Table 2. Persistence of HBV Infection in the Patients With Acute Hepatitis Who Did or Did Not Receive Lamivudine or Steroid

Treatment	Total	Genotypes/Subgenotypes							
		Aa (n = 8)[a]	Ae (n = 32)[a]	Ba (n = 21)[a]	Bj (n = 10)[a]	Cs (n = 10)[a]	Ce (n = 167)[a]	D (n = 3)[a]	G (n = 5)[a]
Total (n = 256)	3/256 (1.2%)	0	2/32 (6%)c	1/21 (5%)	0	0	0	0	0
Lamivudine (n = 36)[b]	0/36 (0%)	0/1 (0%)	0/9 (0%)	0/2 (0%)	0	0/1 (0%)	0/19 (0%)	0/2 (0%)	0/2 (0%)
Steroid (n = 16)[b]	0/16 (0%)	0	0/3 (0%)	0	0	0/1 (0%)	0/12 (0%)	0	0
Neither	3/210 (1.4%)	0/7 (0%)	2/23 (9%)c	1/19 (5%)	0/10 (0%)	0/8 (0%)	0/139 (0%)	0/1 (0%)	0/3 (0%)

[a]Exclusive of 40 patients with fulminant hepatitis and 5 without clinical data available.
[b]Six patients received steroid along with lamivudine.
[c]$P < .05$, Ae vs. non-Ae.

Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis. Table 3 compares demographic, clinical, and virological characteristics between the 40 patients with fulminant and the 261 with acute self-limited hepatitis for whom analysis was feasible. Patients with fulminant hepatitis were significantly older (44.7 ± 16.3 vs. 36.0 ± 14.3 years, $P = .0017$), less predominantly male (43% vs. 71%, $P = .0005$) and less often positive for HBeAg (23% vs. 60%, $P < .0001$) than those with acute hepatitis. Peak ALT and total bilirubin levels were higher for fulminant than acute hepatitis ($P < .0001$), reflecting severe hepatic lesions. Notably, the median HBV DNA level was lower in patients with fulminant than acute hepatitis (4.89 vs. 5.19 log copies/mL, $P = .0178$); the frequency of undetectable HBV DNA at the presentation was higher in fulminant hepatitis (25% vs. 10%, $P = .0086$). Lamivudine or steroid was given significantly more often to patients with fulminant hepatitis.

There were marked differences in the distribution of genotypes between patients with fulminant and acute hepatitis. HBV/Ae was less frequent (0% vs. 13%, $P = .0121$), whereas Bj was more often (30% vs. 4%, $P < .0001$) in patients with fulminant than acute hepatitis. Although HBV/Ce tended to be less frequent in patients with fulminant than acute hepatitis (55% vs. 65%), the difference fell short of being significant.

Precore stop-codon mutation (G1896A) and core-promoter double mutation (A1762T/G1764A) were more

Table 3. Comparison Between Patients With Fulminant and Acute Self-Limited Hepatitis Who Were Infected With HBV

Features	Fulminant (n = 40)	Acute (n = 261)	P Value
Age (years)	44.7 ± 16.3	36.0 ± 14.3	.0017
Men	17 (43%)	186 (71%)	.0005
HBeAg positive	9 (23%)	157 (60%)	<.0001
ALT (IU/L)	4315 ± 2889	2284 ± 1221	<.0001
Total bilirubin (mg/dL)	20.5 ± 16.4	8.3 ± 7.3	<.0001
HBV DNA (log copies/mL)			
Median	4.89	5.19	.0178
(range)	(2.00-8.44)	(2.00-9.06)	
<2.00 (undetectable)	10 (25%)	27 (10%)	.0086
Treatment			
Lamivudine	16 (40%)	37 (14%)	.0003
Steroid	9 (23%)	16 (6%)	.0022
Genotypes/subgenotypes			
Aa	1 (2.5%)	9 (3%)	NS
Ae	0 (0%)	33 (13%)	.0121
Ba	1 (2.5%)	21 (8%)	NS
Bj	12 (30%)	10 (4%)	<.0001
Cs	1 (2.5%)	10 (4%)	NS
Ce	22 (55%)	170 (65%)	NS
D	2 (5%)	3 (1%)	NS
G	1 (2.5%)	5 (2%)	NS
Mutations[a]			
nt 1753 and/or nt1754[b]	11/30 (37%)	28/234 (12%)	.0003
A1762T/G1764A	15/30 (50%)	39/234 (17%)	<.0001
G1896A	16/30 (53%)	21/234 (9%)	<.0001
G1899A	7/30 (23%)	8/234 (3%)	<.0001

[a]Exclusive of 37 patients in whom precore region and core-promoter could not be amplified by PCR.
[b]T1753C/A/G and/or T1754C/A/G.

Table 4. Multivariate Analysis for Factors Independently Associated With Fulminant Hepatitis

Factors	Odds Ratio	95% Confidence Interval	*P* Value
Age (yr)			
<34[a]	1		
≥34	3.472	1.094-11.023	.0347
Sex			
Male	1		
Female	2.272	0.780-6.613	.1323
HBeAg			
Positive	1		
Negative	3.344	1.065-10.506	.0387
ALT (IU/L)			
<2200[a]	1		
≥2200	2.094	0.683-6.414	.1957
Total bilirubin (mg/dL)			
<10.0[a]	1		
≥10.0	18.818	4.320-81.980	<.0001
HBVDNA (log copies/mL)			
<5.00[a]	1		
≥5.00	1.042	0.367-2.961	.9383
Treatment			
Lamivudine (−)	1		
Lamivudine (+)	2.650	0.814-8.625	.1056
Steroid (−)	1		
Steroid (+)	2.515	0.668-9.472	.1728
Genotypes/Subgenotypes			
Non-Bj	1		
Bj	7.001	1.737-28.228	.0062
Mutations			
nt 1753 and/or 1754[b]			
Absent	1		
Present	2.316	0.698-7.683	.1700
A1762T/G1764A			
Absent	1		
Present	1..013	0.295-3.478	.9841
G1896A			
Absent	1		
Present	4.157	1.265-13.657	.0189
G1899A			
Absent	1		
Present	2.525	0.534-11.949	.2427

[a]Median values.
[b]T1753C/A/G or T1754C/A/G.

frequent in patients with fulminant than acute hepatitis (53% vs. 9% and 50% vs. 17%, respectively, $P < .0001$ for each). Likewise, mutations in core-promoter at nt 1753 or nt 1754, and G1899A mutation were more frequent in patients with fulminant than acute hepatitis ($P = .0003$ and $P < .0001$, respectively).

Factors Independently Associated With the Development of Fulminant Hepatitis. Various factors found in association with fulminant hepatitis were evaluated for the independence in multivariate analysis (Table 4). Age 34 years or older (odds ratio 3.47 [95% confidence interval 1.09-11.02], $P = .035$), HBV/Bj (7.00 [1.74-28.23], $P = .006$), HBeAg-negative (3.34 [1.07-10.51], $P = .039$), total bilirubin ≥10.0 mg/dL (18.82 [4.32-81.98], $P < .0001$) and G1896A (4.16 [1.27-13.66], $P = .019$) were independent risk factors for the development of fulminant hepatitis.

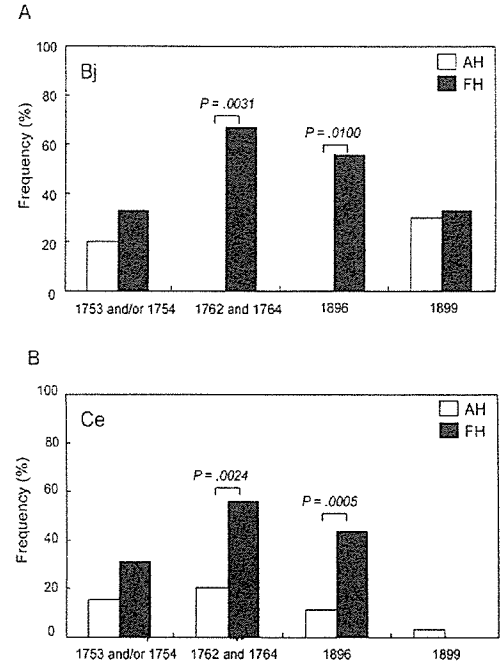

Fig. 2. Frequencies of precore and core-promoter mutations compared between patients with fulminant and acute self-limited hepatitis who were infected with HBV/Bj (A) or Ce (B).

In view of the majority of Japanese patients who were infected with Bj or Ce, mutations in the precore region and core-promoter were compared between those with fulminant and acute self-limited hepatitis for each subgenotype (Fig. 2). G1896A and A1762T/G1764A were significantly more frequent in patients with fulminant than acute hepatitis infected with either HBV/Bj or Ce (56% vs. 0% and 67% vs. 0% for Bj or 44% vs. 11% and 56% vs. 22% for Ce, respectively, $P \leq .01$ for all). For the patients infected with HBV/Bj, in particular, precore and core-promoter mutations were highly frequent in those with fulminant hepatitis (56% and 67%, respectively), whereas they occurred in none of those with acute hepatitis. G1899A was equally frequent in both patients with fulminant and acute hepatitis infected with HBV/Bj; it was rarely seen in those with Ce. Mutations involving nt 1753 or nt 1754 tended to be more frequent in patients with fulminant than acute hepatitis.

***Replication of the Wild-Type HBV as Well as Precore and Core-Promoter Mutants* In Vitro.** Full-length HBV DNA of the wild-type HBV/Bj from a patient with chronic hepatitis B was incorporated with G1896A or A1762T/G1764A mutation *in vitro*. Another plasmid was constructed with HBV/Bj_58 carrying G1896A from a fulminant patient. Figure 3 compares

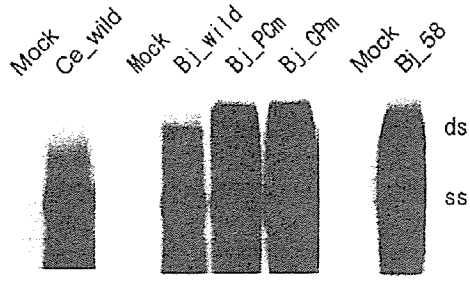

Fig. 3. Southern blot analysis for replicative activity of the wild-type HBV clones (HBV/Ce_wild and Bj_wild), as well as mutants with precore (Bj_PCm) or core-promoter (Bj_CPm) mutation, and Bj_58 with precore stop-codon mutation obtained from a patient with fulminant hepatitis.

densities of migration patterns of the wild-type, precore, and core-promoter mutants in Southern blotting analysis. The wild-type HBV/Bj displayed a band for single-stranded (ss) HBV DNA and an additional band for double-stranded (ds) HBV DNA. Of note, the densities of these bands were far greater for HBV/Bj mutants incorporated with precore or core-promoter mutation, as well as Bj_58 with the precore mutation, thereby indicating much enhanced replicative activity of precore or core-promoter mutant *in vitro*. Although the intracellular HBV DNA level for the wild-type HBV/Bj was comparable with that for the wild-type Ce (Fig. 3), the extracellular HBV DNA level in culture media was approximately threefold higher for Bj than Ce ($P < .01$) (Sugiyama M et al., manuscript in submission).

Discussion

A nationwide survey of genotypes/subgenotypes in patients with acute HBV infection from Japan during the past 2 decades has examined their influence on fulminant and chronic outcomes. The study was feasible in a country where mass vaccination has not been performed because of an extremely high efficacy of immunoprophylaxis on babies born to carrier mothers; it has decreased the persistent HBV carrier rate from 1.4% to 0.3%.[26] Acute HBV infection keeps increasing, however, predominantly through promiscuous sexual contacts in Japan.

