Evaluation of the Impact of Preoperative Values of Hyaluronic Acid and Type IV Collagen on the Outcome of Patients with Hepatocellular Carcinoma After Hepatectomy

Junji Ueda^{1,2}, Hiroshi Yoshida¹, Yasuhiro Mamada¹, Nobuhiko Tanai¹,
Masato Yoshioka¹, Atsushi Hirakata², Youichi Kawano¹, Tetsuya Shimizu¹,
Tomohiro Kanda¹, Hideyuki Takata² and Eiji Uchida¹

¹Department of Gastrointestinal Hepato-Biliary-Pancreatic Surgery, Nippon Medical School, Tokyo, Japan

²Department of Surgery, Nippon Medical School Tama Nagayama Hospital, Tokyo, Japan

Background: Recently, some reports have revealed a relationship between post-hepatectomy prognosis in hepatocellular carcinoma (HCC) and hepatic fibrosis markers. We evaluated the relationship between these markers of hepatic fibrosis, clinicopathological findings, and prognosis.

Methods: Three hundred and sixty patients underwent hepatectomy for HCC in the Nippon Medical School Hospital between 1993 and 2013. We divided these patients into two groups: normal serum hyaluronic acid (HA) levels and abnormal levels. We also divided patients into groups with normal serum type IV collagen levels and abnormal levels.

Results: The overall survival rate and recurrence-free survival rate of the normal group were significantly higher than those of the abnormal group. In the normal hyaluronic acid group, serum albumin and prothrombin time were significantly higher than in the abnormal group, and age, hepatitis C virus antibody (HCV)-Ab positivity, Child-Pugh grade B, liver cirrhosis, indocyanine green retention rate at 15 min (ICGR15), type IV collagen level, and type IV collagen 7s level were significantly lower than those in the abnormal group. In the normal type IV collagen group, HCV-Ab positivity, liver cirrhosis, ICGR15, HA level, and type IV collagen 7s level were significantly lower than those in the abnormal group, and the serum albumin level was significantly higher than that in the abnormal group. Multivariate analysis independently revealed the significant effect of serum type IV collagen on the overall survival rate as well as the significant effect of serum HA on the recurrence-free survival rate in patients who underwent hepatectomy for HCC.

Conclusions: Preoperative examinations of serum hyaluronic acid levels and type IV collagen levels are imperative for hepatic resection for HCC because these markers are significantly associated with liver function and prognosis.

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Key words: hyaluronic acid, type IV collagen, hepatocellular carcinoma, liver cirrhosis

Introduction

Hepatocellular carcinoma (HCC) is the fifth-most prevalent carcinoma in the world. HCC is the third-most common cause of cancer-related death worldwide^{1,2}. From a clinical point of view, the critical problem is recurrence of HCC after treatment³. Despite improving techniques in non-surgical approaches, hepatectomy still represents a

potentially curative treatment for HCC. Unfortunately, tumor recurrence is the major cause of worsening results in long-term survival, with an expected 5-year intra-hepatic recurrence rate of up to 70 %³. Thus, the identification of new markers for recurrence and survival is important to predict the possible outcome of patients with HCC⁴.

Correspondence to Junji Ueda, MD, PhD, Department of Surgery, Nippon Medical School Tama Nagayama Hospital, 1-7-1 Nagayama, Tama, Tokyo 206-8512, Japan

E-mail: junji0821@nms.ac.jp

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Table 1 Clinical and pathological characteristics of the study

Age (yr)		66.7 (±9.63)
Sex	male/female	263/97
Infection	HBV/HCV/none	70/224/77
Child-Pugh	A (5)/A (6)/B (7) B (8)/B (9)/C (10-)	212/109/36 3/0/0
ICG 15R (%)		18.2 (±12.3)
Tumor size (mm)		45.5 (±35.7)
Differentiation	wel, mod/por	316/44
Background	NL, CH/LC	174/186
pStage	I/II/III/IV	48/129/104/74

(±standard deviation SD)

HBV: hepatitis B virus, HCV: hepatitis C virus, ICG: indocyanine green, wel: well differentiated hepatocellular carcinoma, mod: moderately differentiated hepatocellular carcinoma, por: poorly differentiated hepatocellular carcinoma, NL: normal liver, CH: chronic hepatitis, LC: liver cirrhosis, pStage: pathological stage, T-bil: total bilirubin, Alb: albumin, PT: prothrombin time, AFP: alfa-fetoprotein, AFP L3: Lectin-reactive alpha-fetoprotein, DCP: des-γ-carboxy prothrombin

Hyaluronic acid (HA) is one of the oldest serum parameters of hepatic fibrosis. HA is known as mucopolysaccharide. Several reports indicate that HA is a biomarker for fibrosis and cirrhosis in liver disease⁵.

