

Clinical utility of serum Fucosylated-hemopexin in Japanese patients with Hepatocellular Carcinoma

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is a common clinical problem all over the world. Fucosylated-hemopexin (Fuc-Hpx) is a newly reported glycoprotein for the diagnosis of HCC, however, its clinical implication is not referred. The aim of this study was to elucidate the clinical utility of Fuc-Hpx in Japanese patients with HCC.

Methods: The sera from 331 HCC patients, 45 with liver cirrhosis (LC), 85 with chronic hepatitis (CH), and 22 healthy people were examined for the expression of Fuc-Hpx; the level was compared with clinical parameters as well as hemopexin (Hpx) expression. The expressions of Fuc-Hpx in 12 HCC tissues and corresponding adjacent non-cancerous liver tissues were also examined.

Results: No correlation was observed between Hpx and Fuc-Hpx level. The median Fuc-Hpx levels in healthy people, CH, LC, and HCC patients were 3.8, 3.7, 6.1, and 7.6 AU/ml, respectively (CH vs. LC, $p=0.002$; CH vs. HCC, $p<0.001$; LC vs. HCC, $p=0.02$). Multivariate analysis revealed that low albumin, low prothrombin time, and the presence of HCC were significantly correlated with high Fuc-Hpx ($p=0.013$, $p=0.001$, and $p<0.001$, respectively). Among the HCC patients, albumin was correlated with high Fuc-Hpx; however, none of the tumor factors, such as tumor size, tumor number, and tumor stage, was correlated with Fuc-Hpx level. The expression of Fuc-Hpx in cancer tissue was not different from that in non-cancerous tissue.

Conclusions: Fuc-Hpx is a valuable biomarker for HCC but it might be a marker for hyper-carcinogenic liver rather than a marker for tumor-bearing.

Keywords: Fucosylated-hemopexin, Hepatocellular carcinoma, Glycosylation, Biomarker, hyper-carcinogenicity,

INTRODUCTION

HCC is the fifth most common cancer and its very poor prognosis makes it the third leading cause of cancer death worldwide.^{1,2} HCC accounts for over 90% of common primary liver cancer in Japan. More than 80% of HCC cases develop in patients suffering from long-lasting viral hepatitis. Recently, rising rates of diabetes, obesity, and nonalcoholic steatohepatitis (NASH) have become increasingly important risk factors of future HCC incidence trends globally, particularly in developed countries.^{3, 4} Although HCC without hepatitis virus infection, which is difficult to survey, is increasing and the percentage of cases with viral hepatitis is decreasing in Japan, the majority of HCC patients (over 80%) still suffer from either hepatitis C or hepatitis B virus infection.⁵ Many of these patients were under surveillance programs for the diagnosis of HCC, resulting in smaller tumor size at diagnosis.

While modalities of imaging diagnoses have been improving and therapeutic options have progressed, a major problem in HCC surveillance is the lack of reliable biomarkers.⁴ Alpha-fetoprotein (AFP) is the best available biomarker with high sensitivity for HCC surveillance, but the low specificity of AFP led the American Association for the Study of Liver Disease (AASLD) Practice Guideline Committee to recommend that surveillance has to be based on ultrasound examination.⁶ Des- γ -carboxy prothrombin (DCP) is used widely as an HCC biomarker in Japan, but it is not popular in other countries including the United States. DCP is more closely related to tumor size with high sensitivity in the diagnosis of large HCC than AFP, but the sensitivity is low for the diagnosis of small HCC.⁷ It is known that the fucosylation of glycoprotein often emerges during carcinogenesis.⁸⁻¹⁵ The fucosylated AFP (AFP-L3) was highly specific and correlated with biological malignancy and prognosis of HCC patients.¹⁶⁻¹⁹ Recent glycan analysis demonstrated the increasing fucosylation of serum glycoproteins, not only AFP but also haptoglobin, fetuin A, hemopexin (Hpx), kininogen, α -1 antitrypsin, and Golgi protein 73 (GP73) with the

development of HCC.^{8, 15}

Hpx is a 60-kDa glycoprotein that is one of the acute-phase reactant proteins. Besides its classical functions, such as binding and transportation of free heme in peripheral blood, a wide range of other properties of the hemopexin molecule have been described, such as antioxidant activity.²⁰ Hpx is produced in the liver and secreted in serum. A report from the United States demonstrated that the fucosylated form of hemopexin (Fuc-Hpx) was a good serum marker for HCC and its capacity for the diagnosis of HCC was superior to that of AFP.^{8, 9, 21} However, the profile of glycosylation is known to be different by age, race, or resident country.²² In addition, HCC surveillance has become popular, so the size of HCC at diagnosis is smaller in Japan than in other countries.^{23, 24} Thus, the aim of this study is to evaluate the clinical utilities of Fuc-Hpx in Japanese HCC patients.

MATERIAL & METHODS

Human subjects

Human serum samples were obtained from newly developed HCC patients (n=331), those with chronic hepatitis (CH: n=85), or those with liver cirrhosis (LC: n=45), who were admitted to Okayama University Hospital between 2002 and 2009, as well as from healthy volunteers (n=22). The serum was collected at the time of admission, meaning that no intervention had been performed. The characteristics of the patients are summarized in Table 1. Healthy subjects did not have a past history of liver disease, cancer, or metabolic or hormone disorder that required medication. Age is shown as median and interquartile range. The median age of HCC patients was older than that of others ($p<0.001$). For etiology, patients with hepatitis B virus surface antigen positivity were classified HBV, and those with hepatitis C virus antibody were classified as HCV. Alcohol-induced liver injury, NASH, autoimmune hepatitis, or liver disease of unknown origin were classified as others. Over 80% of the patients suffered from viral infection in both the HCC and the non-HCC groups, and hepatitis C viral infection was more prevalent in HCC patients than in non-HCC patients (73% vs. 49%, $p<0.001$). The changes of Fuc-Hpx between before and after curative treatments of HCC were examined in 21 cases. Nine cases were treated by local curative treatments (5 surgical resection and 4 radiofrequency ablation). The others were treated by liver transplantation.