Fulminant hepatitis developed rather frequently in 40 of the 301 (13%) patients. This is likely due to selection bias because the study included only patients who were hospitalized for acute hepatitis B. Exclusion of subclinical cases of acute HBV infection would have overestimated the incidence of fulminant hepatitis. Regardless of such a selection bias, influence of HBV genotypes/subgenotypes was evident in comparison with the 40 patients with fulminant and the 261 with acute self-limited hepatitis. Remarkably, none of the 33 patients infected with HBV/Ae developed fulminant hepatitis. In sharp contrast, 12 of the 22 (55%) patients infected with HBV/Bj developed it. Furthermore, both precore (G1896A) and core-promoter (A1762T/G1764A) mutations were detected significantly more frequently in patients with fulminant than acute self-limited hepatitis. In infection with HBV/Bj, in particular, the frequency of core-promoter mutation was much higher in the patients with fulminant (67%) than that reported in those with chronic hepatitis (16%).[27] Precore and core-promoter mutations are very frequent in patients with fulminant hepatitis from Asia[28-30] and the Middle East.[31] The failure in detecting these mutations in Western countries[32-35] could be attributed to frequent HBV/Ae and rare Bj there. In multivariate analysis, HBeAg-negative, HBV/Bj, and the precore stop-codon mutation for G1896A were independent risk factors for the development of fulminant hepatitis (Table 4). Various mutations at nt 1753 for enhanced HBV replication,[36] as well as those adjacent at nt 1754 prevailing in patients with fulminant hepatitis,[37] occurred more frequently in patients with fulminant than acute self-limited hepatitis. Host factors, such as age and total bilirubin, contributed to the development of fulminant hepatitis as well (Table 4).

In vitro replication analysis demonstrated the intracellular HBV DNA level of the wild-type HBV/Bj comparable with that of the wild-type Ce (Fig. 3). The extracellular HBV DNA level of HBV/Bj-clone, however, was much higher than those of the other genotypes, indicating its strong inclination to be secreted from cells (Sugiyama et al., manuscript in submission). Such a high concentration of HBV/Bj in the circulation of patients would rapidly and extensively promote infection of hepatocytes.

Enhanced replication capacities of precore (G1896A) and core-promoter (A1762T/G1764A) mutants for HBeAg-minus and -reduced phenotypes, respectively, were demonstrated in a replication model *in vitro* (Fig. 3). These observations were concordant with those in previous reports[38,39]; however no data are available on the replication of HBV/Bj *in vitro*, either of the wild-type or variants with these mutations. Extremely high intracellular and extracellular expressions of viral DNA were observed for the HBV/Bj clone with precore stop-codon mutation from a patient with fulminant hepatitis. These results might implicate high replication due to mutations of precore region and core-promoter in the induction of fulminant hepatitis. In support of this view, Bocharov et al.[40] have proposed that enhanced HBV replication would efficiently stimulate immune reactions, represented by the cytotoxic T lymphocyte response, suggesting that enhanced replication by HBV/Bj or precore/

core-promoter mutation might lead to fulminant hepatitis.

That HBV DNA levels were lower in patients with fulminant than acute hepatitis, despite a high replication capacity of HBV/Bj incriminated in the development of fulminant hepatic failure, may seem surprising. Because destruction of hepatocytes proceeds swiftly in patients with fulminant hepatitis, hepatic mass for HBV to thrive would have been extremely reduced in them at presentation. As a consequence, some patients with fulminant hepatitis B are without serum HBsAg; they are diagnosed by high-titered IgM anti-HBc.[41] On the contrary, HBV DNA levels were higher in the patients with HBV/Ae than Bj (Table 1); those with Ae tend to delay reducing HBV DNA, some of whom have chronic outcome. Combined, correlating HBV DNA levels with the clinical outcome in acute HBV infection would be difficult.

A wide variation has been seen in the rate of persistence after acute HBV infection in adulthood. No chronic outcomes of acute hepatitis B were seen in female recipients of red blood cells contaminated with HBV (0/28)[42] or patients in an acupuncture-associated outbreak (0/35).[43] In marked contrast, they ranged from 0.2% (14/715) in Greece[44] through 2.7% (1/37) in university students in Taiwan[45] to 10.4% (5/8) in Alaskan Eskimos[46] and 12.1% (7/58) in Germany.[47] HBV genotypes are implicated in a high rate of persistence in European countries where HBV/A is predominant.[48] In Japan, also, adulthood infection tends to persist longer with HBV/A than B or C (23% 3/13 vs. 13% 1/8 or 12% 3/25).[49] In the current series on 256 patients with acute hepatitis B in Japan who were followed rigorously, HBV infection persisted in only three (1%), representing 2 of the 32 (6%) with HBV/Ae and 1 of the 21 (5%) with Ba. Hence, 99% of patients lost their HBsAg by 6 months. Persistence of HBV observed in the patients with HBV/Ae (6%) is less frequent than that in 4 of the 31 (13%) patients with Ae from a hospital in metropolitan Tokyo.[49] The difference would be ascribable, at least in part, to lamivudine given to some patients in this study (18%). All patients treated with lamivudine recovered from acute hepatitis, whereas none of the three patients with chronic outcome had received antiviral treatment during their acute phase of illness, indicating that lamivudine might be able to prevent the chronic outcome. Likewise, some patients from metropolitan Tokyo, in whom HBV persisted,[49,50] had received immunosuppressants in the acute phase of infection before referral to their hospital.

Using cell culture and chimeric mice models for the replication system of different genotype/subgenotype clones, we have observed that the replication of HBV is the highest for HBV/Bj or C and the lowest for Aa/Ae (Sugiyama M et al., manuscript in submission). It is probable that the propensity of HBV/A infection to chronicity would be due to less intensive immune response against its slow viral dynamics. Taken together, the infection with HBV/A appears to persist longer than those with the other genotypes; this needs to be confirmed by further investigation in patients from various countries.

In conclusion, persistence of HBV after acute infection is rare and occurs more often in patients infected with HBV/Ae than others. Fulminant outcome is frequent in hospitalized patients and associated with HBV/Bj accompanied by the lack of serum HBeAg as well as high replication due to precore stop-codon mutation (G1896A), a finding supported by an *in vitro* replication model.

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Usefulness of elastometry in evaluating the extents of liver fibrosis in hemophiliacs coinfected with hepatitis C virus and human immunodeficiency virus

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Abstract

The newly developed elastometer, FibroScan®, was utilized to evaluate liver fibrosis in hepatitis C virus (HCV)- and human immunodeficiency virus (HIV)-coinfected 33 hemophiliacs and HIV-uninfected 24 patients with chronic hepatitis C. Chronicity in the liver was categorized into 4 stages by abdominal ultrasound (AUS): 1 (normal or fatty liver); 2 (chronic liver disease, mild); 3 (moderate); and 4 (severe). Stiffness of the liver was significantly increased as AUS stages advanced: 5.4 ± 2.2 ($N=3$) versus 7.5 ± 2.7 ($N=9$), in stage 1; 4.9 ± 1.7 ($N=2$) versus 9.9 ± 6.0 ($N=10$), in stage 2, 13.5 ± 4.7 ($N=5$) versus 12.9 ± 5.9 ($N=6$), in stage 3, and 22.0 ± 9.5 ($N=14$) versus 28.1 ± 21.3 ($N=8$), in stage 4, in non-HIV group and in HIV group, respectively ($P=0.004$ and 0.007). Stiffness was correlated with AUS stages ($r=0.740$, $P<0.001$), platelet counts (PLT; $r=-0.642$, $P=0.001$) and 7S domain of type IV collagen (IV-coll; $r=0.480$, $P=0.024$) in non-HIV group, while in HIV group, with IV-coll ($r=0.801$, $P<0.001$), AUS stages ($r=-0.603$, $P<0.001$), procollagen type III peptides (P-III-P; $r=0.621$, $P=0.001$), PLT ($r=-0.480$, $P=0.005$), and hyaluronic acid ($r=0.433$, $P=0.027$). FibroScan® is absolutely noninvasive and can be the alternative to liver biopsy, especially in patients with bleeding tendency.

Keywords: Liver fibrosis; HCV; HIV; Stiffness

1. Introduction

It has been well documented that coinfection of hepatitis C virus (HCV) and human immunodeficiency virus (HIV) might accelerate the progression of liver fibrosis, as compared with HCV infection alone [1]. However, especially in patients with hemophilia coinfected with HCV and HIV, it is practically difficult to perform liver biopsy, the established gold standard to evaluate the extents of liver fibrosis, because of their bleeding tendency. Moreover, tiny biopsied specimens, corresponding to only one forty thousandth of entire liver volume, may sometimes cause misleading explanations. Although rough estimation of liver fibrosis can be performed by abdominal ultrasound (AUS), it would be somewhat subjective and dependent on technical experience. In addition, several serum markers for liver fibrosis such as procollagen type III peptides (P-III-P), 7S domain of type IV collagen (IV-coll), and hyaluronic acid, have been commercially available, however, those substances might just reflect overproduction of collagen in vivo.

Recently, a new noninvasive device to quantify liver fibrosis, FibroScan®, was developed by Echosens (Paris, France). This device is based on one-dimensional transient elastography, using both ultrasound (5 MHz) and low-frequency (50 Hz) elastic waves, whose propagation velocity is directly proportional to elasticity [2]. Usefulness of this device to assess the extents of liver fibrosis in patients with chronic hepatitis was already reported from France [3], and from Japan [4]. In this study, we attempted to evaluate its usefulness

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in hemophilacs coinfected with HCV and HIV, in whom liver biopsy is ordinarily contraindicated, as well as in non-hemophiliacs infected by HCV alone.

2. Patients and methods

Hemophiliacs coinfected with HCV and HIV or non-hemophiliacs infected by HCV alone, who consulted Division of AIDS Research Center or Division of Gastroenterology, International Medical Center of Japan, from February to April, 2004, were randomly enrolled in this study, after informed consent was given. The patient received elastometry with FibroScan® version 2 (FibroScan® 502: Echosens, Paris, France), as previously reported [4], at the same time with B-mode AUS, by two hepatologists (N.M. and M.I.) with over 15-year experience. The elasticity of the liver was measured 10 times and the median value, which was automatically calculated, was considered as Stiffness (KPa). AUS findings of the liver were categorized into 4 stages, based on the extents of surface irregularity, dullness of the edge, heterogeneity of internal echogenecity, blurriness of hepatic veins, and imbalance in size of both lobes (hypertrophy of the caudate lobe and/or atrophy of the right lobe): (1) normal or fatty liver; (2) chronic liver disease, mild; (3) moderate; and (4) severe. On the same day, serum levels of albumin, alanine aminotransferase (ALT), and liver fibrosis markers (P-III-P, IV-coll, and hyaluronic acid), were measured. HCV genotype and peripheral platelet counts (PLT) were also determined. Only in HIV group, CD4 counts and HIV RNA concentrations were determined. The correlation between Stiffness and AUS stage, PLT, or serum liver fibrosis markers were investigated.

In patients, where the age of blood transfusion or the first infusion of clotting factor concentrates could be identified, annual progression rate of liver fibrosis (R-value: KPa/year) was arbitrarily calculated, according to the following equation. R-value = [Stiffness − 5.4]/[Age (at the elastometry) − Age (of the HCV infection)], where 5.4 is the mean value of Stiffness in patients with normal AUS finding (data shown in Section 3).

2.1. Statistical analysis

The data were shown as mean ± standard deviation. The correlation between the Stiffness and AUS stage was determined by Kruskal–Wallis test. The correlation between the parameters was determined by Pearson's correlation coefficient or Spearman's correlation coefficient. The comparisons between the data were analyzed by Mann–Whitney U-test or Fisher's exact test.