Type IV collagen codistributes with laminin in the basement membranes of vessels and bile ducts, and there is also “free” type IV collagen that is not associated with laminin and is distributed in the sinusoids as small, discrete, discontinuous deposits. During the transition from early to late fibrosis, trichrome (stains primarily collagen I) and collagen IV showed the steepest increase and appeared to be the most useful discriminators between the early and late stages of fibrosis⁶.

The aim of this study was to evaluate the relationship between serum HA and type IV collagen levels, clinicopathological findings, and prognosis in patients who underwent hepatic resection for HCC.

Materials and Methods

Consecutive patients who underwent hepatectomy for HCC between February 1993 and September 2013 at the Nippon Medical School Hospital were included in this study. This study was approved by the Human Ethics Review Committee of the Nippon Medical School.

Indications for hepatectomy were based on the results of preoperative and intraoperative imaging and liver function assessment.

Pathological diagnoses and clinicopathological factors were established using the general guidelines for primary liver cancer of the Liver Cancer Study Group of Japan⁷.

All patients were followed up with examination of α-fetoprotein (AFP) and des-γ-carboxyprothrombin (DCP) levels, ultrasonography, dynamic computed tomography (CT), and magnetic resonance imaging (MRI). Recurrences were diagnosed as the appearance of a lesion that is typical of HCC, as confirmed by ultrasound imaging,

CT, and MRI. If recurrence was detected, the patient received further treatment.

Serum HA levels and type IV collagen levels were measured within 4 weeks before surgery. We divided these patients into two groups: normal serum HA levels and abnormal levels. We also divided patients into groups with normal serum type IV collagen levels or with abnormal serum type IV levels. The normal HA level was under 50 ng/mL. The normal type IV collagen level was under 140 ng/mL.

Categorical variables were compared using the χ^2 test and Student's t-test. Multiple logistic regression analyses were performed to confirm the relationship between variables, serum HA level, and type IV collagen level. Recurrence-free survival (RFS) and overall survival (OS) curves were determined with the Kaplan-Meier method. Multivariable Cox proportional hazards regression analyses were performed.

Results

During the study, 360 consecutive patients underwent hepatectomy for HCC. The median age was 66.7 years, and 263 of the patients were men; 70 patients were hepatitis B surface antigen (HBs-Ag) positive, and 224 patients were hepatitis C virus antibody (HCV-Ab) positive. Child-Pugh grade A was present in 324 cases, and grade B was present in 36 cases. The mean tumor diameter was 45.5 mm. In the analysis of type IV collagen, 63 patients were excluded because their preoperative serum type IV collagen level had not been determined. The remaining 297 patients were analyzed (Table 1).

Regarding HA level, a comparison of the two groups is shown in Table 2. In the normal HA group, age, HCV-Ab positivity, Child-Pugh grade B, liver cirrhosis, indocyanine green retention rate at 15 min (ICGR15), type IV collagen level, and type IV collagen 7s level were signifi-

Table 2 Comparison of the background characteristics of the two hyaluronic acid level groups

Hyaluronic Acid		Normal (n=58)	Abnormal (n=302)	p-value
Age (yr)		64.3	67.1	0.042
Sex	male/female	48/10	215/87	0.0975
Tumor diameter (mm)		49.3	44.3	0.3399
HBV	+/-	16/42	54/246	0.1325
HCV	+/-	24/34	200/101	0.0005
Child-Pugh	A/B,	57/1	262/40	0.0399
Differentiation	wel, mod/por	43/7	223/37	0.999
Background of liver	NL, CH/LC	42/12	104/174	0.0001
T-bil (mg/dL)		0.747	0.847	0.234
Alb (g/dL)		4.12	3.75	0.001
PT (%)		91.1	84.6	0.0034
ICG15R (%)		10.9	19.6	0.0001
AFP (ng/mL)		13,248	17,262	0.8993
L3 (%)		9.09	17.6	0.114
DCP (mAU/mL)		1,577	5,292	0.237
Type IV collagen (ng/mL)		157	250	0.0001
Type IV collagen 7s (ng/mL)		4.73	8.37	0.0308

Fig. 1 **A:** A high serum hyaluronic acid (HA) concentration was associated with shorter overall survival (OS) ($P=0.0005$) and recurrence-free survival (RFS) ($P=0.0351$). In the normal HA group, OS and RFS were significantly longer than in the abnormal group. **B:** A high serum type IV collagen concentration was also associated with shorter OS ($P=0.0039$) and RFS ($P=0.00294$). In the normal type IV collagen group, OS and RFS were significantly longer than in the abnormal group.

cantly lower than in the abnormal group. Moreover, in the normal HA group, serum albumin level and prothrombin time were significantly higher than in the abnormal group. Anatomical resection of HCC was performed more often in the normal HA group than in the abnormal group. High serum HA concentrations were as-

sociated with shorter OS ($P=0.0005$) and RFS ($P=0.0351$). (Fig. 1A).

