Lymphatic vessel endothelial hyaluronan receptor-1 is a novel prognostic indicator for human hepatocellular carcinoma

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Abstract. Angiogenesis is an important mechanism of tumor development, growth and metastasis in hepatocellular carcinoma (HCC). The poor prognosis of HCC patients has been associated with a failure to detect recurrences following surgery. In the present study, we investigated the association between the patient characteristics and the expression of angiogenic genes to identify early biomarkers of HCC. A comprehensive angiogenic gene expression profile was obtained by paired TaqMan gene array analysis of primary HCC nodules and adjacent non-HCC liver tissue from 12 patients. A total of 14 genes were found to be differentially expressed in HCC liver nodules (>2-fold change); the genes encoding collagen type XVα1, IVα1 and IVα2 were upregulated and the genes associated with vessel growth, neuropilin 2 (*NRP2*) and lymphatic vessel endothelial hyaluronan receptor-1 (*LYVE-1*) were downregulated. The histopathological analysis revealed that the evolution of HCC nodules from well to poorly differentiated was associated with a 5-fold decrease in *LYVE-1* expression, reaching its lowest level early during the transition. The significance of this gene as a biomarker of postoperative survival was demonstrated by a 2-fold decrease in overall survival (OS) rates in the low expression group compared to the high expression group. The multivariate and univariate Cox regression analyses identified *LYVE-1* expression as a significant independent prognostic parameter of OS [hazard ratio (HR)=3.067; 95% confidence interval (CI): 1.507-6.273; P=0.0021]. Thus, the results of this study suggested that *LYVE-1* expression may constitute a novel early biomarker of postoperative survival in HCC patients.

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Introduction

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer worldwide (1). The median survival time of patients with unresectable tumors and untreated patients with less advanced disease is <4 months and <1 year, respectively (2-6). The total survival rate of HCC patients is 3-5% (7), due to the high rate of recurrence following resection and the resistance to chemotherapy.

This type of cancer is particularly aggressive as a result of its high degree of vascularization. Multiple angiogenic and anti-angiogenic factors released by the tumor and host cells are involved in this process (8). The microvascular density of HCCs correlates with disease prognosis and postoperative disease recurrence (9-12). Angiogenesis, the formation of new blood vessels from preexisting vasculature, is crucial in the development, growth and metastasis of various neoplasms, including HCCs (13,14). Although angiogenesis constitutes a promising avenue for the identification of markers and novel therapeutic approaches, the ramifications of the signaling pathways are complex and have not yet been fully elucidated, particularly with respect to vascularization.

This study aimed to identify angiogenic genes that are deregulated by HCC and determine their potential as predictors of postoperative survival. Liver tissue samples and nodules from three groups of HCC patients were used to perform TaqMan gene array analysis and to identify the most promising biomarker of HCC in terms of patient characteristics, survival rates and tissue histology.

Materials and methods

Paired analysis of angiogenic gene expression in HCC nodules and non-HCC liver tissue. A preliminary experiment was conducted, using tissue samples from 12 HCC patients to identify the affected angiogenesis-related target genes to be investigated in this study. All the patients were Japanese and they had undergone surgical HCC resection between October, 2008 and October, 2009 at the Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, Japan. The majority of the patients were male, with moderately differentiated HCC histology and negative for intrahepatic metastases (IM), portal vein invasion (Vp)

Table I. Characteristics of the 12 HCC patients who provided liver samples for the identification of angiogenic genes deregulated by HCC.

Characteristics	Frequency	Percentage
Age (years)		
Mean (range)	12 (51-81)	-
Gender		
Male	10	83
Female	2	17
Tumor size (cm)		
Mean (range)	2.4 (1.5-4.2)	-
Histology		
Well differentiated	1	8
Moderately differentiated	11	92
IM		
Positive	2	17
Negative	10	83
Vp		
Positive	2	17
Negative	10	83
Vv		
Negative	12	100
Macroscopic findings		
SNIM	2	17
SN	6	50
SNEG	4	33
Child-Pugh classification		
A	12	100
Liver status		
Cirrhosis	6	50
Chronic hepatitis	5	42
Normal	1	8
Infection		
HBV	3	25
HCV	7	58
HCV+HBV	1	8
Negative	1	8

HCC, hepatocellular carcinoma; IM, intrahepatic metastasis; Vp, portal vein invasion; Vv, venous invasion; SNIM, small nodular type with indistinct margin; SN, simple nodular type; SNEG, simple nodular type with extranodular growth; HBV, hepatitis B virus; HCV, hepatitis C virus.

or venous invasion (Vv). Half of the patients had liver cirrhosis or chronic hepatitis resulting from viral infection (Table I). The patients provided written informed consent according to the institutional regulations. This study was approved by the Ethics Committee and Institutional Review Board of the Tokyo Women's Medical University.