3. Results

Thirty-three hemophiliacs coinfected with HCV and HIV (HIV group; hemophilia A/B = 23 patients/10 patients; all males) and 24 non-hemophiliacs infected by HCV alone (non-HIV group; 12 males and 12 females), were enrolled in this study, and received B-mode AUS and elastmetry at the same time. The demographic features and laboratory data of both groups were shown in Table 1. All the patients in HIV group have been successfully treated by highly active antiretroviral therapy (HAART) since 1996, and in 23 patients, HIV RNA concentrations were maintained less than 50 copy/ml. Seven out of 21 (33%) patients in HIV group were infected by HCV with uncommon genotype in Japan (1a or 3a). The patients were classified into 4 stages on AUS: stage 1: 3 and 9; stage 2: 2 and 10; stage 3: 5 and 6; stage 4: 14 and 8, in non-HIV group and HIV group, respectively. Apparently, in non-HIV group, most of the patients were older and had more advanced stages of chronic liver disease, as compared with those in HIV group. Stiffness of the liver in each AUS stage was shown as follows: 5.4 ± 2.2 and 7.5 ± 2.7, in stage 1; 4.9 ± 1.7 and 9.9 ± 6.0, in stage 2, 13.5 ± 4.7 and 12.9 ± 5.9, in stage 3, 22.0 ± 9.5 and 28.1 ± 21.3, in stage 4,

Table 1
Demographic features and laboratory data of non-HIV group and HIV group

Parameters	Non-HIV group (N = 24)	HIV group (N = 33)	P-value
Age	69 ± 13	39 ± 11	0.000[a]
Gender (M/F)	12/12	33/0	0.000[b]
Albumin (g/dl)	4.0 ± 0.4	4.3 ± 0.3	0.004[a]
ALT (U/l)	60 ± 45	67 ± 54	NS[a]
PLT ($\times 10^4/\mu l$)	12.5 ± 5.2	19.0 ± 8.6	0.003[a]
CD4 counts (μl^{-1})	n.d.	442 ± 230	
P-III-P (U/ml)	1.2 ± 0.3	0.9 ± 0.3	0.003[a]
IV-coll (ng/ml)	8.3 ± 3.1	6.2 ± 2.7	0.013[a]
Hyaluronic acid (ng/ml)	335 ± 399	210 ± 190	NS[a]
HCV genotype (1/1a/1a + 1b/1b/2a/2b/3a/3a + 2b)	2/0/0/8/1/2/0/0	4/4/1/5/3/2/1/1	
AUS stage (1/2/3/4)	3/2/5/14	9/10/6/8	0.031[c]

n.d.: not determined, NS: not significant. The abbreviations used are: ALT, alanine aminotransferase; PLT, platelet counts; P-III-P, procollagen type III peptides; IV-coll, 7S domain of type IV collagen; AUS, abdominal ultrasound.

[a] Mann–Whitney U-test.

[b] Fisher's exact test.

[c] Pearson's χ^2-test.

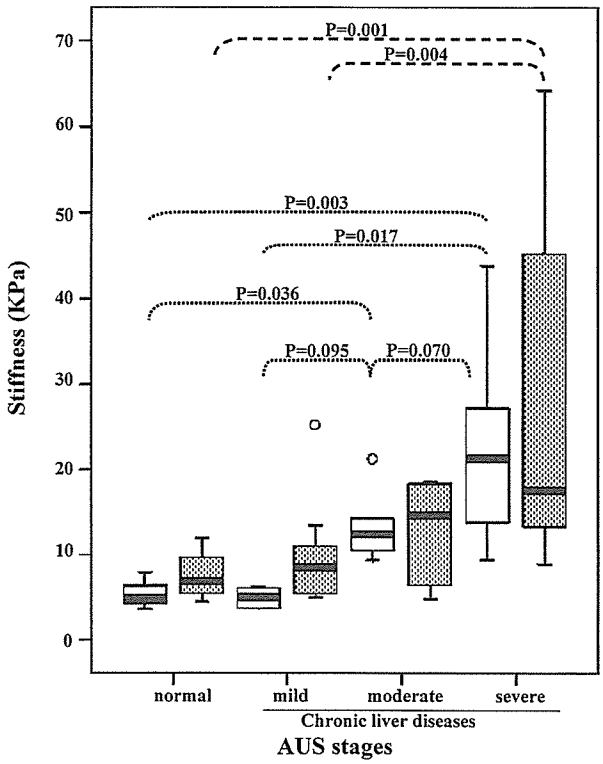

Fig. 1. Box plots of Stiffness for each AUS stage in non-HIV group and HIV group. The *top* and *bottom* of the boxes are the first and third quartiles, respectively. The length of the box represents the interquartile range within which 50% of the values were located. The *line* through the middle of each box represents the median. The *error bars* show the minimum and maximum values. Open boxes and dotted boxes show the data of non-HIV group and those of HIV group, respectively. The *P*-value was calculated by Mann–Whitney *U*-test.

in non-HIV group and in HIV group, respectively ($P=0.004$ and 0.007, by Kruskal–Wallis test in each group). There was no significant difference between non-HIV group and HIV group, in each AUS stage. Box plots of Stiffness for each AUS stage were demonstrated in Fig. 1. In non-HIV group, there existed significant difference in Stiffness between stages 4 and 2 ($P=0.017$), between stages 4 and 1 ($P=0.003$), and between stages 3 and 1 ($P=0.036$). In addition, there was marginal difference between stages 4 and 3 ($P=0.070$), and between stages 3 and 2 ($P=0.095$). While, in HIV group, Stiffness in stage 4 was significantly higher than that in stage 1 ($P=0.001$), and that in stage 2 ($P=0.004$).

To examine usefulness of elastometry in differentiating advanced stage of chronic liver disease from non-advanced one, similar analysis was conducted after patients of stages 2 and 3 were combined together. Chronicity in the liver was simply categorized into 3 modified AUS stages: normal (stage 1), non-advanced (stage 2+3) and advanced (stage 4). Stiffness of the liver in each modified AUS stage was shown as follows: 5.4 ± 2.2 ($N=3$) and 7.5 ± 2.7 ($N=9$), in stage 1 (normal), 11.1 ± 5.7 ($N=7$) and 11.1 ± 6.0 ($N=16$), in stage 2+3 (non-advanced), 22.0 ± 9.5 ($N=14$) and 28.1 ± 21.3 ($N=8$), in stage 4 (advanced), in non-HIV group and in HIV group, respectively ($P=0.003$ and 0.004, by Kruskal–Wallis test in each group). Again, there was no significant difference between non-HIV group and HIV group, in each modified AUS stage. In addition, as compared with other liver fibrosis markers such as P-III-P, IV-coll, hyaluronic acid, and PLT, Stiffness was found to be the most useful in differentiating advanced stage of chronic liver disease from non-advanced one, both in non-HIV group and in HIV group (Fig. 2).

3.1. Correlation between Stiffness and other parameters for liver fibrosis

To evaluate usefulness of elastometry, we investigated correlation between Stiffness and conventional liver fibrosis markers such as P-III-P, IV-coll, and hyaluronic acid, including AUS stage and PLT. In non-HIV group, Stiffness was significantly correlated with AUS stage ($r=0.740$, $P<0.001$), PLT ($r=-0.642$, $P=0.001$), and IV-coll ($r=0.480$, $P=0.024$). On the other hand, in HIV group, Stiffness was significantly correlated with IV-coll ($r=0.801$, $P<0.001$), AUS stage ($r=0.603$, $P<0.001$), P-III-P ($r=0.621$, $P=0.001$), PLT ($r=-0.480$, $P=0.005$), and hyaluronic acid ($r=0.433$, $P=0.027$).

3.2. Correlation between platelet counts and other parameters for liver fibrosis

It has been well established that PLT are inversely proportional to the extents of liver fibrosis [5]. In this study, we investigated correlation between PLT and conventional liver fibrosis markers, AUS stage or Stiffness. In non-HIV group, PLT were significantly correlated with AUS stage ($r=-0.647$, $P=0.001$), IV-coll ($r=-0.643$, $P=0.001$), Stiffness ($r=-0.642$, $P=0.001$), P-III-P ($r=-0.526$, $P=0.012$), and hyaluronic acid ($r=-0.424$, $P=0.049$). On the other hand, in HIV group, PLT were significantly correlated with Stiffness ($r=-0.480$, $P=0.005$), AUS stage ($r=-0.415$, $P=0.016$), and IV-coll ($r=-0.417$, $P=0.031$).

3.3. Annual progression rate of liver fibrosis

We attempted to evaluate whether HIV coinfection may affect progression of liver fibrosis in patients with chronic hepatitis C. In non-HIV group, the age of blood transfusion was identified in 9 patients. Two of them had received interferon (IFN) therapy: IFN monotherapy and IFN-α2b/ribavirin, each, without sustained virological response (SVR). On the other hand, in HIV group, they had received multiple infusions of clotting factor concentrates, it was practically impossible to determine the time of the first infection of HIV or HCV. We speculated the age of the first infusion of such concentrates as the time of HCV infection, in 23 patients, in order to arbitrarily calculate annual progression rate of liver fibrosis (*R*-value). Twelve out of them had history of IFN therapy: IFN monotherapy, IFN-α2b/ribavirin and Pegylated IFN-α2a in 3, 5 and 4 patients,

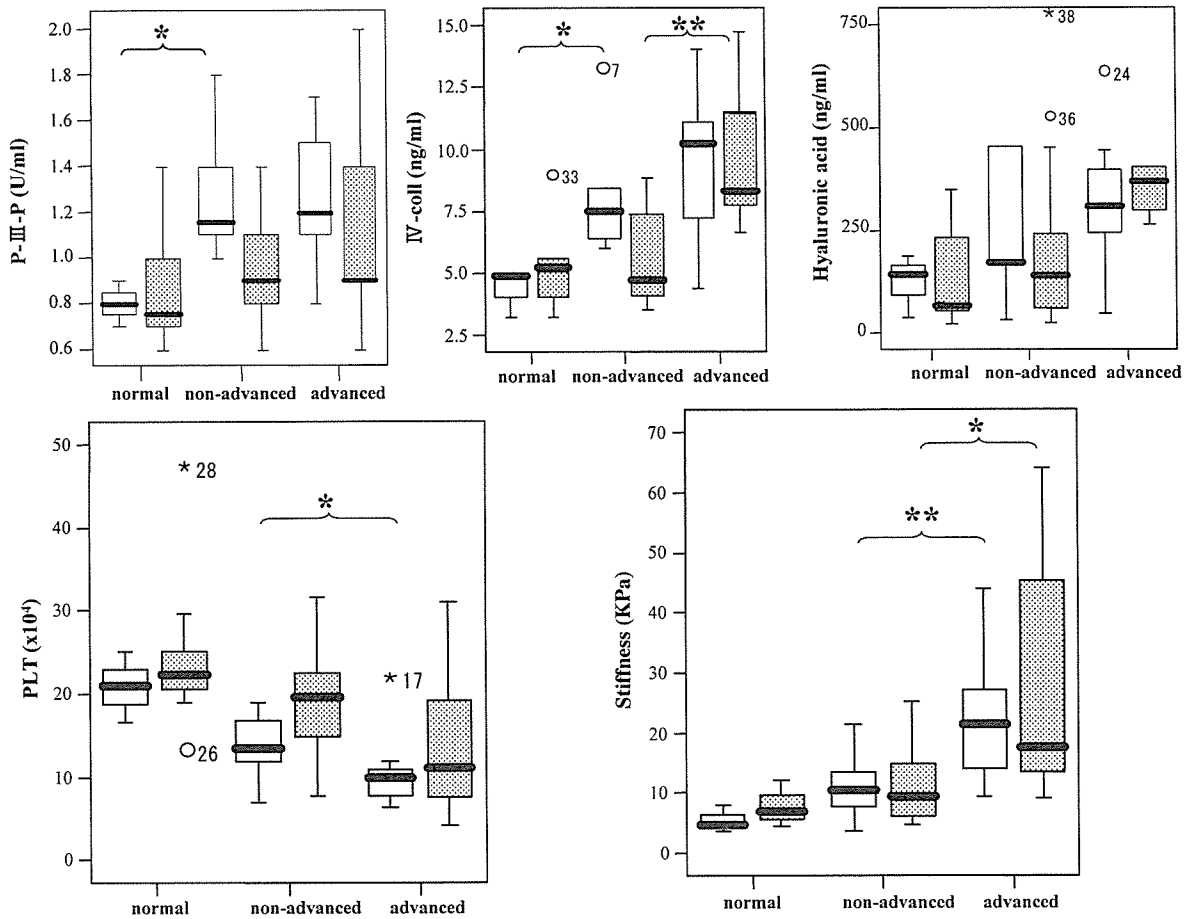

Fig. 2. Box plots of liver fibrosis markers (P-III-P, IV-coll, hyaluronic acid), PLT and Stiffness for each modified AUS stage in non-HIV group and HIV group. Open boxes and dotted boxes show the data of non-HIV group and those of HIV group, respectively. The *P*-value was calculated by Mann–Whitney *U*-test ($^{*}P<0.05$, $^{**}P<0.01$).

respectively. Four of them (33%) obtained SVR. There was no significant difference in *R*-value between non-HIV group and HIV group (0.32 ± 0.17 versus 0.38 ± 0.70; $P=0.125$, by Mann–Whitney *U*-test). In HIV group, there was no significant difference in *R*-value between IFN-treated ($N=12$) and untreated ($N=11$) patients (0.34 ± 0.50 versus 0.43 ± 0.90, $P=0.710$). Furthermore, in HIV group treated by IFN, there was no significant difference in *R*-value between patients with SVR ($N=4$, 0.08 ± 0.10) and those without SVR ($N=7$, 0.52 ± 0.60; $P=0.252$).

