion			0.292
Absent	47	41	
Present	22	28	
Microscopic intrahepatic metastasis			0.8469
Absent	51	53	
Present	18	17	

HBV, hepatitis B virus; HCV, hepatitis C virus; PIVKA-II, protein induced by vitamin K absence or antagonist II; well/moderately, well or moderately differentiated hepatocellular carcinoma; poorly, poorly differentiated hepatocellular carcinoma.

Table II. Association between clinicopathological factors and EphA2 expression.

Characteristics	Low expression (n=69)	High expression (n=70)	p-value
Age (years)			0.2349
<65	35	34	
≥65	34	42	
Gender			0.8283
Male	57	56	
Female	12	14	
HBV infection			0.6689
Present	14	12	
Absent	55	58	
HCV infection			0.999
Present	42	43	
Absent	27	27	
Child-Pugh grade			0.6596
A	56	59	
B	13	11	
Cirrhosis			0.8650
Absent	38	40	
Present	31	30	
α-fetoprotein (ng/ml)			0.2829
<100	43	50	
≥100	26	20	
PIVKA-II (mAU/ml)			0.1519
<40	27	19	
≥40	42	51	
Tumor size (cm)			0.8656
<3	33	32	
≥3	36	38	
Tumor multiplicity			0.2500
Single	54	48	
Multiple	15	22	
Macroscopic portal invasion			0.9999
Absent	62	62	
Present	7	8	
Stage (TNM)			0.3472
I/II	65	62	
IIIA/IIIB	4	8	
Histological grade			0.3309
Well/moderately	45	37	
Poorly	24	33	
Microscopic portal vein invasion			0.0786
Absent	49	39	
Present	20	31	
Microscopic intrahepatic metastasis			0.4353
Absent	54	50	
Present	15	20	

HBV, hepatitis B virus; HCV, hepatitis C virus; PIVKA-II, protein induced by vitamin K absence or antagonist II; well/moderately, well or moderately differentiated hepatocellular carcinoma; poorly, poorly differentiated hepatocellular carcinoma.

Table III. Univariate analysis of disease-free survival.

Characteristics	n	Hazard ratio	p-value
Age (years)			0.2987
<65	63	Ref.	
≥65	76	1.239	
Gender			0.3658
Male	113	Ref.	
Female	26	0.785	
HBV infection			0.3692
Present	26	Ref.	
Absent	113	0.798	
HCV infection			0.2871
Present	85	Ref.	
Absent	54	1.244	
Child-Pugh grade			0.5886
A	115	Ref.	
B	24	0.8681	
Cirrhosis			0.3820
Absent	78	Ref.	
Present	61	1.194	
α-fetoprotein (ng/ml)			0.1128
<100	93	Ref.	
≥100	46	1.395	
PIVKA-II (mAU/ml)			0.4261
<40	46	Ref.	
≥40	93	1.193	
Tumor size (cm)			0.5323
<3	65	Ref.	
≥3	74	0.881	
Tumor multiplicity			0.0143
Single	102	Ref.	
Multiple	37	1.736	
Macroscopic portal invasion			0.2075
Absent	124	Ref.	
Present	15	1.477	
Stage (TNM)			0.3271
I/II	127	Ref.	
IIIA/IIIB	12	1.410	
Histological grade			0.1678
Well/moderately	82	Ref.	
Poorly	57	1.321	
Microscopic portal vein invasion			0.0042
Absent	88	Ref.	
Present	51	1.801	
Microscopic intrahepatic metastasis			0.0007
Absent	104	Ref.	
Present	35	2.185	
EFNA1			0.0113
Low expression	69	Ref.	
High expression	70	1.701	
EPHA2			0.4044
Low expression	69	Ref.	
High expression	70	1.185	

Ref., reference; HBV, hepatitis B virus; HCV, hepatitis C virus; PIVKA-II, proteininduced by vitamin K absence or antagonist II; well/moderately, well or moderately differentiated hepatocellular carcinoma; poorly, poorly differentiated hepatocellular carcinoma.