The tissue samples collected from primary HCC nodules and non-HCC liver tissue of each patient were immediately snap-frozen and stored at -80˚C until further use. The samples were then homogenized and total RNA was isolated using the RNeasy® Mini kit (Qiagen, Valencia, CA, USA). Subsequently, complementary DNA (cDNA) was synthesized using 2 μg of total RNA and High Capacity RNA-to-cDNA Master Mix (Applied Biosystems Inc., Foster City, CA, USA) according to the manufacturer's protocol. We used the TaqMan® Array Gene Expression 96-well Human Angiogenesis Plate (Applied Biosystems Inc.) to determine the angiogenic gene profiles of the specimens in each sample set. A total of 92 angiogenesis- or lymphangiogenesis-associated gene assays and 4 control endogenous gene assays were performed in each plate. The target genes investigated in this study are listed in Table II. The gene expression level was analyzed using a 7500 Real-Time PCR system (Applied Biosystems Inc.). Polymerase chain reaction (PCR) using TaqMan® Gene Expression Master Mix (Applied Biosystems Inc.) was performed under the following conditions: 2 min at 50˚C, 10 min at 95˚C, followed by 40 cycles of 30 sec at 95˚C and 1 min at 60˚C. Data were analyzed using SDS software, version 1.4 (Applied Biosystems Inc.) and gene expression levels were compared using the $\Delta\Delta$Ct method (15). Significantly upregulated or downregulated genes were screened using a cut-off P-value of <0.01.

Paired analysis of lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1) expression in HCC nodules and non-HCC liver tissue. Archived liver tissue samples (primary HCC tumors; >95% HCC cells and non-HCC tissue from the same patient) from HCC patients were tested for *LYVE-1* expression. The 58 complete sets were obtained from Japanese patients who had undergone surgical HCC resection between December, 1993 and May, 2007 at the Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, Japan. Similar to the 12-patient group, the archived samples were collected primarily from males with moderately differentiated HCC histology and cirrhosis or chronic hepatitis resulting from viral infection (Table III). The patients provided written informed consent in accordance with institutional regulations.

The formalin-fixed paraffin-embedded (FFPE) samples were preserved using the general protocol of the Institute of Pathology, Tokyo Women's Medical University, Japan. Each FFPE specimen was cut into 10-μm sections, deparaffinized in xylene and rehydrated in graded ethanols. The tissues were dissected and total RNA was isolated using the RNeasy® FFPE kit (Qiagen). Subsequently, cDNA was synthesized using High-Capacity cDNA Reverse Transcription kits (Applied Biosystems Inc.) with 1 μg of total RNA, according to the manufacturer's protocol. The expression of *LYVE-1* and β-2 microglobulin (*B2M*), which was used as endogenous control, were measured using a StepOne™ Real-Time PCR system (Applied Biosystems Inc.). The TaqMan® primers/probe for *LYVE-1* (Assay ID: Hs00272659_m1) and *B2M* (Assay ID: Hs99999907_m1) were purchased from TaqMan® Gene Expression Assays (Applied Biosystems Inc.). PCR was performed using TaqMan® Fast Master Mix under the following conditions: 20 sec at 95˚C, followed by 40 cycles of 1 sec at 95˚C and 20 sec at 60˚C. Data were analyzed using StepOne™ software, version 2.1 and the gene expression level was quantified by the $\Delta\Delta$Ct method.

Table II. List of the angiogenic genes included in the gene array plate[a].