4. Discussion

In this study, we attempted to validate Stiffness measured by the newly developed elastometer, FibroScan® 502, in evaluating the extents of liver fibrosis in hemophiliacs coinfected with HIV and HCV (HIV group). Non-hemophiliacs infected by HCV alone (non-HIV group), could be regarded as a control, since hemophilia per se may not affect inflammation or fibrosis in the liver. It has been recently established that Stiffness is strongly correlated with liver fibrosis markers as well as *F* score evaluated by liver biopsy, in non-HIV group [3,4]. Liver biopsy could not be practically performed in clinical settings, however, such correlation was similarly, and even more significantly, confirmed also in HIV group. In this study, correlation between Stiffness and other parameters for liver fibrosis was rather weak in non-HIV group where more than half of the patients might have liver cirrhosis, probably because the variations of such liver fibrosis markers were greater in more advanced stage of chronic liver disease as shown in Fig. 2, as well as described previously [4]. Although AUS findings might be somewhat subjective, Stiffness in stage 4 was significantly higher than those in stages 1 and 2, both in HIV group and in non-HIV group, in this study (Fig. 1). In addition, when the patients of stages 2 and 3 were combined together, Stiffness was the only tool in differentiating advanced stage of chronic liver disease from non-advanced one, both in non-HIV group and in HIV group, as shown in Fig. 2. Accordingly, measurement of Stiffness could be a very useful tool to identify patients, at least, with advanced liver fibrosis. In contrast, PLT, which have been widely accepted as one of the very sensitive markers for liver fibrosis [5], seemed to be less reliable in HIV group than in

non-HIV group. Possibility of influence of HAART regimens on PLT counts should be further investigated in a future study.

It has been occasionally described that HIV coinfection increases the risk of cirrhosis and even hepatocellular carcinoma (HCC) in HCV-infected patients [6]. In addition, whether introduction of HAART or IFN therapy could affect these situations would be undoubtedly our major concern. Kramer et al. [7] recently reported that HIV coinfection accelerated liver fibrosis in pre-HAART era, but not in HAART era, while it did not affect the risk of HCC in HCV infected U.S. veterans in both eras. Similarly, Marine-Barjoan et al. [8] found that early HAART may slow liver fibrosis progression in coinfected French patients. On the contrary, Martinez-Sierra et al. [9] showed that the immune response to HAART did not influence liver fibrosis progression rate in coinfected Spanish patients. Moreover, it has been recently pointed out that HAART regimens including nevirapine, one of nonnucleoside reverse-transcriptase inhibitors, may be associated with faster liver fibrosis progression in coinfected patients [10], especially with advanced stages of liver fibrosis [11]. In this study, we arbitrarily calculated annual progression rate of liver fibrosis, using Stiffness and duration of HCV infection. Interestingly, there was no significant difference between non-HIV group and HIV group receiving HAART. This may be quite consistent with our previous findings that plasma levels of TGF-β and IL-13, known cytokines to play pivotal roles in liver fibrosis, were significantly decreased in coinfected patients under HAART, as compared with those in patients infected HCV alone (unpublished results). Furthermore, we could not get any definitive results that IFN therapy may counteract progression of liver fibrosis, because of the small numbers of patients examined and short terms of follow-up after IFN therapy. Further regular check-ups of the elasticity of the liver would be prerequisite to settle these controversial issues.

In conclusion, FibroScan® is absolutely noninvasive and can be the alternative to liver biopsy, especially in patients with bleeding tendency. It is strongly suggested that HIV/HCV coinfection may not accelerate liver fibrosis progression in HCV-related chronic liver disease, at least in HAART era.

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Fucosylated haptoglobin is a novel marker for pancreatic cancer: A detailed analysis of the oligosaccharide structure and a possible mechanism for fucosylation

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Changes in oligosaccharide structures have been reported in certain types of malignant transformations and, thus, could be used for tumor markers in certain types of cancer. In the case of pancreatic cancer cell lines, a variety of fucosylated proteins are secreted into their conditioned media. To identify fucosylated proteins in the serum of patients with pancreatic cancer, we performed western blot analyses using Aleuria Aurantica Lectin (AAL), which is specific for fucosylated structures. An ~40 kD protein was found to be highly fucosylated in pancreatic cancer and an N-terminal analysis revealed that it was the β chain of haptoglobin. While the appearance of fucosylated haptoglobin has been reported in other diseases such as hepatocellular carcinoma, liver cirrhosis, gastric cancer and colon cancer, the incidence was significantly higher in the case of pancreatic cancer. Fucosylated haptoglobin was observed more frequently at the advanced stage of pancreatic cancer and disappeared after an operation. A mass spectrometry analysis of haptoglobin purified from the serum of patients with pancreatic cancer and the medium from a pancreatic cancer cell line, PSN-1, showed that the α 1-3/α 1-4/α 1-6 fucosylation of haptoglobin was increased in pancreatic cancer. When a hepatoma cell line, Hep3B, was cultured with the conditioned media from pancreatic cancer cells, haptoglobin secretion was dramatically increased. These findings suggest that fucosylated haptoglobin could serve as a novel marker for pancreatic cancer. Two possibilities were considered in terms of the fucosylation of haptoglobin. One is that pancreatic cancer cells, themselves, produce fucosylated haptoglobin; the other is that pancreatic cancer produces a factor, which induces the production of fucosylated haptoglobin in the liver.

Key words: haptoglobin; pancreatic cancer; fucosylation; tumor marker; mass spectrometry; oligosaccharide; lectin; fucosyltransferase

Pancreatic cancer is currently one of the leading causes of cancer-related deaths and the overall 5-year survival has been reported to be less than 5%.[1,2] One of the reasons for its poor prognosis is that an early diagnosis is quite difficult and a high-risk population for pancreatic cancer has not yet been identified. Carbohydrate Antigen 19-9 (CA19-9) and carcinoembrionic antigen (CEA) are commonly used as markers of pancreatic cancer, but false positives are a problem in the diagnosis.[3] To increase the specificity of a diagnosis, a combination of tumor markers would be desirable. To this end, novel markers for pancreatic cancer, which have different characteristics from those of CA19-9 or CEA, are required. Oligosaccharides are known to be one of the most important post-translational modifications, and many studies have shown that changes in oligosaccharide structures occur during inflammation and tumorigenesis.[4] This oligosaccharide heterogeneity has been applied to tumor markers for the differential diagnosis for Hepatocellular Carcinoma (HCC). Alpha-fetoprotein (AFP), a well-known tumor marker for HCC, contains 1 asparagine-linked oligosaccharide.[5] However, serum levels of AFP also increase in certain patients with chronic hepatitis and liver cirrhosis. α 1-6 fucosylated AFP (AFP-L3 fraction) has been applied to the clinical diagnosis of HCC. α 1-6 fucosylated AFP, which is produced *via* α 1-6 fucosyltransferase (FUT8), is specifically found in the serum of patients with HCC and can be diagnosed by measuring the Lentil Lectin-(LCA) binding portion of AFP.[6,7] Moreover, AFP-L3 has been reported as a marker for a poor prognosis of HCC.[8] Changes in fucosylation patterns, as the result of different levels of expression for various fucosyltransferases, have been reported in certain diseases including various types of cancers.[9–12]

To identify potentially novel tumor markers of pancreatic cancer, we conducted a search for fucosylated proteins that are increased in the serum of patients with pancreatic cancer. The findings showed that the haptoglobin β chain was highly fucosylated and the oligosaccharide structures of haptoglobin purified from the serum of patients with pancreatic cancer were examined in detail. Furthermore, we investigated the mechanisms associated with the increased levels of fucosylated haptoglobin in pancreatic cancer.

Material and methods

Serum samples

Serum samples of patients with pancreatic cancer (n = 49, male 31, female 18, mean age 62 years), HCC (n = 23, male 17, female 6, mean age 69 years), liver cirrhosis (n = 12, male 9, female 3, mean age 63 years), gastric cancer (n = 10, male 5, female 5, mean age 59 years) and colon cancer (n = 17, male 10, female 7, mean age 61 years) were obtained from Osaka National Hospital, Osaka University Hospital and Osaka Medical Center for Cancer and CVD. The present project was approved by the ethics committees of the participating hospitals. Serum samples of healthy vol-

Abbreviations: AAL, Aleuria Aurantia Lectin; AFP, α-Fetoprotein; AOL, Aspergillus Oryzae Lectin; CA19-9, Carbohydrate Antigen 19-9; CBB, Coomassie Brilliant Blue; CEA, Carcinoembrionic Antigen; ConA, Concanavalin A; FUT8, α 1-6 Fucosyltransferase; HCC, Hepatocellular Carcinoma; LC-ESI-MS, Liquid Chromatography-Electrospray Ionization Mass Spectrometry; LCA, Lentil Lectin; MALDI-TOF-MS, Matrix assisted Laser Desorption Ionization-Time of Flight–Mass Spectrometry; PBS, Phosphate Buffered Saline; TBS, Tris-Buffered Saline.

†These authors equally contributed to this work.

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unteers (n = 30, male 16, female 14, mean age 34 years) were obtained in our laboratory.

Cell culture

Human pancreatic carcinoma cell lines (PK8, PANC-1, PSN-1, KMP4, KLM-1 and MIAPaCa2) and a human hepatoma cell line, Hep3B, were grown in RPMI-1640 (Nacalai Tesq, Kyoto, Japan) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 100 μg/ml kanamycin at 37°C in 5% CO_2. These cell lines were obtained from the ATCC (American Type Culture Collection), or the Institute of Development, Aging and Cancer, Tohoku University.

Identification of fucosylated proteins in the serum of patients with pancreatic cancer

A 0.5 μl aliquot of serum proteins from patients with pancreatic cancer and normal controls were electrophoresed on 8% polyacrylamide gels in duplicate. One gel was used for the aleuria aurantica lectin (AAL) blot analysis, which preferentially recognized α 1-3/α 1-6 fucosylated proteins.[13] The other was stained with Coomassie Brilliant Blue (CBB) after transferring onto a PVDF membrane. All procedure of AAL lectin blot analyses was described previously.[14] Bands strongly stained with AAL were subjected to N-terminal amino-acid sequence.