HCC tissue samples and the corresponding adjacent liver tissue samples were obtained from 12 patients who received liver transplantation. Informed consent was obtained from all patients, and the study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki and was approved by our institutional review board.

Diagnosis of HCC

In accordance with the American Association for the Study of Liver Disease (AASLD, 2005)

Practice Guidelines, we confirmed the diagnosis of HCC by at least 2 dynamic imaging modalities. Typical findings were confirmed as hyperattenuation at the arterial phase and hypoattenuation at the portal phase in dynamic computed tomography (CT) or magnetic resonance imaging (MRI), and tumor staining on angiography. The nodules without these findings were diagnosed by histological examination via US-guided, fine-needle biopsy. Stage was based on the General Rules for the Clinical and Pathological Study of Primary Liver Cancer. The diagnosis of CH and LC was based on liver histology, or clinical and laboratory data including the findings of ultrasound, CT or MRI.

Sample preparation from human liver tissues

Human liver samples were extracted from 50 mg of frozen tissues. Briefly, samples were homogenized with 250 µl reagent mixed CelLyticTM-MT (SIGMA-ALDRICH, St. Louis, USA) containing protease inhibitor. The lysed samples were centrifuged for 10 minutes at 4°C, 12,000-20,000 X g, to pellet the tissue debris. The supernatant was harvested in a clean tube and used for the following studies. The protein's concentration in each sample was measured by the Bradford method.

Measurement of Hpx

Serum Hpx concentrations were measured by enzyme-linked immunosorbent assay (ELISA). We used the AssayMax Human Hemopexin ELISA kit (ASSAYPRO, St. Charles, USA). The samples were measured in duplicate according to the manufacturer's instructions. A microplate reader (BIO-RAD Model680, BIO-RAD Laboratories, Tokyo, Japan) was used for reading absorbance at 450 nm.

Lectin ELISA for Fuc-Hpx

We performed lectin-ELISA for quantitative analysis of Fuc-Hpx in accordance with the method reported by Metha et al. with some modification.⁹ Briefly, the rabbit anti-human hemopexin antibody (ASSAYPRO, St. Charles, USA) was incubated with 10 mmol/L sodium periodate to remove the fucosylation of the captured antibody at 4°C for 1 hr. under dark conditions. An equal volume of ethylene glycol was added and the oxidized antibody was diluted to a concentration of 10 µg/ml with sodium carbonate buffer (pH9.5). 1 µg of antibody was added to each well of the ELISA plate and incubated overnight at 4°C. The plate was washed 5 times with 0.1% Tween 20/PBS7.4 (PBS-T) and then blocked overnight with 3% bovine serum albumin/PBS.

For analysis, 50 µl of serum was diluted in 50 µl of PBS with 1 µl of Immunoglobulin Inhibiting Reagent (BIORECLAMATION, New York, USA) and incubated at room temperature for 45 minutes. The samples were added to the plate and incubated at 37°C for 1 hr, followed by washing with lectin incubation buffer (10 mM Tris pH8.0, 0.15 M NaCl, 0.1% Tween20) 5 times. After that, AAL lectin (VECTOR LABORATORIES, Burlingame, USA) diluted 250 times by lectin incubation buffer was applied and incubated at room temperature for 1 hr. After 5 washes with PBS-T, AP-streptavidin (VECTOR) diluted 1000 times by PBS was applied and incubated at room temperature for 1 hr. After washing 5 times, color was developed using phosphatase substrate (KPL, Baltimore, USA), and the optical density (OD) at 630 nm was measured. The concentration is expressed as arbitrary unit (AU) based on the relative concentration against a standard HCC sample and normal stock serum. Control curve of lectin-ELISA was shown at Figure 1.

Statistical analysis

JMP (version 8.02) software package (SAS Institute, Cary, North Carolina, USA) was used for the analyses. Continuous variables are shown as median and interquartile range. The Wilcoxon rank sum test was used to compare the continuous data and the chi-squared test was used

to compare categorical data. Statistical significance was set at $p < 0.05$. Univariate analysis was performed in all patients except healthy volunteers to identify the potential factors correlated with Fuc-Hpx in liver diseases. Variables at $p < 0.05$ in the univariate analysis were further analyzed to identify independent factors correlated with Fuc-Hpx by multivariate analysis. The variables used in the analysis included age, sex, etiology, presence of HCC, platelet count (Plt), prothrombin time (PT), albumin (Alb), total bilirubin (T-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and Child-Pugh grade. For the analysis in HCC patients, tumor markers such as AFP, AFP-L3, DCP, tumor size, tumor number, presence of portal vein tumor thrombosis (Vp), and tumor stage (Stage) were added. The optimal cut-off values of most variables were set at approximate values of medians. Those of AFP, AFP-L3, and DCP were 20 ng/ml, 10%, and 40 mAU/ml, respectively. Paired t-test was used for the analysis of Fuc-Hpx expression levels between HCC and adjacent liver tissues. Correlation analysis was verified at r^2 value by Pearson correlation coefficient. Diagnostic abilities in differentiating HCC from liver disease without HCC were evaluated using the areas under the Receiver Operating Characteristic (ROC) curve (AUROC). Sensitivity, specificity, and accuracy were analyzed by McNemar test, and positive predictive value (PPV) and negative predictive value (NPV) were analyzed by Fisher's exact test. All test were two sided between Fuc-Hpx and another marker, and $p < 0.05$ was considered significant.