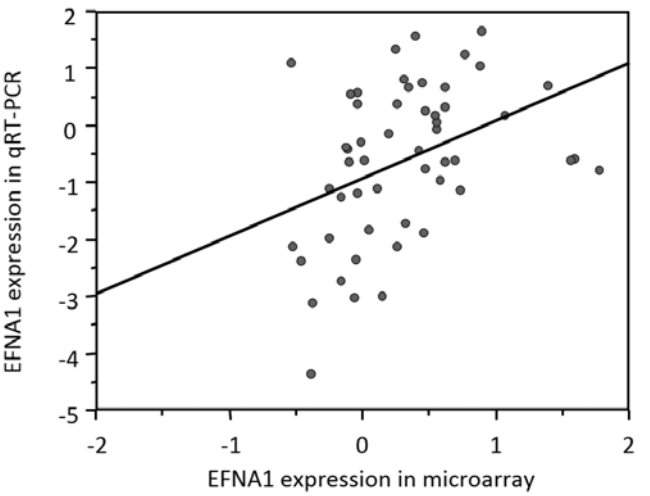

Figure 3. Correlation of *EFNA1* expression according to microarray data and quantitative reverse transcription PCR (qRT-PCR). qRT-PCR data were significantly correlated with the microarray data in all 53 of the hepatocellular carcinoma samples that were tested.

patients with high *EFNA1* expression had significantly shorter disease-free survival (DFS) than those with low *EFNA1* expression based on both microarray (Fig. 4A) and qRT-PCR (Fig. 4B) data. *EPHA2* expression was not correlated with the prognosis for HCC after curative resection (Fig. 4C). Univariate analysis for survival revealed that tumor number, microscopic vascular invasion, microscopic intrahepatic metastasis, and *EFNA1* expression were significantly associated with DFS based on microarray data (Table III). Multivariate Cox regression analysis clarified that only *EFNA1* expression remained an independent prognostic factor (Table IV).

Discussion

EFNA1 expression was previously reported to be associated with prognosis in early squamous cell cervical carcinoma (31) and colorectal cancer (32). However, the prognostic impact of *EFNA1* in HCC patients remains unknown. The present study