Gene symbol	Assay ID
18S	Hs99999901_s1
GAPDH	Hs99999905_m1
HPRT1	Hs99999909_m1
GUSB	Hs99999908_m1
FGA	Hs00241027_m1
PLG	Hs00264877_m1
CXCL12	Hs00171022_m1
EDIL3	Hs00174781_m1
EPHB2	Hs00362096_m1
FGF1	Hs00265254_m1
FGF2	Hs00266645_m1
FGF4	Hs00173564_m1
PDGFB	Hs00234042_m1
PTN	Hs00383235_m1
PROK1	Hs00260905_m1
TGFA	Hs00608187_m1
TGFB1	Hs99999918_m1
TNF	Hs00174128_m1
TNFSF15	Hs00270802_s1
ITGA4	Hs00168433_m1
IFNB1	Hs01077958_s1
IFNG	Hs00174143_m1
CXCL10	Hs00171042_m1
IL12A	Hs00168405_m1
CD44	Hs00153304_m1
CDH5	Hs00174344_m1
CXCL2	Hs00601975_m1
SERPINB5	Hs00184728_m1
FLT1	Hs00176573_m1
SEMA3F	Hs00188273_m1
ANGPTL3	Hs00205581_m1
CEACAM1	Hs00236077_m1
HEY1	Hs00232618_m1
ITGAV	Hs00233808_m1
PECAM1	Hs00169777_m1
LYVE-1	Hs00272659_m1
FOXC2	Hs00270951_s1
COL4A1	Hs00266237_m1
COL4A2	Hs01098873_m1
COL15A1	Hs00266332_m1
HSPG2	Hs00194179_m1
COL18A1	Hs00181017_m1
CSF3	Hs99999083_m1
GRN	Hs00963711_g1
THBS2	Hs01568063_m1
LECT1	Hs00993254_m1
ANGPTL4	Hs01101127_m1
ITGB3	Hs01001469_m1
SERPINC1	Hs00166654_m1
PRL	Hs00168730_m1
MMP2	Hs00234422_m1
ANG, RNASE4	Hs02379000_s1
ANGPT1	Hs00181613_m1
ANGPT2	Hs00169867_m1
FST	Hs00246256_m1
HGF	Hs00300159_m1
IL8	Hs00174103_m1
LEP	Hs00174877_m1
MDK	Hs00171064_m1
TYMP	Hs00157317_m1
VEGFA	Hs00900054_m1
VEGFB	Hs00173634_m1
VEGFC	Hs00153458_m1
CTGF	Hs00170014_m1
FBLN5	Hs00197064_m1
THBS1	Hs00962914_m1
SERPINF1	Hs00171467_m1
PF4	Hs00427220_g1
VASH1	Hs00208609_m1
ADAMTS1	Hs00199608_m1
ANGPTL1	Hs00559786_m1
AMOT	Hs00611096_m1
TEK	Hs00176096_m1
TIE1	Hs00178500_m1
TNMD	Hs00223332_m1
TIMP2	Hs00234278_m1
TIMP3	Hs00165949_m1
ANGPTL2	Hs00765775_m1
KIT	Hs00174029_m1
TNNI1	Hs00913333_m1
NRP2	Hs00187290_m1
KDR	Hs00176676_m1
ENPP2	Hs00196470_m1
FIGF	Hs00189521_m1
FN1	Hs01549940_m1
COL4A3	Hs01022527_m1
F2	Hs01011995_g1
BAI1	Hs01105174_m1
CHGA	Hs00900373_m1
ANGPT4	Hs00211115_m1
PDGFRA	Hs00998026_m1
PDGFRB	Hs00387364_m1
FLT4	Hs01047677_m1
NRP1	Hs00826128_m1
S1PR1	Hs01922614_s1
PROX1	Hs00896294_m1

[a]The table presents the gene symbol and assay ID associated with each well.

Table III. Characteristics of the 58 HCC patients investigated for the histology of HCC nodules and non-HCC liver tissue and survival curves.

Characteristics	Frequency	Percentage
Age (years)		
Mean (range)	63 (39-81)	-
Gender		
Male	45	77
Female	13	23
Histology		
Well differentiated	7	12
Moderately differentiated	44	73
Poorly differentiated	9	15
Child-Pugh classification		
A	53	88
B	7	12
Liver status		
Cirrhosis	23	38
Chronic hepatitis	35	58
Normal	2	3
Viral infection		
HBV	17	28
HCV	30	50
Negative	13	22

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus.

Histological analysis of the nodules. All the HCC specimens, including the fresh specimens from the 12 patients, were histologically evaluated according to the general rules for the clinical and pathological study of primary liver cancer (16). The clinicopathological parameters of the specimens, including tumor diameter, liver status, IM, Vp, Vv and histopathological classification were obtained.

Correlations between LYVE-1 expression, HCC differentiation and patient survival. We analyzed archived HCC samples from 103 HCC patients. Those archived samples had been primarily collected from males with moderately differentiated HCCs and cirrhosis or chronic hepatitis resulting from viral infection (Table IV). The patients were Japanese and had undergone surgical HCC resection between December, 1993 and May, 2007 at the Department of Surgery, Institute of Gastroenterology, Tokyo Women's Medical University, Japan. The patients provided written informed consent in accordance with institutional regulations.

Statistical analysis. We used Wilcoxon signed-rank tests to compare gene expression levels between HCC nodules and non-HCC liver tissue. The correlation between *LYVE-1* expression levels in HCC nodules and the degree of nodule differentiation was assessed using Steel-Dwass tests. Disease-free survival (DFS) and overall survival (OS) were calculated by the Kaplan-Meier method and differences in survival curves were analyzed using log-rank tests. The follow-up time was defined as the time from the date of

Table IV. Characteristics of the 103 HCC patients investigated for survival curves.