RESULTS

Relationship between serum Hpx and Fuc-Hpx

To determine the effect of Hpx concentration on Fuc-Hpx level, we measured both Hpx and Fuc-Hpx expressions in 18 samples simultaneously (Figure 2). No correlation was observed between Hpx and Fuc-Hpx ($p=0.89$). The level of Hpx was not significantly different between non-HCC (median 648 AU/ml, range 488-750) and HCC groups (772 AU/ml, 483-1022, $p=0.16$), whereas Fuc-Hpx level was higher in HCC group (6.8 AU/ml, range 4.9-11.0) than in non-HCC group (2.6 AU/ml, range 0.9-4.8, $p<0.001$). Because total Fuc-Hpx level was closely correlated with the percentage of Fuc-Hpx ($R^2=0.6$, $p<0.001$) and no difference of AUROC of total and percentage of Fuc-Hpx was observed in this study population (0.84 and 0.77, respectively), we used total Fuc-Hpx level in the following analysis.

Serum Fuc-Hpx level in liver diseases

To confirm the Fuc-Hpx expression in various liver diseases, we measured it in large populations. The median value in HCC group ($n=331$) was 7.6 AU/ml (range 5.6-10.8), which was significantly higher than that of non-HCC group ($n=130$, median 4.6 AU/ml, range 2.5-7.1, $p<0.001$). A progressive increase of Fuc-Hpx was observed from that of healthy controls (median 3.8 AU/ml, range 0.1-5.8) through CH (3.7 AU/ml, 1.9-6.2) to LC (6.1 AU/ml, 4.1-8.9). Significant difference was observed between HCC group and LC ($p=0.02$) or CH group ($p<0.001$), and between LC and CH groups ($p=0.002$), but no difference was observed between CH group and healthy subjects (Figure 3). We examined Fuc-Hpx level in patients with or without HCC with the same liver function. The median was 7.7AU/ml (range 5.4-10.5) in HCC group, which was significantly higher than that in non-HCC group (median 3.9 AU/ml, range 2.1-6.7, $p<0.001$) in Child-Pugh grade A patients. In Child-Pugh grade B/C patients, no difference was observed between the groups, and

the median was 7.8 AU/ml (range 6.2-11.1), and 6.6 AU/ml (range 5.5-11.2) in HCC group and in non-HCC group respectively. We measured to compare Fuc-Hpx levels in 21 HCC cases before and after curative therapy. Fuc-Hpx levels in all 9 cases but one who received local curative treatments did not decrease after the treatments. The median of Fuc-Hpx level before and after the treatments were 5.23 and 6.77 AU/ml, respectively. On the other hand, in 9 out of 12 HCC cases who received liver transplantation, the median of Fuc-Hpx level significantly decreased from 10.2 to 4.87 AU/ml ($p=0.02$). Significant difference was observed between local curative treatment and liver transplantation ($p=0.001$).

Factors correlated with serum Fuc-Hpx

We evaluated the relationship between serum Fuc-Hpx and clinical parameters in patients with liver diseases (Table 2). Fuc-Hpx in elderly patients and HCV-infected patients was high. High AST (≥ 40 IU/l) and T-Bil (≥ 1.0 mg/dl), and low Plt ($\leq 10 \times 10^4/\mu\text{l}$), PT (< 100 %) and Alb (≤ 3.5 g/dl), were also correlated with high serum Fuc-Hpx level. In addition, the presence of HCC was significantly associated with high Fuc-Hpx ($p<0.001$). On multivariate analysis, low Alb, low PT, and the presence of HCC were significantly correlated with high Fuc-Hpx ($p=0.013$, $p=0.001$, and $p<0.001$, respectively).

The relationship between Fuc-Hpx and tumor factors in combination with three variables that showed correlation with Fuc-Hpx on multivariate analysis was examined in HCC patients (Table 3). None of the tumor factors such as tumor size, tumor number, Vp, or STAGE was correlated with Fuc-Hpx level. Fuc-Hpx was high in patients with high DCP (≥ 40 mAU/mL), while AFP and AFP-L3 were not correlated with Fuc-Hpx. On multivariate analysis, Alb was the only factor correlated with serum Fuc-Hpx level ($p=0.027$).

Utility of Fuc-Hpx for the diagnosis of HCC

The accuracy, sensitivity, and specificity of Fuc-Hpx for the diagnosis of HCC were 69%, 71%, and 63% at a cut-off of 5.95 AU/ml, respectively (Table 4). The diagnostic accuracies of AFP and DCP in the same serum samples were 56% and 58%, sensitivities were 46% and 47%, and specificities were 87% and 91% at cut-offs of 20 ng/ml and 40.0 mAU/ml, respectively. We showed ROC curve of three individual markers in Figure 4. AUROC of Fuc-Hpx for the diagnosis of HCC was 0.739, which was inferior to those of AFP (0.791) but was superior to those of DCP (0.723).

The levels of AFP and DCP gradually increased as the stage progressed, but no correlation was observed between Fuc-Hpx and the stage. The sensitivity of Fuc-Hpx were superior to those of the others in both stage I and >stage II patients. The clinical utility of Fuc-Hpx was equivalent in both stage I and >stage II patients as well as AFP. AUROC was statistical significant superior to DCP in stage I.

The sensitivities of Fuc-Hpx+AFP and Fuc-Hpx+DCP were 84% and 74%, respectively, and the specificities were 66% and 71%, respectively. Sensitivity was improved, whereas specificity was not improved by combination with AFP or DCP.