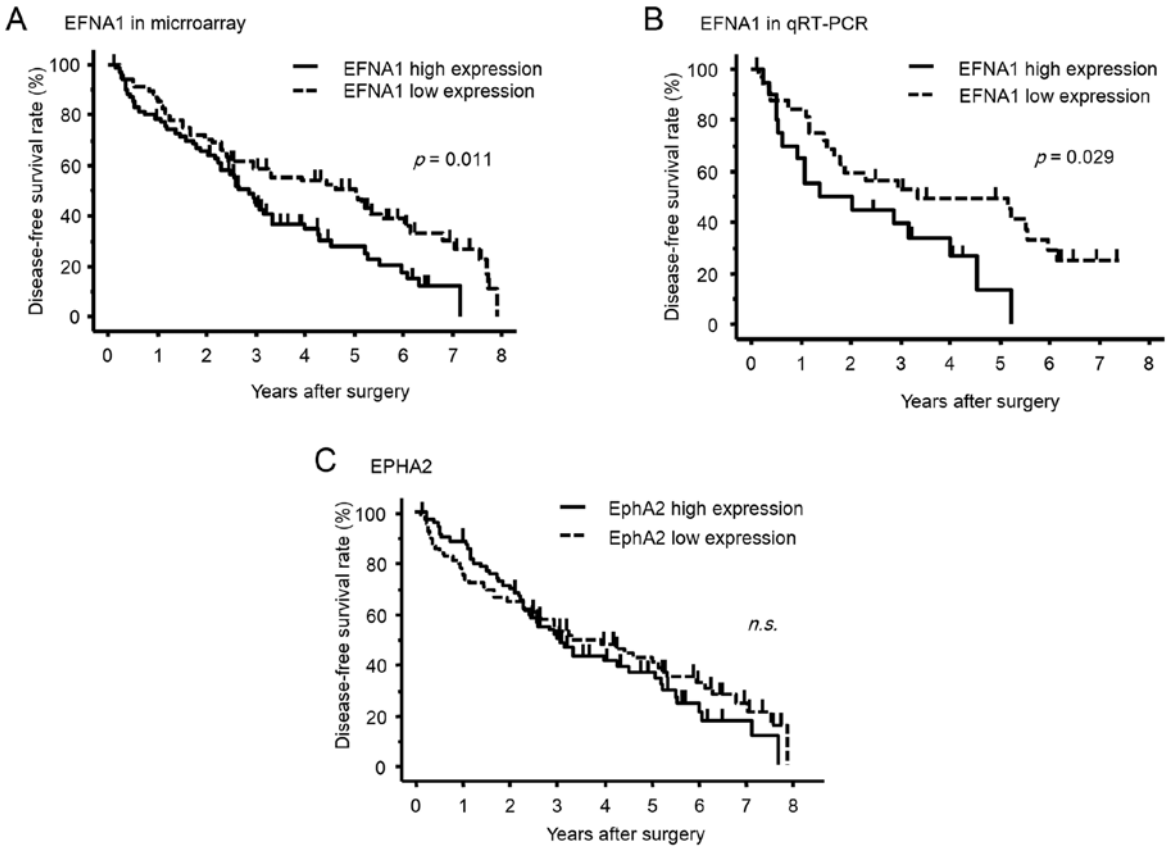

Figure 4. Kaplan-Meier disease-free survival curves for hepatocellular carcinoma patients according to *EFNA1* and *EPHA2* mRNA expression. Patients in the high *EFNA1* mRNA expression group had poorer disease-free survival than those in the low expression group, as shown by both (A) microarray and (C) qRT-PCR analysis. Differences in disease-free survival curves were estimated with the log-rank test for (A) microarray analysis (p=0.011) and (B) qRT-PCR analysis (p=0.029).

Table IV. Multivariate analysis of disease-free survival.

Characteristics	n	Hazard ratio	95% CI	p-value
Tumor multiplicity				0.8701
Single	102	Ref.		
Multiple	37	1.053	0.565-1.965	
Microscopic portal invasion				0.0574
Absent	88	Ref.		
Present	51	1.505	0.987-2.295	
Microscopic intrahepatic metastasis				0.0611
Absent	104	Ref.		
Present	35	1.848	0.972-3.516	
EFNA1 expression				0.0277
Low expression	69	Ref.		
High expression	70	1.605	1.054-2.451	

95% CI, 95% confidence interval; ref., reference.

evaluated the correlation between *EFNA1* mRNA expression levels and prognosis in patients with HCC by microarray analysis of 139 HCC samples and qRT-PCR analysis of 53 samples. The most important finding was that patients with high *EFNA1* expression had a poorer prognosis than those with low *EFNA1* expression. Furthermore, multivariate analysis demonstrated that *EFNA1* expression was an independent prognostic factor for HCC.

HCC is generally known to occur as a hypervascular tumor, but the rapid proliferation of tumor cells continuously

induces local hypoxia in advanced stages. Angiogenesis is an essential process in carcinogenesis and progression, and several angiogenic factors play important roles in HCC. We previously reported that the expression of vascular endothelial growth factor (VEGF) and angiopoietin-2 is associated with microvascular density in HCC. We also found that high nuclear expression of HIF1A is a significant predictive factor for recurrence after curative resection in HCC patients (9). HIF1A is one of the key transcription factors induced by hypoxic conditions. In the absence of oxygen, it binds to hypoxia-response elements, which activates the expression of numerous hypoxia-response genes, such as VEGF, glucose transporter-1, erythropoietin and *EFNA1* (33).

Two previous studies evaluated the association between expression of *EFNA1* and clinical features in patients with HCC. One report revealed that expression of *EFNA1* and *AFP* was strongly associated and that they induced the expression of genes related to the cell cycle, angiogenesis and cell-cell interactions (34). The other report showed that *EFNA1* mRNA was overexpressed in 90% of HCC cells and *EPHA2* expression was significantly correlated with poor survival in HCC patients (35). Both reports indicated that EFNA1 and its receptor, EphA2, promote proliferation and invasiveness in HCC.

Our result show that *EFNA1* mRNA expression was significantly associated with *EPHA2* mRNA expression, based on microarray data. Moreover, *EFNA1* was a novel independent prognostic factor for HCC. However, *EPHA2* was not a significant prognostic factor. EPHA2 is a transmembrane receptor tyrosine kinase that is frequently overexpressed in various cancers and is stimulated and phosphorylated by EFNA1 (36-38). Overexpression of EPHA2 is associated with aggressive phenotypes and decreased differentiation (37,39). Our results also show *EPHA2* expression tends to correlate with microscopic portal invasion. However, there was no association between EPHA2 and prognosis in HCC.