Characteristics	Frequency	Percentage
Age (years)		
Mean (range)	63 (39-81)	-
Gender		
Male	78	76
Female	25	24
Histology		
Well differentiated	19	18
Moderately differentiated	67	65
Poorly differentiated	17	16
Child-Pugh classification		
A	90	87
B	12	12
C	1	1
Liver status		
Cirrhosis	44	43
Hepatitis	56	54
Normal	3	3%
Viral infection		
HBV	24	24
HCV	49	47
HCV+HBV	1	1
Negative	29	28
IM		
Positive	17	16
Negative	86	84
Vp		
Positive	19	18
Negative	84	82
Vv		
Positive	6	6
Negative	97	94
Macroscopic findings		
SNIM	25	24
SN	30	29
SNEG	38	38
Conflict multinodular type	4	4
Massive type	6	6
Tumor size (cm)		
Mean (range)	4.2 (0.8-17)	-

HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; IM, intrahepatic metastasis; Vp, portal vein invasion; Vv, venous invasion; SNIM, small nodular type with indistinct margin; SN, simple nodular type; SNEG, simple nodular type with extranodular growth.

Table V. Differentially expressed genes in HCC and non-HCC tissues.

A, Genes upregulated in primary HCC nodules compared to non-HCC liver tissue.

No.	Gene name	Description	P-value
1	*COL15A1*	Collagen, type XVα1	0.0020
2	*COL4A1*	Collagen, type IVα1	0.0010
3	*COL4A2*	Collagen, type IVα2	0.0034
4	*EDIL3*	EGF-like repeats and discoidin I-like domains 3	0.0098
5	*MDK*	Midkine	0.0005
6	*PDGFB*	Platelet-derived growth factor β polypeptide	0.0010

B, Genes downregulated in primary HCC nodules compared to non-HCC liver tissue.

No.	Gene name	Description	P-value
1	*ANGPTL1*	Angiopoietin-like 1	0.0010
2	*CXCL12*	Chemokine (C-X-C motif) ligand 12	0.0024
3	*CXCL2*	Chemokine (C-X-C motif) ligand 2	0.0010
4	*HGF*	Hepatocyte growth factor	0.0049
5	*LYVE-1*	Lymphatic vessel endothelial hyaluronan receptor-1	0.0010
6	*NRP2*	Neuropilin 2	0.0068
7	*PDGFRA*	Platelet-derived growth factor receptor α polypeptide	0.0005
8	*PLG*	Plasminogen	0.0034

HCC, hepatocellular carcinoma.

surgery to the date of death or the last known follow-up. The correlation of *LYVE-1* expression to the clinicopathological parameters was evaluated using Fisher's exact probability tests or Chi-square tests. Independent prognostic factors were analyzed using the Cox proportional hazards regression model. $P<0.05$ was considered to indicate a statistically significant difference. All tests were two-sided. We used JMP® software, version 9.0.1 (SAS Institute Inc., Cary, NC, USA) to compute all the statistics.

Results

Identification of angiogenic genes deregulated by HCC. The gene array analysis of liver tissue samples collected from the initial 12-patient group identified 14 genes differentially expressed in HCC and non-HCC tissues (Table V). Among these, the genes encoding collagen type XVα1, IVα1 and IVα2, as well as two growth factor-related genes [EGF-like repeats and discoidin I-like domains 3 (EDIL3) and platelet-derived growth factor β polypeptide (PDGFB)] were upregulated by HCC. HCC was also associated with upregulation of the gene encoding neurite growth-promoting factor 2 (midkine, MDK), which is involved in embryonic development and inflammation. By contrast, HCC caused downregulation of genes encoding inflammatory chemokines (CXCL2 and CXCL12) and genes associated with vessel growth, namely neuropilin 2 (*NRP2*) and *LYVE-1* (Table V).

Interpatient variability in LYVE-1 downregulation by HCC. The effect of HCC on *LYVE-1* expression was verified using a larger cohort of 58 patients. *LYVE-1* expression was significantly lower in HCC nodules compared to the corresponding non-HCC liver tissue ($P<0.0001$). Paired analysis of HCC nodule and non-HCC liver tissue samples from each patient revealed a large variability in *LYVE-1* expression between the patients (Fig. 1A).