Fuc-Hpx expression in liver tissue

The expression of Fuc-Hpx in HCC tissue was higher than that in adjacent non-cancerous liver tissue in 4 out of 12 HCC patients, almost equal in one patient, and lower in 7 patients. Median Fuc-Hpx level in HCC tissue was 6.5 AU/ml and was 7.0 AU/ml in adjacent non-cancerous tissue. The difference between them was not statistically significant (Figure 5).

DISCUSSION

Several tumor markers of HCC have been identified, but there is no evidence indicating that the detection of HCC by these markers precedes clinical imaging diagnosis. However, the diagnostic accuracy of the radiological tools is tumor-size-dependent and this approach is expensive.²⁵ Moreover, ultrasound examination is affected by the skill of individual operators. Therefore, it is necessary to find non-invasive, reliable markers for detecting or predicting HCC.

The expression of Fuc-Hpx increased according to the progression of liver disease from hepatitis, cirrhosis, to HCC, and albumin, PT, and the presence of HCC were major factors to determine the expression level. However, we did not observe any correlations between Fuc-Hpx and tumor factors such as tumor size or tumor number. The result is quite different from those of conventional tumor markers such as AFP and DCP. From the analysis of the expression in liver tissues, Fuc-Hpx was produced not only in HCC but also in non-cancerous tissue, meaning that Fuc-Hpx might be a biomarker for hyper-carcinogenic liver rather than a marker for tumor-bearing.

Recently, glycoproteomics and glycomics have been focused on as a post-genomic research field to find diagnostic markers.^{26, 27} Glycosylation is involved in both physiological and pathological events, such as cell growth, migration, differentiation, and tumor invasion. In particular, fucosylation of N-glycan is well known as one of the changes during carcinogenesis of various cancers.^{8, 14, 15, 28} There are several putative mechanisms of elevation of fucosylated proteins in cancers. A tumor marker of hepatocellular carcinoma, AFP-L3, was produced by core-fucosylation of AFP by α -1,6-fucosyltransferase (Fut8), which is overexpressed in advanced liver diseases. However, high expression of Fut8 was also observed in non-cancerous liver cirrhotic tissues as well as HCC tissues.²⁹ α -1,6-fucosylated proteins are normally rare in the blood and are enriched in the bile by proper balance of two secretion pathways of glycoproteins; one is sorting to an apical surface of hepatocytes followed by secretion into bile ducts and the other is

sorting to the basolateral surface followed by secretion into blood vessels.¹³ If hepatocytes become depolarized in hepatocarcinogenesis, these normal secretion pathways cannot work and, thus, fucosylated proteins are elevated in the blood.³⁰

Several reports have been published dealing with the utility of Fuc-Hpx for the diagnosis of HCC.^{8, 9, 21} They reported that Fuc-Hpx is superior to AFP, which has been a standard marker for the detection of HCC. Communale et al. reported that the sensitivity and specificity of Fuc-Hpx for the diagnosis of HCC were high (both 92%) and AUROC curve for Fuc-Hpx was 0.951. In our study, the diagnostic ability in Japanese patients was inferior to the data described above. In previous report, they analyzed 72 HCC patients and 280 patients without HCC; however, 248 out of 280 were non-cirrhotic patients including 20 healthy control. AUROC decreased to 0.8665 when only cirrhotic patients were used as control. We did not include healthy control for AUROC analysis so that the difference of the liver function in non-HCC patients might be one of the reasons for the difference of AUROC between the studies. In addition, the race was different, the median age was higher, and the etiology was different; hepatitis virus infection was a major cause of liver injury in our research, while alcoholism was the main etiology in previous reports. Although it is not clear whether these differences affect the diagnostic utility, it is possible that albumin and PT, which are factors correlated with Fuc-Hpx expression, are different between the studies, which were not precisely indicated in other reports. Despite the differences, Fuc-Hpx expression in HCC patients was high in both studies, indicating that Fuc-Hpx is an effective biomarker for HCC.

Although serum Fuc-Hpx increased in HCC patients, the expression level was not correlated with any tumor factors. Furthermore, Fuc-Hpx levels did not decrease except one case by surgical resection or RFA. On the other hand, 9 out of 12 cases who received liver transplantation, which replaced the hyper-carcinogenic liver with the normal liver, Fuc-Hpx level decreased by the treatment. The result indicated that the major source of Fuc-Hpx in blood is

non-cancerous liver tissue although it might be secreted from HCC by the mechanism described above. Scarce correlation with tumor factors is a disadvantage as a conventional tumor marker. Generally, annual incidence of Hepatocellular carcinoma (HCC) from liver cirrhosis (LC) is known to be 4~8%. On the other hand, the recurrence rate of HCC is reported at an annual rate of 20 %, indicating tumor-bearing liver is hyper-carcinogenic. We interpreted that the difference of Fuc-Hpx between LC and HCC might correspond to hyper-carcinogenic status mentioned above. We inferred that high level of Fuc-Hpx might not be shown under the existence of HCC and be shown under the hyper-carcinogenic liver. We could not refer in this study how effective Fuc-Hpx was as a hyper-carcinogenic marker because we did not prospectively examine. If we assumed that AUROC for the diagnosis of HCC is a surrogate marker of hyper-carcinogenicity, the ability of Fuc-Hpx (0.73) was higher than those of albumin, platelet count and Child-Pugh grade (0.53, 0.66, and 0.67, respectively)

In this study, we demonstrated that Fuc-Hpx could be an effective biomarker of HCC. Future prospective research is necessary to verify the utility of Fuc-Hpx as a marker for hypercarcinogenic liver.