Western blot analysis of haptoglobin and immunoprecipitation

A 0.5 μl aliquot of serum was electrophoresed on an 8% polyacrylamide gel and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membranes were incubated with 5% skim milk in phosphate buffered saline (PBS) overnight and then incubated with 1/1,000 diluted anti human haptoglobin antibody (Dako Cytomation Kyoto, Japan) for 2 hr. After washing 3 times with Tris-buffered saline-T (TBS) (136 mM NaCl, 2.6 mM KCl, 24 mM Tris, 0.05% Tween 20, pH 7.4) for 10 min each, the membrane was incubated with peroxidase-conjugated rabbit IgG for 1 hr. After washing the membrane 3 times with TBS-T for 10 min each, development was performed using an ECL™ Western Blotting Detection Reagents (Amersham Biosciences, Uppsala, Sweden), according to standard protocols. The same membrane was used in an AAL lectin blot analysis.[14] In all AAL lectin blotting experiments, 1 pair of a negative control (a healthy control) and a positive control (a case of pancreatic cancer) was used in the same gel. For the immunoprecipitation of haptoglobin, 5 μl samples of serum from patients with pancreatic cancer and from controls were used. Serum samples were preincubated with normal rabbit serum and proteinG-sepahrose (Amersham Bioscience) followed by incubation with anti-human haptoglobin antibody for 2 hr. Immunoprecipitaed haptoglobin was analyzed by AAL lectin blot, as described earlier.

Purification of the haptoglobin β chain

To purify the haptoglobin β chain, 80 μl of sera in which albumin was depleted by a Montage Albumin Deplete kit (Millipore Corp.) or 2.5 ml of 100-fold concentrated conditioned media from PSN-1 cells were applied to an anti-haptoglobin affinity column that was coupled with 300 μl of anti human haptoglobin antibody, according to standard protocols of HiTrap NHS-activated HP (Amersham Biosciences). The haptoglobin bound to the column was eluted with 5 ml of elution buffer (100 mM Glycine, 0.5 M NaCl, pH 3.0). Thirty microliters of 50-fold concentrated fraction was subjected to SDS-PAGE under reducing conditions and stained with CBB or blotted onto a nitrocellulose membrane followed by lectin blot analyses using AAL or aspergillus oryzae lectin (AOL).[15]

Mass spectrometry

Mass spectrometry was used to identify the structure of the oligosaccharide in haptoglobins. The gels that contained purified haptoglobin were cut into smaller sizes and collected in a 1.5-ml microtube. To remove CBB, 50 mM NH_4HCO_3 (SIGMA, Tokyo Japan) in 30% acetonitrile (MERCK, Darmstadt Germany) was added, followed by washing at room temperature for 20 min using Bio shaker (TAITEC). The samples were then added with 300 μl of acetonitrile and incubated at room temperature for 10 min. After removing the extra acetonitrile, a reduction solution consisting of 10 mM DTT, 10 mM EDTA and 50 mM NH_4HCO_3 was added, followed by incubation at 65°C for 60 min. Samples were then alkylated in a solution consisting of 40 mM idoacetamide, 10 mM EDTA and 50 mM NH_4HCO_3 in the dark for 30 min. After washing twice with 50 mM NH_4HCO_3 for 10 min, an additional 300 μl of acetonitrile was added and the sample was then incubated at room temperature for 10 min. For trypsin digestion, the samples were incubated at 37°C overnight with 0.5 μg of sequencing grade-modified trypsin (Promega, Madison, WI USA) in 50 mM NH_4HCO_3. After the gels were removed, the sample was concentrated and taken to dryness with a Speed Vac (CENTRIFUGAL EVAPORATOR CVE-2000, EYELA). The residues were dissolved in 20 μl of water. A 2-μl aliquot of this solution was used in the Mascot research. The other sample was incubated at 100°C for 10 min with 32 μl of a 20 mM phosphate solution. The samples were then treated with *N*-Glycosidase F rec. [*E. coli*] (Roche, USA) and incubated at 37°C overnight. After boiling at 100°C for 10 min, PA (pyridylamino) modification was performed using a Glyco TAG™ Reagent Kit (TaKaRa, Otsu Japan), according to the standard protocols. The samples were filtered with Sephadex LH-20 and *N*-glycans derived with the PA fraction were collected. The samples were dried with a Speed Vac and then dissolved in 100 μl of water. Liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) and matrix-assisted laser desorption ionization-time of flight–mass spectrometry (MALDI-TOF-MS) were then performed.

RNA extraction and RT-PCR

A human hepatoma cell line (Hep3B) and human pancreatic carcinoma cell lines (PSN-1, KLM-1, MiaPaCa-2, PK8, PK59, PANC-1) were cultured as described earlier. Trizol (1 ml) was added to each 10-cm dish and collected in a 2-ml microtube. After 15 min, 200 μl of chloroform was added to the samples followed, by vortexing for 15 sec. After standing at room temperature for 10 min, the samples were centrifuged at 15,000 rpm for 15 min, and an equal amount of 2-propanol was then added to the supernatant. After an additional 15 min, the samples were centrifuged at 15,000 rpm at 4°C for 15 min and the pellets were washed with 0.5 ml of 75% ethanol twice. The pellets were dried and dissolved in 50 μl of DEPC (diethylpyrocarbonate) treated water. The concentration of RNAs was measured at an absorbance of 260 nm.

According to the SuperScript™(III) Reverse Transcriptase (Invitrogen Corp. Carlsbad, CA UA) protocol, 5 μg of total RNA was incubated with 1 μl of Oligo dT at 70°C for 10 min. The samples were incubated at 42°C for 5 min with a 1st strand cDNA synthesis buffer consisting of 10 μl of 5× First Strand Buffer, 10 μl of dNTP Mixture, 5 μl of 0.1 M DTT and 13 μl of DEPC, at 42°C for 50 min with 1 μl of Reverse Transcriptase, at 99°C for 5 min and at 37°C for 20 min with 1 μl of RNaseH.

The samples served as a template DNA for 30 rounds of amplification using the GeneAmp PCR System 2700. PCR was performed in a standard 100 μl reaction mixture consisting of 10 μl of 10× Ex taq Buffer, 8 μl of dNTP Mixture, 1 μl of sense and antisense primer, 0.5 μl of Ex taq (TaKaRa), 1 μl of cDNA, PCR primers for haptoglobin cDNA were as follow, forward primer, 5′-TTCCCTGGCAGGCTAAGATG-3′(position 562-581); and reverse primer, 5′-GCACCCATCAGCTTCAAACC-3′(position 1363-1382). Amplification was performed at 95°C for 30 sec, at 66°C for 30 sec, at 72°C for 1 min. Finally, an additional extension step was performed at 72°C for 10 min. The amplified PCR products were run on a 1.5% agarose gel containing 0.005% ethidium bromide. To estimate the amount of total cDNA, glyceraldehydes-3-phosphate dehydrogenase with the same cDNA was used as an internal control under identical conditions.

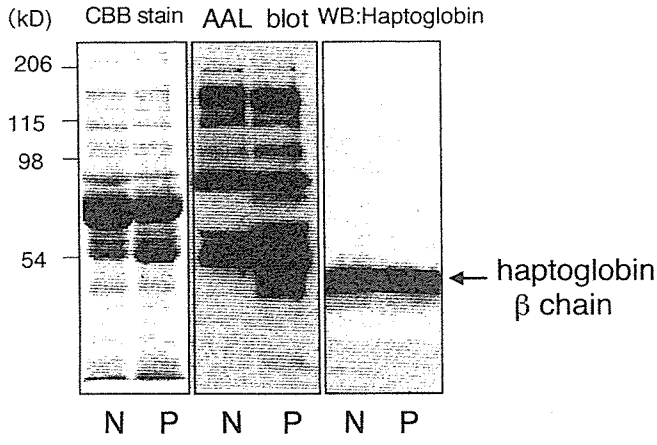

FIGURE 1 – Identification of AAL-binding proteins in serum of pancreatic cancer. 0.5 µl of sera was electrophresed on 8% acrylamide gels, and stained with CBB after blotting onto a PVDF membrane. An AAL blot analysis was performed using the same samples. An approximately 40 kD protein was excised from the membrane and identified as the haptoglobin β chain by its N-terminal amino-acid sequence. N indicates normal controls and P indicates pancreatic cancer. Western blot of haptoglobin in the right panel indicated the approximate position of haptoglobin.

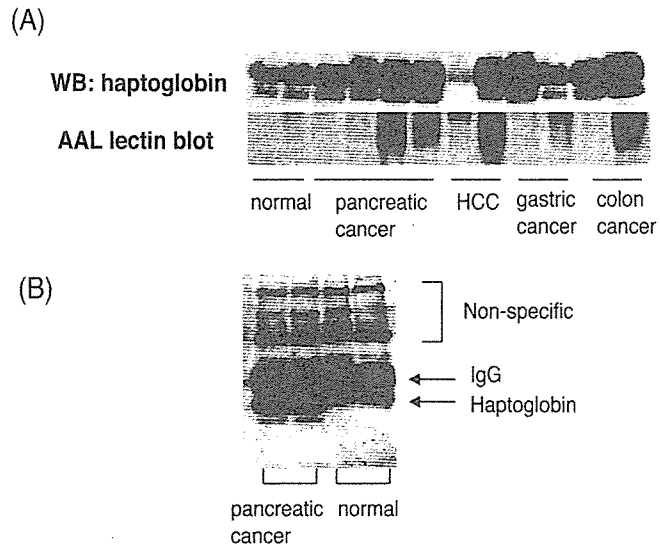

FIGURE 2 – Fucosylation of haptoglobin in the serum of patients with various cancers. (*a*) 0.5 µl of sera was electrophresed on 8% acrylamide gels, and western blot analyses were performed using anti human haptoglobin antibody and AAL lectin. (*b*) Haptoglobin was immunoprecipitated from the serum of patients with pancreatic cancer and healthy controls followed by AAL lectin blot analysis.

Induction of production of fucosylated haptoglobin in hep3B cells

A human hepatoma cell line (Hep3B) and human pancreatic carcinoma cell lines (PSN-1, MiaPaCa-2) were grown in low glucose D-MEM (Nacalai Tesq, Kyoto, Japan) supplemented with 10% FBS, 50 U/ml penicillin and 100 µg/ml kanamycin at 37°C in 5% CO_2. The cells, at sub confluent conditions in 10-cm dishes, were washed twice with PBS and cultured in 10 ml of serum free D-MEM. After 2 days, the media from each run were collected and added to the other cells. After an additional incubation for 2 days, the media were collected. The media were concentrated 100 times and used in a western blot analysis of haptoglobin.

TABLE I – FUCOSYLATION OF HAPTOGLOBIN IN SERUM OF PATIENTS WITH VARIOUS DISEASES

	n	Negative	Positive (%)
Normal	30	29	1 (3)[1]
Pancreatic cancer[2]	49	20	29 (59)
HCC[2,3]	23	18	5 (22)
Liver cirrhosis[2,3]	12	9	3 (25)
Gastric cancer[3]	10	8	2 (20)
Colon cancer[2]	17	10	7 (41)

Statistic analysis was performed according to the program for Stat-view software.
[1]Values in parentheses indicate percentages.–[2]$p < 0.05$ *vs.* normal–[3]$p < 0.05$ *vs.* pancreatic cancer (χ^2 test).

TABLE II – RELATIONSHIP BETWEEN THE INCIDENCE OF FUCOSYLATED HAPTOGLOBIN AND THE CLINICAL STAGE OF PANCREATIC CANCER

Clinical stage	*n*	Positive	Negative
Stage I, II	12	4	8
Stage III, IV	22	15	7

$p = 0.05$, compared with stage I, II and stage III, IV (χ^2 test). Statistic analysis was performed according to the program of Stat-view software.