Correlation between LYVE-1 downregulation and HCC nodule differentiation. Since the only parameter affected by *LYVE-1* expression was the histology of the nodules, this association was further investigated by analysis of HCC nodule samples. The possible contribution of disease severity to interpatient variability in *LYVE-1* expression was assessed using a large number of patients for whom nodule histology reports and archived tissue samples were available for correlation analysis. The loss of nodule differentiation was associated with a decrease in *LYVE-1* expression, which would occur early in the evolution of the disease ($P=0.0006$). The *LYVE-1* expression level was decreased >5-fold between the first two stages ($P<0.0001$) and remained comparable in poorly differentiated HCC nodules ($P=0.91$). These data support an association between *LYVE-1* expression and HCC progression (Fig. 1B).

Correlation between LYVE-1 expression and patient survival. The detrimental effect of *LYVE-1* downregulation on the survival of HCC patients was confirmed in the cohort of the 103 HCCs based on a similar analysis of HCC nodules. Based on a median observation frequency of 2,752 days, this group was characterized by a 5-year DFS rate of 34.1% and a 5-year

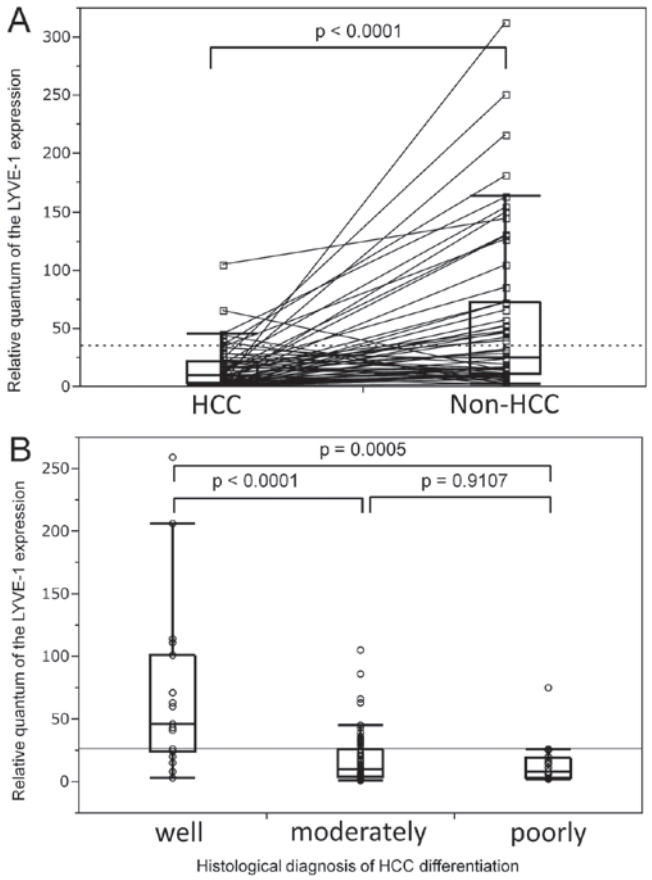

Figure 1. Impact of hepatocellular carcinoma (HCC) on the expression of *LYVE-1* in liver nodules and normal tissue. (A) Correlation between the level of *LYVE-1* gene expression in HCC nodules and corresponding non-HCC liver tissue (n=58). (B) Correlation between the level of *LYVE-1* gene expression in HCC nodules and the histological differentiation of tumor tissue. Well (n=19), moderately (n=67) and poorly (n=17) differentiated tissue.