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FIGURE LEGENDS**Figure 1. Control curve of lectin-ELISA**

The concentration is expressed as arbitrary unit (AU) based on the relative concentration against control curve.

Figure 2. Relationship between serum hemopexin and fucosylated-hemopexin

The data of HCC and non-HCC are plotted by closed circles and open circles, respectively. No correlation was demonstrated between serum Hpx and Fuc-Hpx in both groups.

Figure 3. Serum fucosylated-hemopexin concentration

Serum Fuc-Hpx level increased according to the progression of the liver diseases.

Figure 4. Receiver operating characteristic curve of 3 tumor makers of hepatocellular carcinoma

AUROC of Fuc-Hpx for the diagnosis of HCC was 0.739, which was inferior to those of AFP (0.791) but was superior to those of DCP (0.723).

Figure 5. Fucosylated-hemopexin expression in liver tissue

Fuc-Hpx expressions in HCC tissues and corresponding adjacent non-cancerous liver tissues are shown. Closed circles indicate that Fuc-Hpx was lower in cancer tissue than in non-cancer tissue. Closed triangles indicate that Fuc-Hpx expression was higher in cancer tissue. Closed squares indicate that the expression was at the same level in both tissues.

Figure 1. Control curve of lectin-ELISA

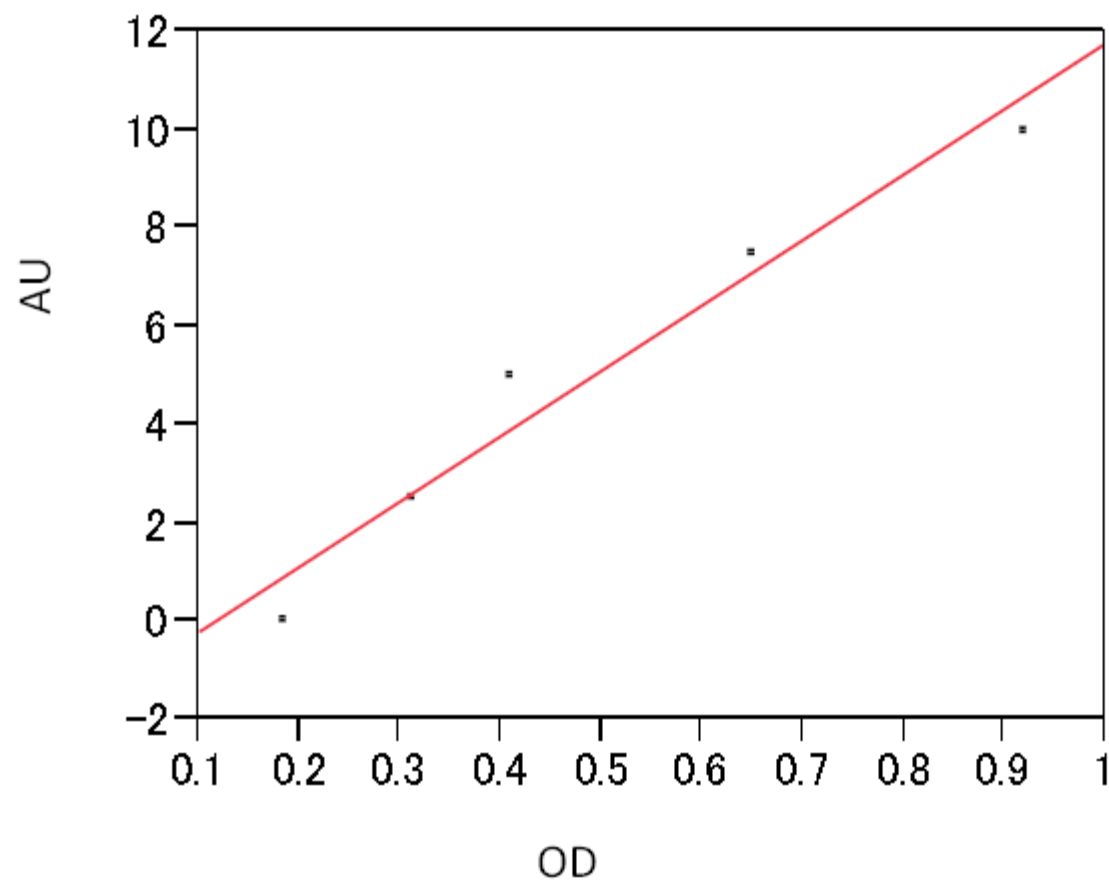


Figure 2. Relationship between serum hemopexin and fucosylated-hemopexin

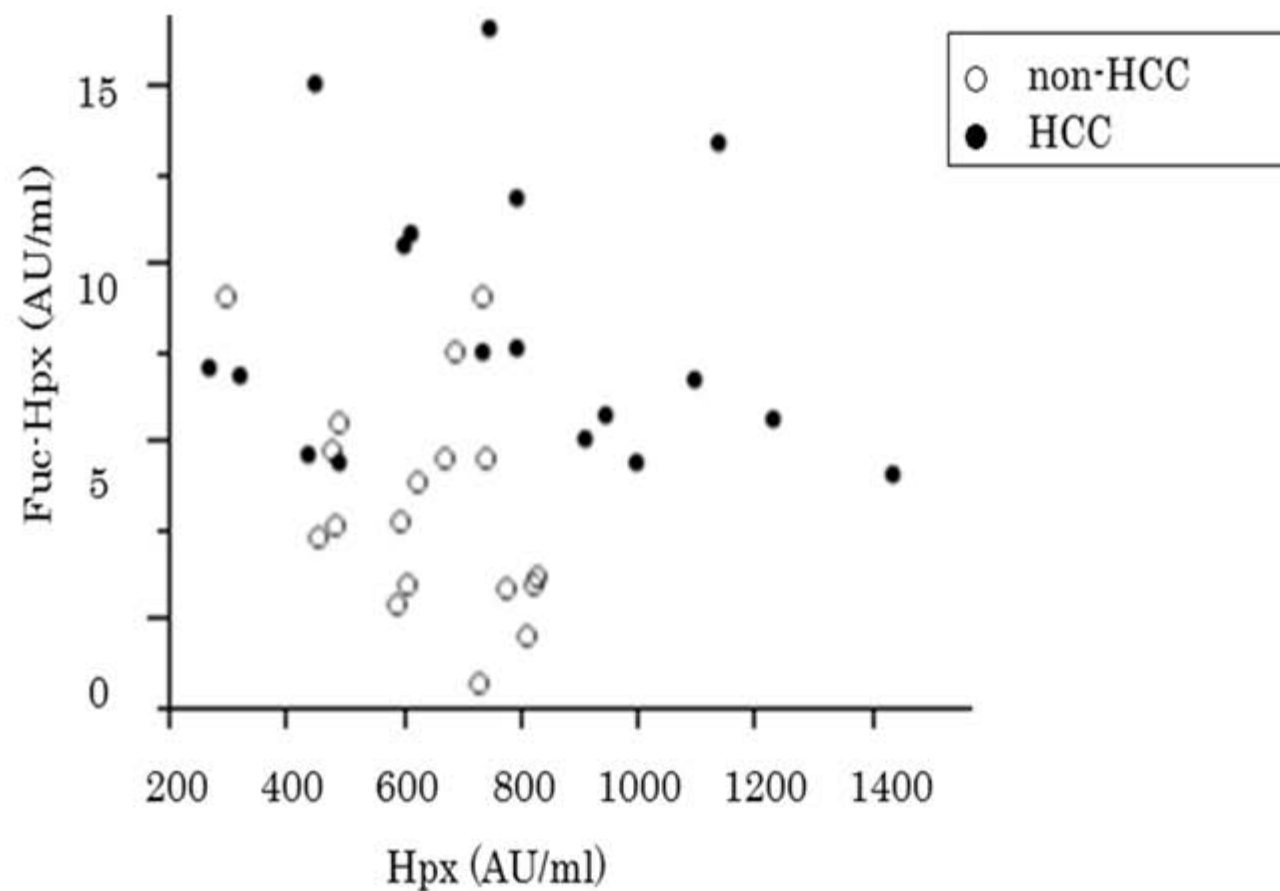
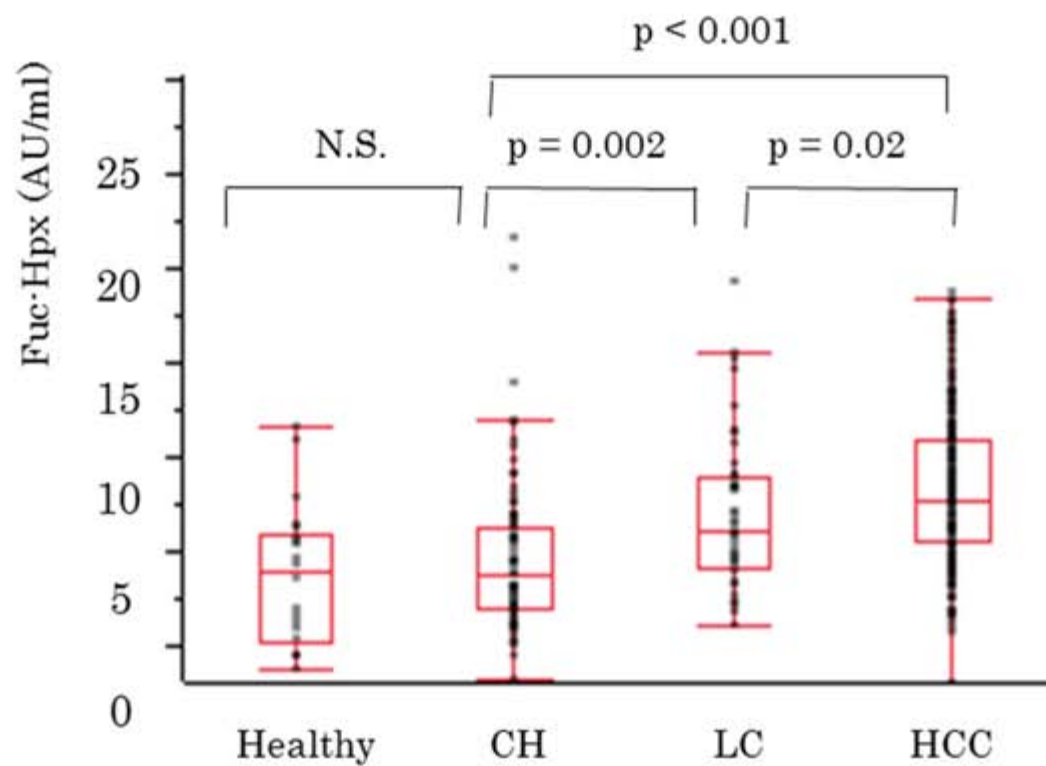