Results

Haptoglobin, as a target protein for fucosylation in the serum of patients with pancreatic cancer

A preliminary study suggested that pancreatic cancer cells produce a variety of fucosylated proteins into the condition medium. To identify fucosylated proteins in the serum of patients with pancreatic cancer, AAL blot analyses were performed. The total binding of serum proteins to AAL was increased in pancreatic cancer as compared with healthy controls. In these proteins, increases in the fucosylation of the ~40 kD band were observed with a high frequency in the serum of patients with pancreatic cancer. The N-terminal amino-acid sequences revealed that the sequence was ILG-GHLDAKG, corresponding to the haptoglobin β chain (Fig. 1). A similar approach was performed in 4 cases of pancreatic cancer and all of the fucosylated proteins of 40 kD identified were the haptoglobin β chain (data not shown). Furthermore, a western blot analysis of haptoglobin was performed to confirm the position of haptoglobin molecular size (Fig. 1).

The appearance of fucosylation of haptoglobin in serum of patients with various cancers

To evaluate the levels of fucosylation of haptoglobin in the serum of patients with pancreatic cancer compared with those of other various cancers, an AAL blot analysis was performed (Fig. 2*a*). The results showed that fucosylated haptoglobin was also increased in HCC, liver cirrhosis (date not shown), gastric cancer and colon cancer. Interestingly, the appearance of the fucosylation was not correlated with total amount of haptoglobin. The immunoprecipitation of haptoglobin, followed by an AAL lectin blot showed more clearly that haptoglobin was strongly fucosylated in patients with pancreatic cancer. The incidence of increase in fucosylated haptoglobin in the serum of patients with various diseases is summarized (Table I). Appearance of fucosylated haptoglobin in the case of pancreatic cancer was significantly higher compared with that of healthy controls and patients with HCC, liver cirrhosis and gastric cancer.

Relationship between fucosylation of haptoglobin and the clinical stage in pancreatic cancer

The appearance of fucosylation in pancreatic cancer was investigated in terms of the clinical background of the subjects (Table II). The fucosylation of haptoglobin was observed in 4/12 cases at stage I and II, and 15/22 cases at stage III and IV, respectively, suggesting that the incidence of haptoglobin fucosylation tended to increase in advanced stages. Interestingly, fucosylated hapto-

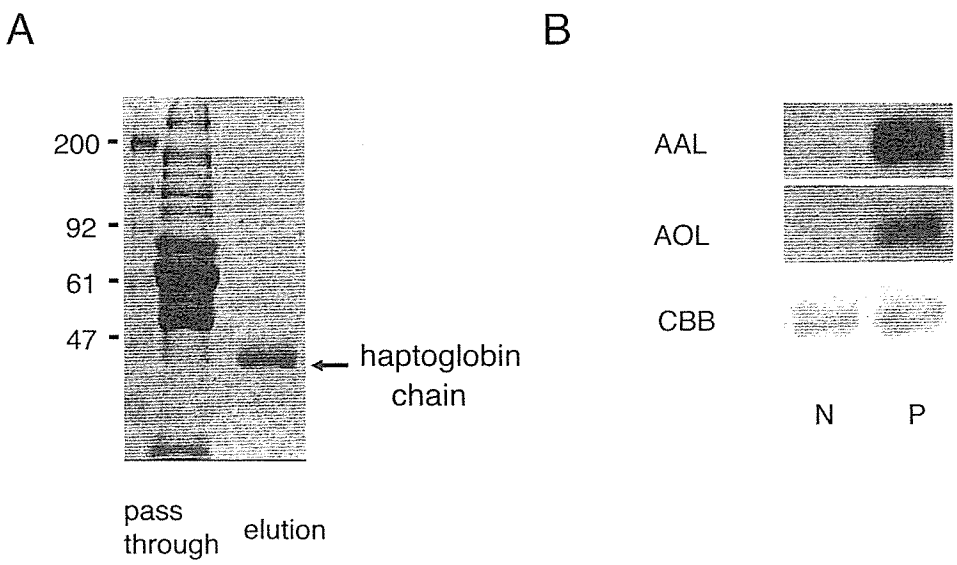

FIGURE 3 – Purification of haptoglobin β chain and analysis of its oligosaccharide structures. (*a*) Haptoglobin was purified from 80 μl of sera with pancreatic cancer and normal individuals by using an anti-haptoglobin affinity column. (*b*) Equal amounts of purified haptoglobin purified from normal controls (N) and pancreatic cancer (P) were electrophresed on 8% polyacrylamide gels, and stained with CBB and analyzed by lectin blot analyses by using AAL and AOL.

globin disappeared after an operation in 2 cases in which it was possible to follow-up.

Analysis of oligosaccharide structures of haptoglobin by lectin blot and mass spectrometry

To determine the oligosaccharide structures, haptoglobin was purified from 80 μl of sera of pancreatic cancer and healthy individuals. The detailed procedure is described in "Material and methods". Five microliters of a 50-fold concentrated fraction was subjected to SDS-PAGE and then stained with CBB. A major band was detected at the expected molecular weight (Fig. 3*a*). To determine the oligosaccharide structures in the purified haptoglobin β chain, lectin blot analyses using AAL and AOL were performed (Fig. 3*b*). AOL specifically interacts with core fucosylation. The results indicate that α 1-3 fucosylation as well as core fucosylation were both increased in the haptoglobin β chain of pancreatic cancer patients.

Thirty microliters of 50-fold concentrated haptoglobin was subjected to SDS-PAGE and the 40 kD band was excised from the gel. This purified protein was confirmed to be the haptoglobin β chain by MALDI-TOF mass spectrometry (data not shown). To determine the oligosaccharide structures of haptoglobin β chain in more detail, LC-ESI-MS was performed (Figs. 4*a*–4*c*). A high level of fucosylation was observed in the case of haptoglobin associated with pancreatic cancer. Furthermore, biantennary chains with disialic acid, which are considered to be the major oligosaccharide structures, were analyzed by MS/MS. This analysis showed that a high level of core fucosylation is associated with pancreatic cancer (data not shown).

To determine the oligosaccharide structures of triantennary structures with trisialic acid, MALDI-TOF-MS was performed. In this experiment, fucose was found to be attached to the α 1-3/α 1-4 position to GlcNAc or α 1-2 position to Galactose. As the result of the mass spectrometry analysis, core fucosylation as well as α 1-3/α 1-4 fucosylation was confirmed to be increased in the haptoglobin β chain purified from serum of patients with pancreatic cancer (Fig. 4*d*).

Mechanisms responsible for the increases in fucosylated haptoglobin

While most haptoglobin is secreted from the liver, the expression of FUT8 in a normal liver is quite low.[16] Therefore, a normal liver does not produce α 1-6 fucosylated haptoglobin. There are 2 possible mechanisms underlying the increased levels of fucosylated haptoglobin in the serum of patients with pancreatic cancer. One is that pancreatic cancer cells, themselves, produce fucosylated haptoglobin. To investigate this possibility, we performed a RT-PCR analysis of haptoglobin using 6 types of pancreatic cancer cells (Fig. 5*a*). The expression of haptoglobin mRNA was observed only in PSN-1 cells. After the purification of haptoglobin from conditioned media of these cells, the oligosaccharide structures were analyzed. Expectedly, binding to AAL and AOL was increased in haptoglobin purified from PSN-1 cells (Fig. 5*b*). Moreover, an LC-ESI-MS analysis indicated that core fucosylation as well as the α 1-3/α 1-4 fucosylation of haptoglobin were observed in the conditioned media of PSN-1 cells (data not shown). The other possibility is that pancreatic cancer produces a factor which induces the production of fucosylated haptoglobin from the liver. To examine this hypothesis further, media from pancreatic cancer cells such as PSN-1 and MIA PaCa-2 were added to a hepatoma cell line, Hep3B. Increase of haptoglobin production was observed in Hep3B cells after addition of the conditioned media of these pancreatic cancer cells (Fig. 6).

Discussion

To find a novel marker for cancers, it is important to identify a protein that is secreted exclusively from cancer cells or to identify a specific modification of a protein that is produced by cancer cells. The best way for the former analysis would be a DNA micro array, and for the latter analysis would be of the detection of modified sugar chains. In the present study, we found that fucosylated haptoglobin was a good serum marker for pancreatic cancer, analyzed the oligosaccharide structure in detail and investigated the mechanism underlying why fucosylated haptoglobin is increased in the serum of patients with pancreatic cancer.

As a result of analyses of various serum samples, we found that fucosylated haptoglobin was observed at high levels in the serum of patients with pancreatic cancer. Haptoglobin is heterotetramer consisting of 2 α subunits and 2 β subunits joined by inter-chain disulfide bonds.[17] There are 4 distinct asparagines residues (Asn 23, 46, 50, 80) in each β-chain and they display oligosaccharide heterogeneity. Recent studies of haptoglobin showed that certain oligosaccharide structures predominate in different diseases. For example, a highly-fucosylated structure is found in breast cancer and ovarian cancer, highly-sialylated structures in Crohn's disease and highly branched structures in alcoholic liver disease.[18–23] Furthermore, the aberrant glycosylation of haptoglobin was found to increase during mouse hepatocarcinogenesis, by our group.[24] In our study, we reported that increases in core fucosylation as well as α 1-3/α 1-4 fucosylation

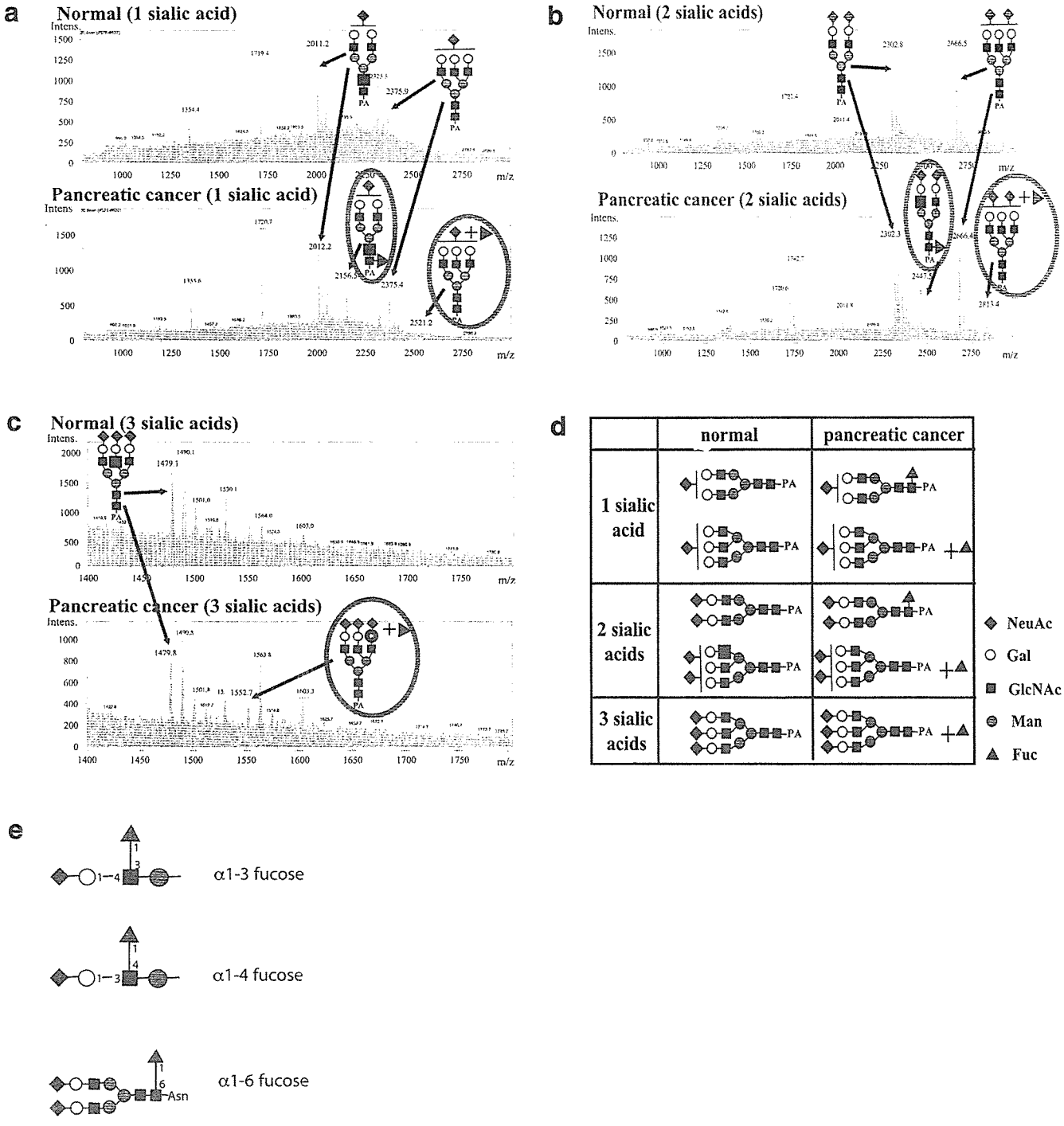

FIGURE 4 – Oligosaccharide structures of haptoglobin by mass spectrometry analysis. (*a*) Biantennary and triantennary chains with 1 sialic acid structure of haptoglobin were examined by LC-ESI-MS. (*b*) Biantennary and triantennary chains with disialic acid structures of haptoglobin were examined by LC-ESI-MS. (*c*) Triantennary chains with tri-sialic acid structures of haptoglobin were examined by LC-ESI-MS. (*d*) Oligosaccharide structures of haptoglobin in the serum of patients with pancreatic cancer were compared with those of normal individuals. Increases in fucosylation levels in haptoglobin were observed in the case of pancreatic cancer. (*e*) Linkages of a fucose residue are indicated.