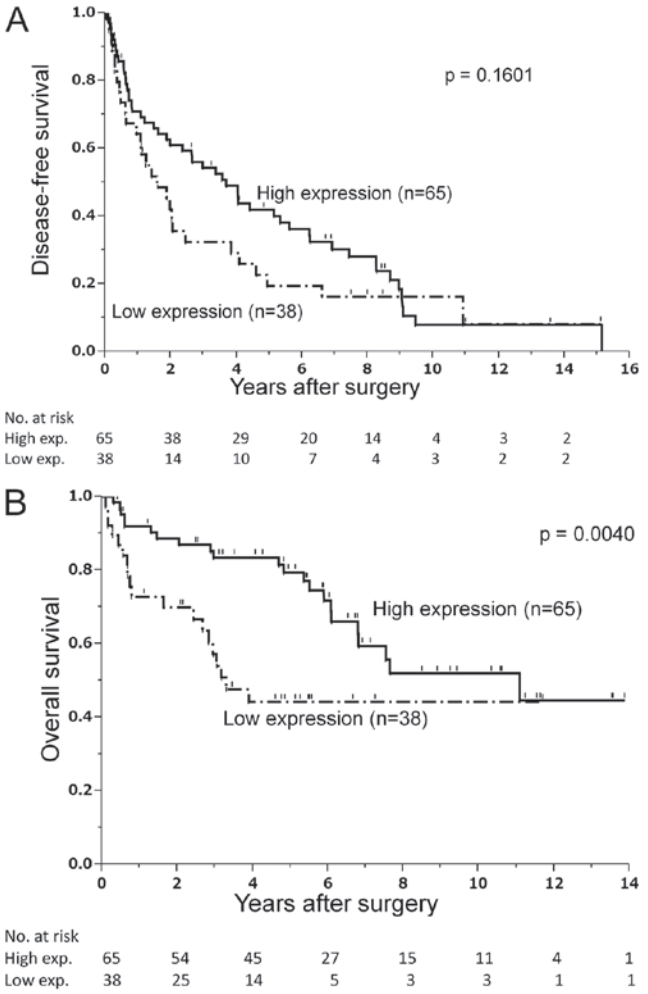

Figure 2. Impact of *LYVE-1* expression in hepatocellular carcinoma (HCC) nodules and non-HCC liver tissue on the survival of HCC patients. (A) Disease-free survival (DFS) of HCC patients with high (n=65) and low (n=38) *LYVE-1* expression in HCC nodules. There was no statistically significant difference in DFS between the two groups (P=0.1601, log-rank test). (B) Overall survival (OS) of HCC patients with high (n=65) and low (n=38) *LYVE-1* expression in HCC nodules. The OS of the high *LYVE-1* expression group was longer compared to that of the low *LYVE-1* expression group (P=0.0040, log-rank test).

OS rate of 66.6% and was used to assess the effect of *LYVE-1* expression on survival by dividing the patients into groups with high expression (>7-fold relative to the lowest value) and low expression (<7-fold relative to the lowest value) in HCC nodules. Fig. 2A shows that DFS was not significantly affected by the *LYVE-1* expression level in HCC nodules. By contrast, the OS curve decayed less rapidly for the high-expression group compared to that for the low-expression group, resulting in 5-year OS rates of 81 and 45%, respectively (P=0.004; Fig. 2B). In fact, all the patients with low *LYVE-1* expression reached the 45% OS plateau phase within 4 years after surgery. Accordingly, these data were confirmed by univariate Cox regression analyses for DFS [hazard ratio (HR)=1.394; 95% confidence interval (CI): 0.864-2.203; P=0.1694] and OS (HR=2.458; 95% CI: 1.298-4.625; P=0.0063). Multivariate Cox regression analyses identified *LYVE-1* expression as a significant independent prognostic parameter of OS (HR=3.067; 95% CI: 1.507-6.273; P=0.0021) (Tables VI and VII).

Specificity of factors affected by LYVE-1 expression in HCC patients. Analyses were performed to determine whether other aspects of the disease were associated with the downregulation of *LYVE-1* expression. The patients were re-examined by comparing the low- and high-expression groups with respect to the general characteristics and the histology of the HCC nodules (Table VIII). The expression of *LYVE-1* did not appear to exert any effect on basic characteristics, such as age, gender ratio, liver status or viral infection and IM, Vp and Vv in neither one of the two groups. With respect to tissue histology, the HCC nodules were significantly less differentiated in the low-expression group (P<0.0064; Table VIII). These data suggest that *LYVE-1* downregulation may be a marker of nodule dedifferentiation in HCC tissues.

Discussion

The field of cancer research has benefited significantly from genetic and functional analyses of oncogenes and tumor suppressor genes (17). Among the 92 angiogenic genes investigated, 14 genes were shown to be significantly deregulated in HCC. Some of these genes (*COL15A1*, *COL4A1*, *COL4A2*, *PDGFB*, *MDK* and *EDIL3*) were upregulated, whereas others (*ANGPTL1*, *CXCL12*, *CXCL2*, *NRP*, *HGF*, *LYVE-1*, *PDGFRA*

Table VI. Uni- and multivariate Cox regression analyses for disease-free survival (DFS) in HCC.

A, Univariate analysis of DFS among the 103 HCC patients.

	Univariate analysis		
Variables	HR	95% CI	P-value
Age ≥65 years	1.230	0.784-1.942	0.3682
Female gender	0.962	0.557-1.588	0.8847
Histopathological grade			
Poor	1.995	1.065-3.488	0.0321[a]
Moderate	1.235	0.778-2.004	0.3744
Child-Pugh classification B or C	0.871	0.384-1.714	0.7083
Cirrhosis	0.898	0.564-1.409	0.6418
Viral infection-positive	0.900	0.553-1.523	0.6860
IM-positive	16.345	7.297-37.151	<0.0001[b]
Vp-positive	3.868	2.059-6.857	<0.0001[b]
Vv-positive	3.999	1.355-9.525	0.0153[a]
Macroscopic findings			
SNEG or massive or conflict multinodular type	3.504	2.164-5.709	<0.0001[b]
Tumor size ≥3 cm	2.608	1.623-4.187	<0.0001[b]
Low *LYVE-1* in HCC	1.394	0.864-2.203	0.1694

B, Multivariate analysis of DFS among the 103 HCC patients.