In the present study, we used tissue microarrays to analyze not only tumor cells, but also many vascular endothelial cells. EFNA1 ligand and its receptor, EPHA2, were expressed and upregulated in both tumor cells and tumor vessels. In line with the results of the present study, hypoxic conditions are known to upregulate the expression of EFNA1 in hepatoma cells *in vitro* (34,35). We previously reported that silencing of *EFNA1* in tumor cells inhibits the migration, invasion and proliferation of tumor cells themselves and also inhibits the migration of endothelial cells in coculture experiment (32). Thus, EFNA-mediated interactions between the endothelium and surrounding cells may be critical for vascular sprouting and the penetration of vessels into tumor tissues.

In conclusion, the present findings strongly suggest that *EFNA1* expression is a useful marker for predicting a high risk of recurrence in HCC patients who have undergone curative resection. Anticancer treatments that target EFNA1 and EPHA2 may be particularly effective, because they could both suppress tumor neovascularization and directly affect tumor cells. It will be critical to the development of novel anticancer therapies to distinguish the effects of inhibiting EFNA1/EPHA2 activity on tumor vasculature versus tumor cells.

References

1. Llovet JM, Burroughs A and Bruix J: Hepatocellular carcinoma. Lancet 362: 1907-1917, 2003.
2. Semenza GL: Hypoxia and cancer. Cancer Metastasis Rev 26: 223-224, 2007.
3. Noda T, Yamamoto H, Takemasa I, *et al*: PLOD2 induced under hypoxia is a novel prognostic factor for hepatocellular carcinoma after curative resection. Liver Int 32: 110-118, 2012.
4. Maxwell PH and Ratcliffe PJ: Oxygen sensors and angiogenesis. Semin Cell Dev Biol 13: 29-37, 2002.
5. Pugh CW and Ratcliffe PJ: Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 9: 677-684, 2003.
6. Semenza GL: Targeting HIF-1 for cancer therapy. Nat Rev Cancer 3: 721-732, 2003.
7. Zhong H, De Marzo AM, Laughner E, *et al*: Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 59: 5830-5835, 1999.
8. 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 16: 4636-4646, 2010.
9. 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 26: 414-423, 2006.
10. Yamada D, Kobayashi S, Yamamoto H, *et al*: Role of the hypoxia-related gene, JMJD1A, in hepatocellular carcinoma: clinical impact on recurrence after hepatic resection. Ann Surg Oncol 19 (Suppl 3): S355-S364, 2012.
11. Hurwitz H, Fehrenbacher L, Novotny W, *et al*: Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med 350: 2335-2342, 2004.
12. Cheng AL, Kang YK, Chen Z, *et al*: Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: a phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 10: 25-34, 2009.
13. Llovet JM, Ricci S, Mazzaferro V, *et al*: Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 359: 378-390, 2008.
14. Vihanto MM, Plock J, Erni D, Frey BM, Frey FJ and Huynh-Do U: Hypoxia up-regulates expression of Eph receptors and ephrins in mouse skin. FASEB J 19: 1689-1691, 2005.
15. Yamashita T, Ohneda K, Nagano M, *et al*: Hypoxia-inducible transcription factor-2alpha in endothelial cells regulates tumor neovascularization through activation of ephrin A1. J Biol Chem 283: 18926-18936, 2008.
16. Bartley TD, Hunt RW, Welcher AA, *et al*: B61 is a ligand for the ECK receptor protein-tyrosine kinase. Nature 368: 558-560, 1994.
17. Holzman LB, Marks RM and Dixit VM: A novel immediate-early response gene of endothelium is induced by cytokines and encodes a secreted protein. Mol Cell Biol 10: 5830-5838, 1990.
18. Brantley DM, Cheng N, Thompson EJ, *et al*: Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo. Oncogene 21: 7011-7026, 2002.
19. Pandey A, Shao H, Marks RM, Polverini PJ and Dixit VM: Role of B61, the ligand for the Eck receptor tyrosine kinase, in TNF-alpha-induced angiogenesis. Science 268: 567-569, 1995.
20. Cheng N, Brantley DM, Liu H, *et al*: Blockade of EphA receptor tyrosine kinase activation inhibits vascular endothelial cell growth factor-induced angiogenesis. Mol Cancer Res 1: 2-11, 2002.
21. Deroanne C, Vouret-Craviari V, Wang B and Pouyssegur J: EphrinA1 inactivates integrin-mediated vascular smooth muscle cell spreading via the Rac/PAK pathway. J Cell Sci 116: 1367-1376, 2003.
22. Ogawa K, Pasqualini R, Lindberg RA, Kain R, Freeman AL and Pasquale EB: The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. Oncogene 19: 6043-6052, 2000.
23. Abraham S, Knapp DW, Cheng L, *et al*: Expression of EphA2 and Ephrin A-1 in carcinoma of the urinary bladder. Clin Cancer Res 12: 353-360, 2006.
24. Brantley-Sieders DM, Fang WB, Hwang Y, Hicks D and Chen J: Ephrin-A1 facilitates mammary tumor metastasis through an angiogenesis-dependent mechanism mediated by EphA receptor and vascular endothelial growth factor in mice. Cancer Res 66: 10315-10324, 2006.