was found in the haptoglobin β chain purified from serum of patients with pancreatic cancer compared to normal controls by LC-ESI-MS and MALDI-TOF-MS. Furthermore, we described 2 possibilities for the fucosylation of haptoglobin found in the serum of patients with pancreatic cancer. A pancreatic cancer cell line, PSN-1 actually produced fucosylated haptoglobin, suggesting that pancreatic cancer itself produces fucosylated (especially α 1-6 fucosylated) haptoglobin. To prove this possibility, the immunohistochemistry of haptoglobin was undertaken. Infiltrating lymphocytes could express ectopic haptoglobin in pancreatic cancer tissues. Secondly, pancreatic cancer produces a factor that induces the production of fucosylated (especially α 1-3 fucosylated) haptoglobin from the liver. To demonstrate this hypothesis, it will be necessary to identify a factor produced by pancreatic cancer. As shown in Figure 6, it would be difficult to know whether or not fucosylated haptoglobin is increased in a normal

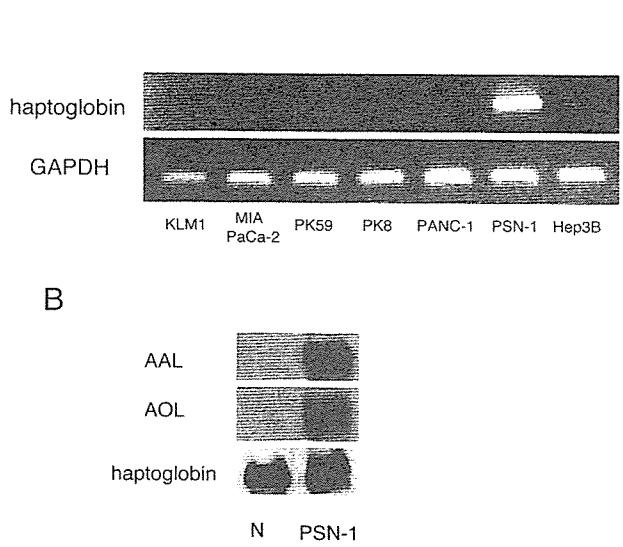

FIGURE 5 – Expression of haptoglobin mRNA and its oligosaccharide structure of pancreatic cancer cells. (*a*) The expression of haptoglobin mRNA was investigated RT-PCR. (*b*) Haptoglobin was purified from the conditioned media of PSN-1 cells and its oligosaccharide structure was analyzed by lectin blot analysis. As expected, binding to AAL and AOL was increased in haptoglobin purified from PSN-1 cells.

liver, because Hep3B is a cancer cell line and secretes high levels of fucosylated haptoglobin. Fucosylated haptoglobin disappeared after an operation, indicating that both of these 2 possibilities could exist *in vivo*.

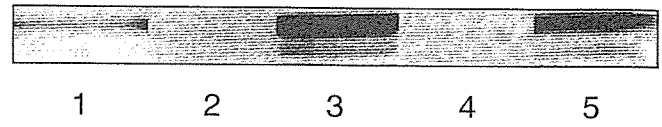

FIGURE 6 – Pancreatic cancer cells induce haptoglobin production in Hep3B cells. Hep3B, PSN-1 and MIAPaCa-2 cells were cultured in serum-free medium for 2 days. After collecting the medium, media from PSN-1 or MIAPaCa-2 cells were added to Hep3B cells and the suspension was further incubated for 2 days. Each sample was electrophoresed on 8% acrylamide gels, and a western blot analysis using an anti-human haptoglobin antibody was performed. Lane 1, Hep3B cells with no treatment, lane 2, PSN-1 cells with no treatment, lane 3, Hep3B cells after addition of the conditioned medium from PSN-1 cells, lane 4, MIA PaCa-2 cells with no treatment and lane 5, Hep3B cells after addition of the conditioned medium from Mia PaCa-2 cells. Increases in haptoglobin production were observed in Hep3B cells when cultured with conditioned media from pancreatic cancer cells.

In conclusion, we reported on the potential use of haptoglobin as a target protein for fucosylation in the serum of patients with pancreatic cancer. We also found that the α 1-3/α 1-4 fucosylation as well as the α 1-6 fucosylation of haptoglobin was specifically detected in pancreatic cancer, as evidenced by mass spectrometry. We conclude that there are 2 possibilities for the fucosylation of haptoglobin in pancreatic cancer. Further studies will be required to verify the clinical use of fucosylated haptoglobin as a tumor marker in terms of comparison with inflammatory diseases such as chronic pancreatitis (a preliminary study in the cases of chronic pancreatitis showed 25% positive (1/4 cases) for fucosylated haptoglobin in their serum).

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Should aged patients with chronic hepatitis C be treated with interferon and ribavirin combination therapy?

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Abstract

The aim of this study was to investigate the efficacy and safety of combination therapy of interferon and ribavirin for aged patients with chronic hepatitis C.

Methods: This study was conducted at Osaka University Hospital and institutions participating in the Osaka Liver Disease Study Group on 329 patients with chronic hepatitis C receiving interferon and ribavirin combination therapy (group A, under 60 year old, $n=199$; group B, 60–64 year old, $n=64$; group C, over 65 year old (mean age, 67.8 ± 2.2 year old, $n=66$)). Of the 293 patients who were tested for HCV serotype and HCV viral loads, 215 had HCV-RNA with serotype 1 and high viral loads (1H) and the other 78 had HCV-RNA with serotype 2 or low viral loads (non-1H).

Results: In per-protocol analysis, the overall SVR rate of 1H patients was 28% (51/184). Among the 1H patients, the SVR rate was significantly lower in group C (16%) and group B (17%) than in group A (34%) ($p<0.05$). The overall SVR rate of non-1H patients was 85% (57/67). No significant difference was found in the SVR rate among group C (79%), group B (100%), and group A (84%). On the other hand, the discontinuance of both drugs due to side effects was 29% (19/66) in group C, 20% (13/64) in group B, and 11% (21/199) in group A, with the discontinuance rates being higher in the older group ($p=0.002$).

Conclusions: In aged chronic hepatitis C patients, interferon and ribavirin combination therapy can be recommended for the non-1H patients who showed a high SVR rate of approximately 65%, but not for the 1H patients.

Keywords: Chronic hepatitis C; Aged patient; Interferon and ribavirin combination therapy

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1. Introduction

Hepatitis C virus (HCV) is estimated to infect up to 170 million people worldwide [1]. Long persistence of HCV infection can lead to progression of liver fibrosis causing liver cirrhosis and ultimately hepatocellular carcinoma (HCC) [2,3]. In Japan, it is estimated that two million people are infected with HCV, and more than 30,000 patients die of HCC every year, with approximately 80% being caused by HCV infection [4]. It has been reported that HCV carriers in Japan tend to be old [5], and liver fibrosis progresses in aged patients. Moreover, the risk of HCC increases with progression of liver fibrosis and older age, with the occurrence of HCV-related HCC reaching a peak at around the age of 65 years old [3]. Past studies have made clear that interferon (IFN) therapy is effective for eliminating HCV, and IFN therapy significantly reduces the progression of liver fibrosis [6,7] and the risk of HCC, especially among virologic or biochemical responders [8–10]. Furthermore, recently, several groups have reported that IFN therapy, specially the SVR group, improved the survival of patients with HCV [11,12], also in aged patients [13].

The combination therapy with IFN and ribavirin has been reported to be effective for eliminating HCV compared with IFN monotherapy [14–16], but additional side effects of ribavirin, such as hemolytic anemia, which is not found in IFN monotherapy have been reported, leading to discontinuance of the treatment [17]. For aged patients, sufficient informed consent should be obtained before the start of stronger antiviral therapy with possible severe side effects, because the function of the organs is generally poor, and the adverse effects of IFN therapy have been observed more frequently in older patients [18].

The question arises of whether aged patients with chronic hepatitis C should be treated with the combination therapy of IFN and ribavirin, while IFN monotherapy has been shown to be effective even in aged patients. In this study, we conducted a multi-center, retrospective study of patients with chronic hepatitis C treated by IFN and ribavirin combination therapy, and examined the efficacy and prevalence of side effects to clarify the adaptation of anti-viral treatment for aged patients.

2. Patients and methods

2.1. Patients

The current study was conducted at Osaka University Hospital and the institutions of the Osaka Liver Disease Study Group. The 329 patients with chronic hepatitis C included in this study were treated with combination IFN-α-2b and ribavirin between January 2001 and April 2004. All patients had HCV RNA detectable in serum by the polymerase chain reaction (PCR) method, had elevated ALT (above the upper limit of the normal) and had been histologically proven to have chronic hepatitis. None of the patients were positive for hepatitis B surface antigen and anti-human immunodeficiency virus antibody or had other forms of liver disease (alcoholic liver disease, hepatotoxic drugs, autoimmune hepatitis). This study protocol was carried out according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained from each patient.

2.2. Determination of HCV RNA levels

Serum HCV-RNA levels were quantified using branched DNA (bDNA) probe assay (version 2; Chiron, Dai-ichi Kagaku, Tokyo) [19,20] or combined PCR assay (Amplicor-HCV monitor assay) [21]. In this study, a high viral load was designated as the condition of a serum HCV-RNA level of more than 10^6 equivalents/ml by bDNA assay or more than 10^5 copies/ml serum by Amplicor-HCV monitor assay [22].

2.3. Treatment schedule

The 329 patients were treated with 10 MU ($n = 79$) or 6 MU ($n = 243$) or 3 MU ($n = 7$) IFN-α-2b intramuscularly every day for the first 2 weeks and the three times a week for the following 22 weeks in combination with ribavirin at a daily dose of 600 or 800 mg, depending on body weight (<60 or ≥60 kg, respectively). The starting doses of ribavirin were 800 mg per day for 178 patients, 600 mg per day for 148 patients, and 400 mg per day for three patients. The ribavirin dose was decreased or stopped in 91 patients (28%) due to side effects. The ribavirin dose of 200 mg was reduced if the hemoglobin value was below 10 g/dl. The ribavirin was stopped if Hb fell bellow 8.5 g/dl. One hundred and five patients continued only IFN therapy for 24 weeks after the combination therapy, because the combination therapy of IFN-α-2b and ribavirin for 48 weeks was not covered by medical insurance in Japan at that time. Patients with persistently undetectable HCV RNA 6 months after completion of treatment were considered to have achieved a sustained virological response.