Next, we analyzed type IV collagen levels. A comparison of the background characteristics of the two groups is shown in Table 3. In the normal type IV collagen group, HCV-Ab positivity, liver cirrhosis, ICG15, HA

Table 3 Comparison of the background characteristics of the two type IV collagen level groups

Type IV collagen		Normal (n=68)	Abnormal (n=227)	p-value
Age (yr)		64.8	66.4	0.2659
Sex	male/female	51/17	165/62	0.9124
Tumor diameter (mm)		43.0	48.1	0.2979
HBV	+/-	17/51	42/185	0.3022
HCV	+/-	33/35	153/74	0.0058
Child-Pugh	A/B	65/3	195/32	0.1796
Differentiation	wel, mod/por	59/9	201/26	0.6981
Background of liver	NL, CH/LC	49/19	99/128	0.0001
T-bil (mg/dL)		0.756	0.873	0.234
Alb (g/dL)		4.01	3.79	0.0012
PT (%)		87.9	84.6	0.1681
ICG15R (%)		14.6	19.0	0.0079
AFP (ng/mL)		2,156	25,755	0.4827
L3 (%)		13.5	18.3	0.2916
DCP (mAU/mL)		2,178	5,347	0.323
Hyaluronic acid (ng/mL)		94.3	266	0.0001
Type IV collagen 7s (ng/mL)		4.96	7.75	0.0001

Table 4 Univariate analysis for overall survival rate

Univariate analysis (OS)	P value	Hazard ratio	95% CI
Age	0.4196	1.008	0.989–1.027
Tumor diameter (mm)	0.0001	1.010	1.006–1.013
Vascular invasion (–)	0.0012	0.598	0.437–0.817
Number of tumor	0.9958	1.001	0.704–1.335
Stage	0.0085	1.523	1.317–1.860
T-bil (mg/dL)	0.7680	1.035	0.825–1.298
Alb (g/dL)	0.0001	0.489	0.360–0.666
PT (%)	0.5182	0.996	0.986–1.007
ICG15R (%)	0.0670	1.012	0.999–1.025
AFP (ng/mL)	0.0397	1.000	1.000–1.000
L3 (%)	0.0003	1.018	1.008–1.027
DCP (mAU/mL)	0.0001	1.000	1.000–1.000
Hyaluronic acid (ng/mL)	0.0001	1.001	1.000–1.001
Type IV collagen (ng/mL)	0.0001	1.002	1.001–1.002
Type IV collagen 7s (ng/mL)	0.7766	0.998	0.986–1.010

level, and type IV collagen 7s level were significantly lower than in the abnormal group. Moreover, in the normal type IV collagen group, the serum albumin level was significantly higher than in the abnormal group. A high serum type IV collagen concentration was also associated with shorter OS ($P=0.0039$) and RFS ($P=0.00294$) (**Fig. 1B**).

Factors that were associated with OS in univariate analysis were tumor diameter, vascular invasion, stage, serum albumin, serum AFP, serum AFP L3, serum DCP, serum HA, and serum type IV collagen (**Table 4**). These factors were included in the multivariate analysis. The multivariate analysis revealed that high preoperative se-

rum type IV collagen levels independently predicted poor OS (**Table 5** hazard ratio (HR) 1.003, 95 percent confidence interval 1.000 to 1.006; $P=0.0326$). Factors associated with RFS in univariate analysis included tumor diameter, tumor number, stage, serum albumin, serum DCP, serum HA, and serum type IV collagen (**Table 6**). These factors were included in the multivariate analysis. The analysis revealed that high preoperative serum HA levels and tumor diameter independently predicted poor RFS (**Table 7**; HR 1.001, 95 % confidence interval 1.000 to 1.002; $P=0.0121$, HR 1.010, 95 % confidence interval 1.003 to 1.016; $P=0.0035$, respectively).

Table 5 Multivariate analysis for overall survival rate

Multivariate analysis (OS)	<i>P</i> value	Hazard ratio	95% CI
Tumor diameter (mm)	0.2145	1.005	0.997–1.014
Vascular invasion	0.3361	1.581	0.622–4.018
Stage	0.3899	1.501	0.595–3.785
Alb (g/dL)	0.1644	0.630	0.328–1.209
AFP (ng/mL)	0.2723	1.000	1.000–1.000
L3 (%)	0.0730	1.012	0.999–1.025
DCP (mAU/mL)	0.0400	1.000	1.000–1.000
Hyaluronic acid (ng/mL)	0.9746	1.000	0.999–1.001
Type IV collagen (ng/mL)	0.0057	1.003	1.000–1.006