Figure 3. Serum fucosylated-hemopexin concentration



**Figure 4. Receiver operating characteristic curve
of 3 tumor makers of hepatocellular carcinoma.**

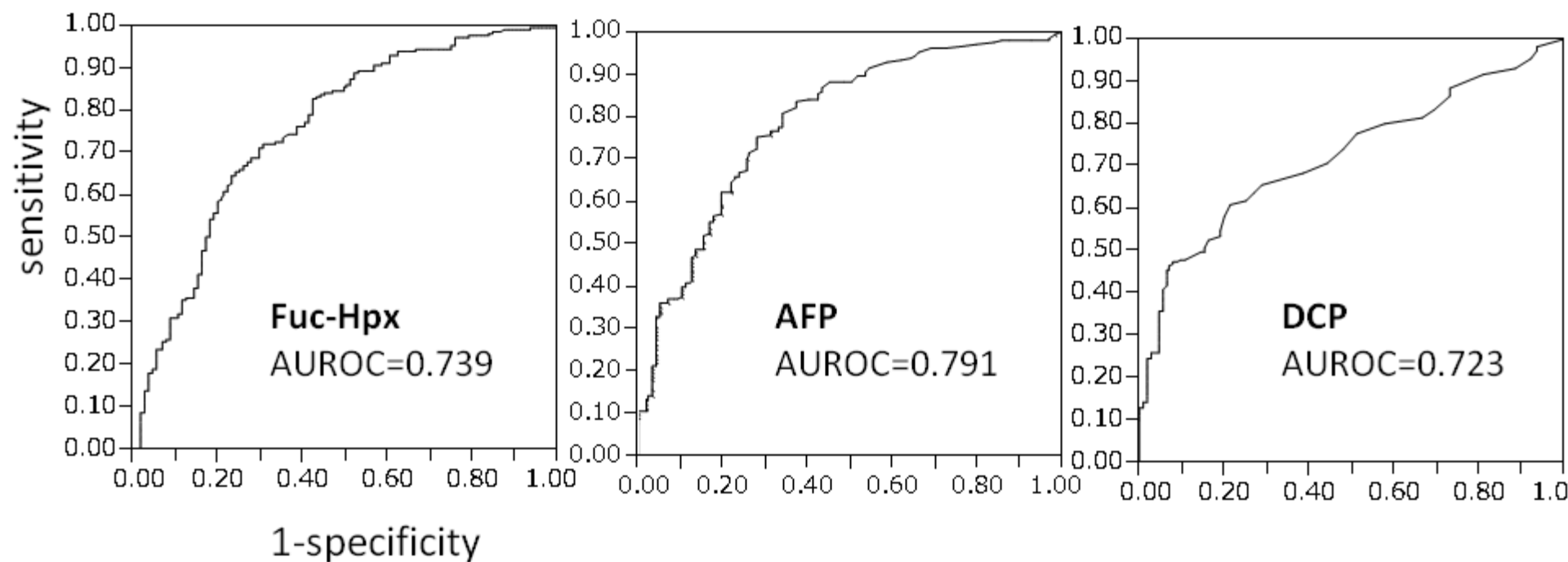


Figure5. Fucosylated-hemopexin expression in liver tissue

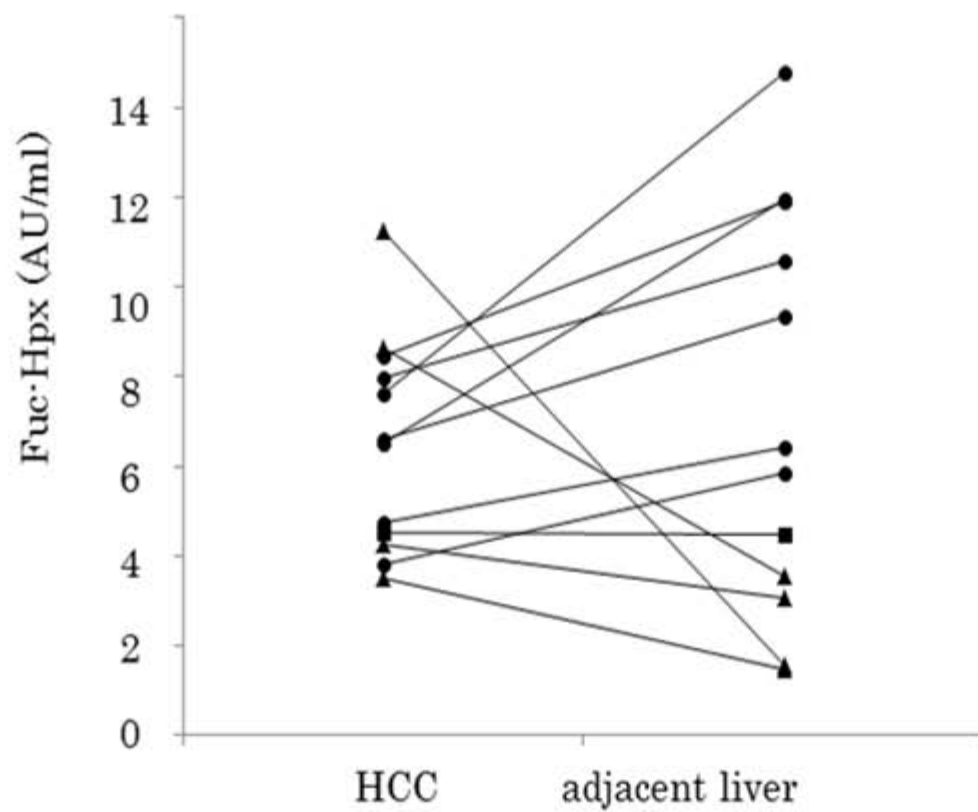


Table 1. Baseline characteristics

Disease	Healthy control	non-HCC		HCC	<i>p</i> value
		CH	LC		
Number of patients	22	85	45	331	
Age (years)	65 (60-71)	50 (41-55)	59 (48-66)	71 (64-76)	<0.001
Sex Male (%)	72	61	71	66	N.S.
Etiology (%) HBV/HCV/others		36/ 60/ 4	29/ 29/ 42	15/ 73/ 14	<0.001
Child-Pugh grade A / B or C (%)		94/ 6	53/47	79/ 21	<0.001
Stage I/II/III/IV (%)				31/35/20/14	

CH, chronic hepatitis; LC, liver cirrhosis; HCC, hepatocellular carcinoma; HBV, positive for hepatitis B virus surface antigen; HCV, positive for hepatitis C virus antibody; others, alcohol-induced liver injury, NASH, autoimmune hepatitis, or liver disease of unknown origin.