	Multivariate analysis		
Variables	HR	95% CI	P-value
Poor histopathological grade	1.043	0.522-1.985	0.9003
IM-positive	8.902	3.687-21.910	<0.0001[b]
Vp-positive	1.450	0.673-2.951	0.3309
Vv-positive	1.880	0.567-5.256	0.2809
Macroscopic findings			
SNEG or massive or conflict multinodular type	2.192	0.995-4.764	0.0516
Tumor size ≥3 cm	1.071	0.517-2.197	0.8531

DFS, disease-free survival; HCC, hepatocellular carcinoma; HR, hazard ratio; CI, confidence interval; IM, intrahepatic metastasis; Vp, portal vein invasion; Vv, venous invasion; SNEG, simple nodular type with extranodular growth; *LYVE-1*, lymphatic vessel endothelial hyaluronan receptor-1. [a]P<0.05; [b]P<0.01.

and *PLG*) were downregulated, suggesting that they may be involved in the mechanism of carcinogenesis or tumor growth. Among these genes, *LYVE-1* was one of the most strongly downregulated genes in HCC nodules, compared to adjacent non-HCC tissue. This gene is of particular interest, as the triad of *glypican-3*, *LYVE-1* and *survivin* was previously demonstrated to provide a reliable diagnosis of early HCC (18). The present study demonstrates the potential of *LYVE-1* deregulation as an independent biomarker of postsurgical outcome in HCC patients.

In the present study, the clinicopathological findings revealed a significant correlation between *LYVE-1* expression and the histology of HCC nodules. From a dynamic perspective, the gradual loss of differentiation may be associated with *LYVE-1* downregulation occurring early during this process. *LYVE-1* expression levels in poorly or moderately differentiated nodules were comparable and were decreased by >5-fold compared to the levels in well-differentiated nodules. These data are consistent with those of a previous study, demonstrating that *LYVE-1* expression decreases progressively in HCC nodules transitioning from a polyclonal cirrhotic to a monoclonal cirrhotic phenotype (19). In addition, our study suggests that *LYVE-1* may be an early marker of HCC tumorigenesis.

Table VII. Univariate and multivariate Cox regression analyses for overall survival (OS) in HCC.

A, Univariate analysis of OS among the 103 HCC patients.

	Univariate analysis		
Variables	HR	95% CI	P-value
Age ≥65 years	1.067	0.576-2.006	0.8381
Female gender	1.470	0.748-2.762	0.255
Histopathological grade			
Poor	4.449	2.251-8.422	<0.0001[b]
Moderate	0.777	0.419-1.462	0.4274
Child-Pugh classification B or C	2.285	0.977-4.737	0.0559
Cirrhosis	1.985	1.072-3.760	0.0292[a]
Viral infection-positive	0.854	0.437-1.794	0.6620
IM-positive	7.273	3.241-15.483	<0.0001[b]
Vp-positive	8.539	4.004-17.853	<0.0001[b]
Vv-positive	1.624	0.718-2.716	0.2010
Macroscopic findings			
SNEG or massive or conflict multinodular type	4.138	2.163-8.211	<0.0001[b]
Tumor size ≥3 cm	3.439	1.736-7.015	0.0004[b]
Low *LYVE-1* in HCC	2.458	1.298-4.625	0.0063[b]

B, Multivariate analysis of OS among the 103 HCC patients.

	Multivariate analysis		
Variables	HR	95% CI	P-value
Poor histopathological grade	1.523	0.638-3.536	0.3374
Cirrhosis	2.533	1.177-5.517	0.0175[a]
IM-positive	3.993	1.386-11.846	0.0103[a]
Vp-positive	2.676	0.9159-7.396	0.0711
Macroscopic findings			
SNEG or massive or conflict multinodular type	2.317	0.857-6.067	0.0964
Tumor size ≥3 cm	1.083	0.420-2.831	0.8693
Low *LYVE-1* in HCC	3.067	1.507-6.273	0.0021[b]

HCC, hepatocellular carcinoma; HR, hazard ratio; CI, confidence interval; IM, intrahepatic metastasis; Vp, portal vein invasion; Vv, venous invasion; SNEG, simple nodular type with extranodular growth; *LYVE-1*, lymphatic vessel endothelial hyaluronan receptor-1. [a]P<0.05, [b]P<0.01.