2.4. Statistical analysis

Age, histological scores before IFN therapy, serum ALT levels, red blood cell (RBC) count, hemoglobin (Hb), white blood cell (WBC) count and platelet (Plt), and creatinine are expressed as mean ± S.D. Statistical analysis for group comparisons was performed by the χ^2-test. The SVR rate was evaluated using the probability proportional to size analysis (PPS analysis) and the intention-to-treat analysis (ITT analysis). A value of $p < 0.05$ (two-tailed) was considered to indicate significance.

3. Results

3.1. Clinical characteristics before combination therapy

The baseline clinical features of the 329 patients are shown in Table 1. At the start of the treatment, 130 patients were 60

Table 1
Baseline characteristics of patients according to age

	Group A ($n=199$)	Group B ($n=64$)	Group C ($n=66$)	p-value
Age (years old)	49.0 ± 8.7	62.0 ± 1.4	67.8 ± 2.2	
Sex (M/F)	142/54[a]	36/28	43/23	[a]$p<0.05$
HCV serotype (1/2/unknown)	142/51/6	53/10/1	54/12/0	N.S.
HCV-RNA (H/L/unknown)	173/12/14	58/2/4	60/5/1	N.S.
1H/non 1H/unknown	125/53/21	45/8/11	45/17/4	
Fibrosis (F 1/F2/F3/F4/unknown)	75/46/33/6/39	26/15/10/2/11	19/15/17/4/11	N.S.
ALT (IU/L)	112 ± 85[b]	91 ± 49	90 ± 57	$p<0.05$[b]
WBC	5330 ± 1570[b]	4970 ± 1390	4760 ± 1120	$p<0.05$[b]
RBC ($\times 10^4$ μl)	458 ± 47[b]	433 ± 45	431 ± 47	$p<0.01$[b]
Hb (g/dl)	14.6 ± 1.5[b]	14.0 ± 1.2	13.7 ± 1.4	$p<0.01$[b]
Plt ($\times 10^4$ μl)	16.0 ± 7.0[b]	14.9 ± 5.3	14.2 ± 4.9	$p<0.05$[b]

Note: Data are given as the mean ± S.D. N.S., not significant. Group A, patients under 60 years of age (gender of three patients were unknown); group B, patients older than 60 years but under 65 years of age; group C, patients older than 65 years of age; 1H group, patients with genotype 1 and high viral load; non-1H group, patients other than 1H group.

[a] Significant level was compared with group B.

[b] Significant levels were compared with group B and group C.

years old or older. One hundred ninety-nine patients were under 60 years old (group A), sixty-four patients were 60–64 years old (group B) and sixty-six patients were 65 years old or older (group C). No significant difference was found in serotype, viral load and histological stage among the three groups. In aged patients, ALT, RBC, Hb, WBC, and Plt were less than in young patients (ALT, $p<0.05$; RBC and Hb, $p<0.01$; WBC and Plt, $p<0.05$). Among the patients, 215 had HCV-RNA with genotype 1 and high viral loads (1H group) and 114 had HCV-RNA with genotype 2 or low viral loads (non-1H group).

3.2. Initial dosage and treatment duration of interferon

Three kinds of IFN dosage were used in this study. Among group A, 10MU, 6MU, and 3MU were administered for 60 patients, 134 patients, and 5 patients; 12, 52, and none among group B, and 8, 56, and 2 among group C. No significant difference was found in the distribution of IFN dosage among each group. The 24 and 48-week treatments (IFN and ribavirin treatment for 24 weeks followed by IFN monotherapy for 24 weeks) were carried out for 102 patients and 75 patients among group A; 37 and 14 among group B; 32 and 16 among group C. The rates of patients receiving the 48-week treatment were similar for the three groups.

3.3. PPS analysis

On PPS analysis, the overall SVR rate of 1H patients was 28% (51/184). The SVR rates were 34% (40/117) for group A, 17% (6/36) for group B, and 16% (5/31) for group C. Among the 1H patients, the SVR rates of group B and C were significantly lower than that for group A ($p<0.05$). The overall SVR rate of non-1H patients was 85% (57/67). No significant difference was found in the SVR rates among group A (84%; 36/43), group B (100%; 5/5), and group C (79%; 11/14) (Fig. 1).

3.4. ITT analysis

On ITT analysis, the SVR rate was 24% (51/215) in 1H patients, being 32% (40/125) for group A, 13% (6/45) for group B, and 11% (5/45) for group C. Among the 1H patients, the SVR rates of group B and C were significantly lower than that for group A (A versus B; $p<0.05$, A versus C; $p<0.01$).

On the other hand, in the non-1H group, the SVR rate was 73% (57/78), being 77% (41/53) for group A, 63% (5/8) for group B, and 65% (11/17) for group C. No significant difference was found among the groups (Fig. 2).

3.5. Adverse effects

The entire treatment schedule without reduction and discontinuance of both drugs was completed by 174 patients (53%). Sixty-two percent (123/199) of the patients in group A, 42% (27/64) in group B, and 36% (24/66) in group C com-

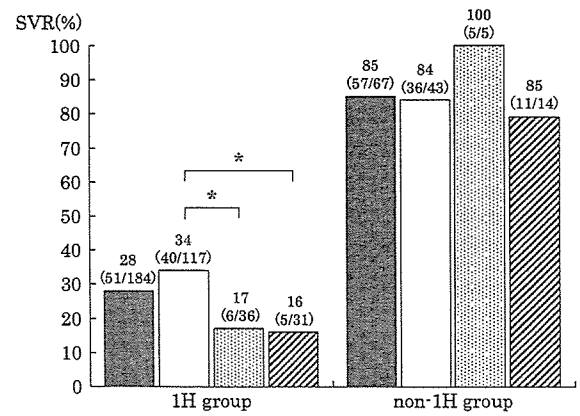

Fig. 1. Efficacy of the combination therapy according to age (PPS analysis). 1H group, patients with genotype 1 and high viral load. Non-1H group, patients not in the 1H group. (■) all patients; (□) group A, patients under 60 years of age; (▨) group B, patients from 60 years and older but under 65 years of age; (▨) group C, patients older than 65 years. Significant levels: *$p<0.05$.

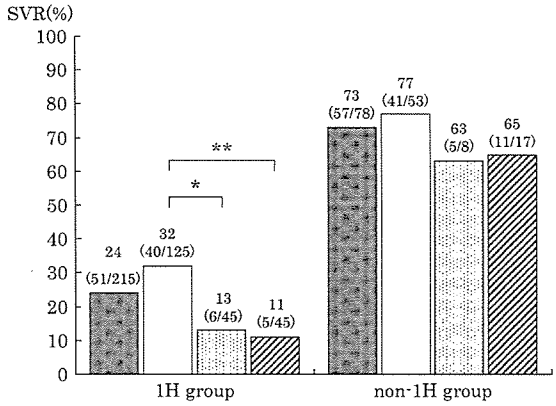

Fig. 2. Efficacy of the combination therapy according to distinction of age (ITT analysis). 1H group, patients with genotype 1 and high viral load. Non-1H group, patients not in the 1H group. (■) all patients; (□) group A, patients under 60 years of age; (▩) group B, patients from 60 years and older but under 65 years of age; (▨) group C, patients older than 65 years. Significant levels: $^{*}p<0.01$; $^{**}p<0.05$.

pleted all treatment schedules (A versus B; $p<0.0001$, A versus C; $p<0.001$). IFN treatment was stopped along with ribavirin in 52 patients (16%), and the IFN dose was decreased in 20 patients (6%). The ribavirin dose was decreased in 72 patients (22%), and stopped without discontinuance of IFN in 20 patients (6%). The discontinuance rate of both drugs was significantly higher in group C (29%, 21/199) and B (20%, 13/64) than group A (11%, 19/66) (Fig. 3).

The reasons for dose reduction and discontinuance of the treatment were anemia, general fatigue, digestive disorder, eczema, neutropenia, and psychological disorder. Among the patients discontinuing both drugs, for those under 60 years old, the major reasons were anemia (32%), general fatigue

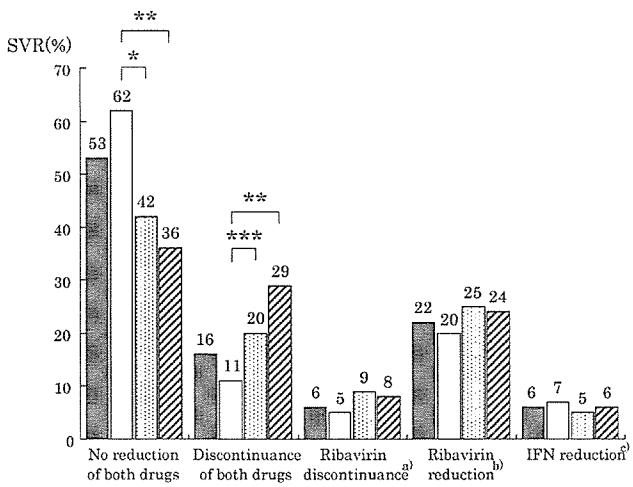

Fig. 3. Dose reduction or discontinuance of IFN and ribavirin. (a) Ribavirin discontinuance without discontinuance of IFN, (b) ribavirin reduction without discontinuance of IFN, and (c) IFN reduction regardless of discontinuance or reduction of ribavirin. (■) all patients; (□) group A, patients under 60 years of age; (▩) group B, patients from 60 years and older but under 65 years of age; (▨) group C, patients older than 65 years. Significant levels: $^{*}p<0.0001$; $^{**}p<0.001$; $^{***}p<0.005$.

(18%), digestive disorder (14%), and psychological disorder (14%). On the other hand, among the patients aged 60 years and older, the discontinuance of therapy due to anemia accounted for approximately 60% (17/28), which was twice as much as those of younger patients, with the difference being significant ($p<0.05$). Other reasons of the discontinuance of therapy among the patients aged 60 years and older were following; digestive disorder (14%), general fatigue (7%), eruption, granulocytopenia, thrombocytopenia, and psychological disorder (4%, respectively). Vascular diseases, such as cerebral bleeding did not appear in this study.

4. Discussion

In Japan, randomized control studies have been performed on the combination therapy of IFN and ribavirin for 24 weeks in patients with chronic hepatitis C, and the combination therapy was approved in 2001. However, the patients in these studies were under 60 years of age. Accordingly, the efficacy and adverse effects of combination therapy for aged patients has been still unclear. Since HCV carriers in Japan are older by 10–20 years than those in the United States and the European countries, it is very important to clarify the actual state of affairs for aged patients with chronic hepatitis C receiving the combination therapy, especially in Japan. These findings should be applicable for patients with chronic hepatitis C in other countries in a few decades, because almost the same efficacy and adverse effects are expected in patients treated by pegylated interferon (peg-IFN) and ribavirin combination therapy. In this study, we examined the efficacy and prevalence of the side effects with the focus on patient age.

The aged patients showed higher rates of discontinuance of IFN and ribavirin and lower rates for no reduction of both drugs than younger patients. The most frequent reason for the discontinuance of both drugs was hemolytic anemia which accounted for 60% of the cases in patients 60 years or older. The progress of anemia was frequently noted in aged patients and resulted in the discontinuation of ribavirin. Hemolytic anemia induced by ribavirin administration has been reported to depend on the plasma ribavirin concentration [23], with a high ribavirin concentration leading to it, and the plasma clearance of ribavirin depending on renal function [24]. A major cause for the advance of anemia in aged patients is due to the fact that renal function is poorer than in younger patients, leading to lower ribavirin clearance. As a result, severe hemolytic anemia can be induced by higher ribavirin concentrations. Therefore, the dosage of ribavirin should be reduced at the beginning of treatment in the aged patients with chronic hepatitis C in order to avoid the discontinuance of ribavirin, because the reduction of ribavirin does not decrease the SVR rate of this therapy.

The SVR difference according to age was observed for 1H patients, but not non-1H patients, when only the patients who completed the treatment were examined (PPS analysis).