Statistical significance was set at $p < 0.05$.

Table 2. Fucosylated-hemopexin expression in patients with liver diseases

Variables		Fuc-Hpx (AU/ml)	Univariate p-value	Multivariate p-value
Age (years)	≤ 65	6.2 (3.5-9.0)	<0.001	0.600
	> 65	7.5 (5.3-10.8)		
Sex	Male	7.2 (4.5-10.2)	0.690	
	Female	6.8 (4.5-9.7)		
Etiology	HBV	6.3 (4.1-9.8)	0.025	0.410
	HCV	7.4 (4.8-10.4)		
	others	6.2 (3.6-8.8)		
Diagnosis	non-HCC	4.6 (2.5-7.1)	<0.001	<0.001
	HCC	7.6 (5.6-10.8)		
Plt (×10 ⁴ /μl)	> 10	6.6 (3.9-9.8)	0.002	0.800
	≤ 10	8.1 (5.3-10.4)		
PT (%)	≥ 100	6.3 (4.0-9.4)	<0.001	0.001
	<100	7.9 (5.6-11.0)		
Albumin (g/dl)	> 3.5	5.8 (3.3-9.0)	<0.001	0.013
	≤ 3.5	8.1 (6.3-11.1)		
T-Bil (mg/dl)	< 1	6.7 (4.0-9.8)	0.031	0.990
	≥ 1	7.3 (5.3-10.5)		
AST (IU/l)	< 40	5.3 (2.8-8.4)	<0.001	
	≥ 40	7.4 (5.4-10.3)		
ALT (IU/l)	< 40	6.5 (4.0-9.4)	0.100	
	≥ 40	7.0 (4.8-10.0)		
Child-Pugh grade	A	6.7 (4.2-9.8)	0.004	
	B+C	7.7 (5.8-11.1)		

Abbreviations of etiology are the same as those indicated in Table 1. Fuc-Hpx, fucosylated-hemopexin; HCC, hepatocellular carcinoma; Plt, platelet count; PT, prothrombin time; T-Bil, total bilirubin ; AST, aspartate aminotransferase; ALT, alanine aminotransferase

Statistical significance was set at $p < 0.05$.

Table 3. Relationship between clinical parameters and fucosylated-hemopexin in HCC patients

Variables		Fuc-Hpx (AU/ml)	Univariate p-value	Multivariate p-value
Albumin (g/dl)	> 3.5	7.1 (4.5-10.3)	<0.001	0.027
	≤ 3.5	8.1 (6.4-11.2)		
PT (%)	≥ 100	7.2 (5.0-9.8)	0.004	0.053
	< 100	8.3 (6.1-11.4)		
AFP (ng/ml)	< 20	7.5 (5.0-10.5)	0.083	
	≥ 20	7.8 (6.0-10.9)		
AFP-L3 (%)	< 10	7.6 (5.5-10.4)	0.379	
	≥ 10	7.8 (6.1-11.2)		
DCP (mAU/ml)	< 40	7.4 (5.0-10.2)	0.021	0.063
	≥ 40	8.1 (6.0-11.2)		
Tumor size (mm)	< 20	7.4 (5.5-10.1)	0.190	
	≥ 20	8.0 (5.5-11.2)		
Tumor number	single	7.4 (5.0-10.4)	0.230	
	multiple	7.7 (6.1-10.9)		
Vp	yes	7.6 (5.5-10.4)	0.800	
	no	7.7 (4.6-11.0)		
Stage	I+II	7.5 (5.3-10.2)	0.420	
	III+IV	7.7 (5.6-11.1)		

HCC, hepatocellular carcinoma; Fuc-Hpx, fucosylated-hemopexin; PT, prothrombin time; AFP, alpha-fetoprotein; AFP-L3, fucosylated AFP; DCP, des-γ-carboxy prothrombin

Statistical significance was set at $p < 0.05$.

Table 4. Utilities of tumor markers for the diagnosis of HCC

		Fuc-Hpx	AFP	DCP
All stage	AUROC	0.739	0.791	0.723
	Sensitivity (%)	71	46**	47**
	Specificity (%)	63	87*	91**
	Accuracy (%)	69	56	58
	PPV (%)	83	91	94
	NPV (%)	46	36	37
Stage I	AUROC	0.720	0.785	0.599*
	Sensitivity (%)	75	45**	28**
	Specificity (%)	63	87**	91**
	Accuracy (%)	69	67	60
	PPV (%)	63	76	76
	NPV (%)	75	63	56
> Stage II	AUROC	0.737	0.802	0.785
	Sensitivity (%)	71	46**	57*
	Specificity (%)	63	87**	91**
	Accuracy (%)	69	56	68
	PPV (%)	83	91	93
	NPV (%)	46	36	51

HCC, hepatocellular carcinoma; Fuc-Hpx, fucosylated-hemopexin; AFP, alpha-fetoprotein; DCP, des-γ-carboxy prothrombin; AUROC, areas under the receiver operating characteristic curve; PPV, positive predictive value; NPV, negative predictive value

*Statistically difference between Fuc-Hpx and the other marker in the given group (p <0.05).

** Statistically difference between Fuc-Hpx and the other marker in the given group (p <0.001).