25. Liu DP, Wang Y, Koeffler HP and Xie D: Ephrin-A1 is a negative regulator in glioma through downregulation of EphA2 and FAK. Int J Oncol 30: 865-871, 2007.
26. Nakamura R, Kataoka H, Sato N, *et al*: EPHA2/EFNA1 expression in human gastric cancer. Cancer Sci 96: 42-47, 2005.
27. Nasreen N, Mohammed KA, Lai Y and Antony VB: Receptor EphA2 activation with ephrinA1 suppresses growth of malignant mesothelioma (MM). Cancer Lett 258: 215-222, 2007.
28. Noblitt LW, Bangari DS, Shukla S, *et al*: Decreased tumorigenic potential of EphA2-overexpressing breast cancer cells following treatment with adenoviral vectors that express Ephrin-A1. Cancer Gene Ther 11: 757-766, 2004.
29. Yamamoto H, Kondo M, Nakamori S, *et al*: JTE-522, a cyclooxygenase-2 inhibitor, is an effective chemopreventive agent against rat experimental liver fibrosis1. Gastroenterology 125: 556-571, 2003.
30. Yoshioka S, Takemasa I, Nagano H, *et al*: Molecular prediction of early recurrence after resection of hepatocellular carcinoma. Eur J Cancer 45: 881-889, 2009.
31. Holm R, de Putte GV, Suo Z, Lie AK and Kristensen GB: Expressions of EphA2 and EphrinA-1 in early squamous cell cervical carcinomas and their relation to prognosis. Int J Med Sci 5: 121-126, 2008.
32. Yamamoto H, Tei M, Uemura M, *et al*: Ephrin-A1 mRNA is associated with poor prognosis of colorectal cancer. Int J Oncol 42: 549-555, 2013.
33. Harris AL: Hypoxia - a key regulatory factor in tumour growth. Nat Rev Cancer 2: 38-47, 2002.
34. Iida H, Honda M, Kawai HF, *et al*: Ephrin-A1 expression contributes to the malignant characteristics of {alpha}-fetoprotein producing hepatocellular carcinoma. Gut 54: 843-851, 2005.
35. Cui XD, Lee MJ, Yu GR, *et al*: EFNA1 ligand and its receptor EphA2: potential biomarkers for hepatocellular carcinoma. Int J Cancer 126: 940-949, 2010.
36. Miyazaki T, Kato H, Fukuchi M, Nakajima M and Kuwano H: EphA2 overexpression correlates with poor prognosis in esophageal squamous cell carcinoma. Int J Cancer 103: 657-663, 2003.
37. Zelinski DP, Zantek ND, Stewart JC, Irizarry AR and Kinch MS: EphA2 overexpression causes tumorigenesis of mammary epithelial cells. Cancer Res 61: 2301-2306, 2001.
38. Zeng G, Hu Z, Kinch MS, *et al*: High-level expression of EphA2 receptor tyrosine kinase in prostatic intraepithelial neoplasia. Am J Pathol 163: 2271-2276, 2003.
39. Walker-Daniels J, Riese DJ, 2nd and Kinch MS: c-Cbl-dependent EphA2 protein degradation is induced by ligand binding. Mol Cancer Res 1: 79-87, 2002.