Table 6 Univariate analysis for recurrence-free survival rate

Univariate analysis (RFS)	<i>P</i> value	Hazard ratio	95% CI
Age	0.8447	0.998	0.985–1.019
Tumor diameter (mm)	0.0001	1.009	1.004–1.012
Vascular invasion (—)	0.0130	0.654	0.978–1.403
Number of tumor	0.2933	0.827	1.082–1.743
Stage	0.3662	1.164	1.123–1.518
T-bil (mg/dL)	0.0736	1.221	0.981–1.520
Alb (g/dL)	0.0239	0.693	0.550–0.920
PT (%)	0.8279	0.999	1.000–1.000
ICG15R (%)	0.2234	1.009	0.998–1.022
AFP (ng/mL)	0.1077	1.000	1.000–1.000
L3 (%)	0.2650	1.006	0.994–1.014
DCP (mAU/mL)	0.0129	1.000	1.000–1.000
Hyaluronic acid (ng/mL)	0.0016	1.001	1.000–1.002
Type IV collagen (ng/mL)	0.0360	1.001	1.000–1.002
Type IV collagen 7s (ng/mL)	0.1233	1.044	0.987–1.102

Table 7 Multivariate analysis for recurrence-free survival rate

Multivariate analysis (RFS)	<i>P</i> value	Hazard ratio	95% CI
Tumor diameter (mm)	0.0123	1.007	1.007–1.020
Vascular invasion (—)	0.3608	1.020	0.602–1.726
Alb (g/dL)	0.4849	0.630	0.387–1.026
DCP (mAU/mL)	0.1667	1.000	1.000–1.000
Hyaluronic acid (ng/mL)	0.0376	1.001	1.000–1.002
Type IV collagen (ng/mL)	0.3192	1.001	1.000–1.002

Discussion

This study revealed that high preoperative serum HA levels and type IV collagen levels predict RFS and OS after hepatectomy for HCC. Specifically, a high HA level independently predicted RFS. Anatomical resection of HCC was performed more often in the normal HA group than in the abnormal group. Anatomical resection of HCC was introduced by Makuchi⁸, who reported that this procedure improved the survival rate by reducing local recurrence. However, some recent reports did not show an improvement in recurrence-free survival follow-

ing anatomical resection of HCC⁹. These findings are controversial. Prospective randomized control studies are required.

High type IV collagen levels independently predicted OS. These markers are very important for hepatic resection for HCC. Interestingly, the effects of tumor number, vascular invasion, stage, and some tumor markers were detected by univariate analysis, but these effects were not detected in multivariate analysis.

We will now review HA and type IV collagen and discuss the mechanism of how these factors affect OS and

RFS.

HA is a high molecular weight (10^6 – 10^7 Da) glycosaminoglycan polymer that is composed of repeating disaccharides: β 1, 3 N-acetyl glucosaminyl- β 1, 4 glucuronide¹⁰. Serum HA levels indicate the severity of underlying chronic liver disease^{11–13}. Serum HA levels reflect liver fibrosis more closely than ICG R15¹⁴. Moreover, because portal pressure is correlated with hepatic fibrosis¹⁵, serum HA may be a biochemical marker of portal pressure and a predictor of postoperative hepatic failure¹⁴. Serum HA concentration reflects the function of hepatic sinusoids and is a sensitive marker of liver endothelial cell dysfunction after liver ischemia¹⁶. Some reports have revealed that HA is a useful marker for the prediction of post-hepatectomy hepatic functional reserve^{15,17,18}. HA is involved in tumorigenesis¹⁰. HA receptors such as CD44 (cluster of differentiation 44) are implicated in the cell signaling cascades associated with cancer initiation and progression¹⁹. The CD44 receptor is a widely distributed transmembrane glycoprotein that plays a critical role in malignant cell activities¹⁰. CD44 has some isoform types. HA/CD44 and HA/CD44v interactions regulate stem cell migration and homing²⁰. CD44s have been implicated in promoting epithelial-mesenchymal transition (EMT)²¹. These mechanisms may contribute to shortening the RFS at a high HA level. Type IV collagen is common in a variety of tumors^{22–26}. A positive correlation between type IV collagen expression and tumor metastasis has been reported in several studies²⁸. Native type IV collagen induced an EMT-like process, increasing matrix metalloproteinase (MMP)-2, focal adhesion kinase (FAK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation, cell migration, and invasion in MCF10A human mammary epithelial cells²⁹. In experiments, shRNA targeting type IV collagen gene-transfected cells formed significantly fewer and smaller lung nodules²⁹.

Conclusion

Preoperative examination of serum hyaluronic acid levels and type IV collagen levels are imperative for the hepatic resection of hepatocellular carcinoma because these markers are significantly associated with liver function and prognosis.

Conflict of Interest: None.

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