The potential of *LYVE-1* as a predictor of postsurgical outcome in HCC patients was clearly demonstrated in terms of the 5-year OS. Logistic regression analyses revealed that low *LYVE-1* expression in HCC nodules was significantly predictive of shorter OS. Since the decrease in *LYVE-1* expression occurs early during the nodule transformation phase, these data suggested that close monitoring of *LYVE-1* expression after surgery may considerably improve survival in HCC patients.

Our understanding of the role of *LYVE-1* in tumorigenesis is evolving rapidly as the dogma is challenged by thorough immunohistochemical examination (20). This marker of lymphatic endothelial cells has been detected in the endothelial cells of the hepatic blood sinusoids of healthy subjects and patients diagnosed with liver cancer and cirrhosis. Notably, this protein is not detected in angiogenic blood vessels of liver tumors and is weakly detected in the microcirculation of regenerative hepatic nodules in cirrhosis, despite the fact that both types of vessels are derived from liver sinusoids. Furthermore, the lymphatics are restricted to the margins of HCCs and the surrounding tissues. This distribution is consistent with the

Table VIII. Association between *LYVE-1* expression in HCC liver nodules and clinicopathological characteristics of the 103 patients.

Clinicopathological characteristics (n)	*LYVE-1* expression		P-value
	High (n=65)	Low (n=38)	
Age (years)			0.1012
<65	34	13	
≥65	31	25	
Gender			0.6387
Male	48	30	
Female	17	8	
Histology			0.0064[a]
Well differentiated	18	1	
Moderately differentiated	38	29	
Poorly differentiated	9	8	
Child-Pugh classification			1.0000
A	57	33	
B or C	8	5	
Liver status			0.1001
Cirrhosis	32	12	
Other	33	26	
Viral infection			1.0000
Positive	47	27	
Negative	18	11	
IM			0.5884
Positive	12	5	
Negative	53	33	
Vp			0.6085
Positive	11	8	
Negative	54	30	
Vv			1.0000
Positive	4	2	
Negative	61	36	
Macroscopic findings			0.2208
SNIM or SN	38	17	
Other	27	21	
Tumor size (cm)			0.2188
<3	41	19	
≥3	24	19	

HCC, hepatocellular carcinoma; IM, intrahepatic metastasis; Vp, portal vein invasion; Vv, venous invasion; SNIM, small nodular type with indistinct margin; SN, simple nodular type ; *LYVE-1*, lymphatic vessel endothelial hyaluronan receptor-1. [a]P<0.01.

LYVE-1 downregulation observed in the highly vascularized HCC nodules compared to non-HCC tissues. Accordingly, the restriction of *LYVE-1* to the periphery of the tumor may translate into progressive decrease, in relative expression with an increase in tumor size, as supported by a previous study demonstrating that *LYVE-1* attenuation in the sinusoidal endothelium was associated with hepatic disease progression (21).

The most common cause of mortality in HCC patients is tumor recurrence following surgery, which may be caused by small metastatic lesions or metachronous multicentric lesions in the case of liver inflammation or cirrhosis. Chronic aggressive hepatitis is a significant risk factor of HCC recurrence following hepatectomy (22). Notably, the expression of *LYVE-1* in the lymphatic endothelium is downregulated by the pro-inflammatory cytokine tumor necrosis factor-α *in vitro* and *in vivo* (23-25), suggesting that *LYVE-1* expression may be suppressed by hepatitis. The fact that inflammation is initiated early during the course of liver disease is consistent with our hypothesis that *LYVE-1* may be an early marker of HCC tumorigenesis.

LYVE-1 is a member of the Link protein superfamily and is similar to the leukocyte hyaluronan receptor CD44, which is known to facilitate tumor cell invasion. Hyaluronan is a key substrate for cell migration among tissues during inflammation, wound healing and neoplasia (26). Recent studies suggested that the ligands of *LYVE-1* receptors may enhance tumor cell adhesion to the vessel wall (27) and open lymphatic intercellular junctions (28), allowing tumor cells to invade the surrounding tissue (29). Therefore, although the overall *LYVE-1* expression is decreased in HCC nodules, the strategic positioning of its receptor at the periphery of the tumor may favor tumorigenesis and metastasis through the facilitation of tumor cell passage in and out of the tumor. This hypothesis is consistent with the recent finding that *LYVE-1* expression may be associated with chemoresistance (30). Therefore, the progressive loss of *LYVE-1* expression during the transformation of HCC nodules may correlate with the severity of inflammation and tumor growth.

To the best of our knowledge, this study is the first to demonstrate a direct correlation between *LYVE-1* expression and tumor dedifferentiation, which strengthens the hypothesis that *LYVE-1* may be a potent independent marker for the clinical prognosis of HCC.

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