–413, 2005
9) 飯野四郎，沖田　極，小俣政男，他：Genotype 1 かつ高ウイルス量のC型慢性肝炎に対するPEG-インターフェロンα-2bとリバビリン48週併用投与の有効性－インターフェロンα-2bとリバビリン6カ月併用療法とのretrospectiveな比較．肝胆膵　49：1099–1121，2004
10) 林　紀夫，熊田博光：肝疾患におけるrecommendation 1．C型肝炎．コンセンサス肝疾患治療2004，アークメディア，pp11–18
11) 熊田博光：B型及びC型肝炎ウイルスの感染者に対する治療の標準化に関する臨床的研究．総括研究報告．厚生科学研究費補助金肝炎等克服緊急対策研究事業（肝炎分野）．平成16年度総括・分担研究報告書，2005，pp1–12
12) Ikeda K, Saitoh S, Kobayashi M et al : Distinction between chronic hepatitis and liver cirrhosis in patients with hepatitis C virus infection. Practical discriminant function using common laboratory data. Hepatol Res 18 : 252–266, 2000
13) 飯野四郎：C型慢性肝炎に対するインターフェロン療法の効果に関するアンケート調査．厚生省非A非B型肝炎研究班 平成7年度研究報告書，1996，pp72–73
14) Akuta N, Suzuki F, Tsubota A et al : Efficacy of interferon monotherapy to 394 consecutive naïve cases infected with hepatitis C virus genotype 2a in Japan: therapy efficacy as consequence of tripartite interaction of viral, host and interferon treatment-related factors. J Hepatol 37 : 831–836, 2002
15) Kasahara A, Hayashi N, Mochizuki K et al : Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Hepatology 27 : 1394–1402, 1998
16) Yabuuchi I, Imai Y, Kawata S et al : Long-term responders without eradication of hepatitis C virus after interferon therapy:characterization of clinical profiles and incidence of hepatocellular carcinoma. Liver 20 : 290–295, 2000
17) 豊田成司，妻神重彦，安田清美，他：インターフェロン無効または再燃したC型慢性肝炎に対するインターフェロンα-2bとSCH10908（リバビリン）の併用投与とインターフェロンα-2b単独投与の比較－二重盲検群間比較法を用いた併用効果の検討．臨床医薬 18：539–563，2002
18) Poynard T, Marcellin P, Lee SS et al : Randomised trial of interferon α 2b plus ribavirin for 48 weeks or for 24 weeks versus interferon α 2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. Lancet 352 : 1426–1432, 1998
19) McHutchison JG, Gordon ST, Schiff ER et al : Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N Engl J Med 1485–1492, 1998
20) Zeuzem S, Hultcrantz R, Bourliere M et al : Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. J Hepatol 40 : 993–999, 2004
21) 堺　隆弘，小俣政男，飯野四郎，他：Ro25-8310（ペグインターフェロンアルファ-2a）のC型慢性肝炎に対する第II相臨床試験．医学と薬学50：655–672，2003
22) Weiland O, Freid MW, Hadzyannis SJ et al : Peginterferon α -2a (40kD) plus ribavirin in treatment-naïve patients with chronic hepatitis C and genotype 2 or 3 infection:individual estimated probability of sustained virological response. J Hepatol 42 (suppl.2) : 226–227, 2005: abstract 619
23) Zeuzem S, Esteban-Mur R, Ferenci P et al : Efficacy of 6 months treatment with PEG-Interferon α -2b plus ribavirin (P/R) in patients infected with hepatitis C with genotype 1 of low viral load (G1LVL) . J Hepatol 42 (suppl.2) : 229, 2005: abstract 625
24) Dalgard O, Bjoro K, Hellum K et al : Short (14 weeks) treatment with pegylated interferon alpha-2b and ribavirin in patients with hepatitis C genotype 2/3 virus infection and early virological response. Hepatology 40 (suppl.1) : 252A, 2004: abstract 197
25) Von Wagner M, Huber M, Berg T et al : Randomized multicenter study comparing 16 vs. 24 weeks of combination therapy with peginterferon alfa-2a plus ribavirin in patients chronically infected with HCV genotype 2 or 3. Hepatology 40 (suppl.1) : 725A, 2004: abstract LB02
26) Bozorg B, Kleiner DE, Pawlotsky JM et al : Early viral kinetics during treatment with low doses of peginterferon alfa-2a and ribavirin in patients with chronic hepatitis C infected with genotype 2 and 3. Hepatplogy 40 (suppl.1) : 340A, 2004: abstract 404

Int. J. Cancer: **118**, 2803–2808 (2006)

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

Noriko Okuyama[1,2†], Yoshihito Ide[1†], Miyako Nakano[1], Tsutomu Nakagawa[1], Kanako Yamanaka[1,2], Kenta Moriwaki[1,2], Kohei Murata[3], Hiroaki Ohigashi[3], Shigekazu Yokoyama[3], Hidetoshi Eguchi[3], Osamu Ishikawa[3], Toshifumi Ito[4], Michio Kato[5], Akinori Kasahara[6], Sunao Kawano[7], Jianguo Gu[1], Naoyuki Taniguchi[1] and Eiji Miyoshi[1,2*]

[1]*Department of Biochemistry, Osaka University Graduate School of Medicine, Osaka, Japan*
[2]*Department of Molecular Biochemistry and Clinical Investigation, Osaka University Faculty of Medicine, School of Allied Health Science, Osaka, Japan*
[3]*Department of Surgery, Osaka Medical Center for Cancer & CVD, Osaka, Japan*
[4]*Department of Gastroenterology, Kansai-Rosai Hospital, Osaka, Japan*
[5]*Department of Gastroenterology, Osaka National Hospital, Osaka, Japan*
[6]*Department of General Medicine, Osaka University Graduate School of Medicine, Osaka, Japan*
[7]*Department of Clinical Laboratory Sciences, Osaka University Faculty of Medicine, School of Allied Health Science, Osaka, Japan*

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.
© *2005 Wiley-Liss, Inc.*

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.

Grant sponsor: Scientific Research on Priority Area 10178104 and 16590245; Grant sponsor: the 21st Century COE program from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant sponsor: Japan Science and Technology Agency (JST).

***Correspondence to:** Department of Biochemistry, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita 565-0871, Japan. Fax +81-6-6879-3429. E-mail: miyoshi34@biochem.med.osaka-u.ac.jp

Received 23 June 2005; Accepted 31 October 2005
DOI 10.1002/ijc.21728
Published online 29 December 2005 in Wiley InterScience (www.interscience.wiley.com).

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 Dèplete 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.

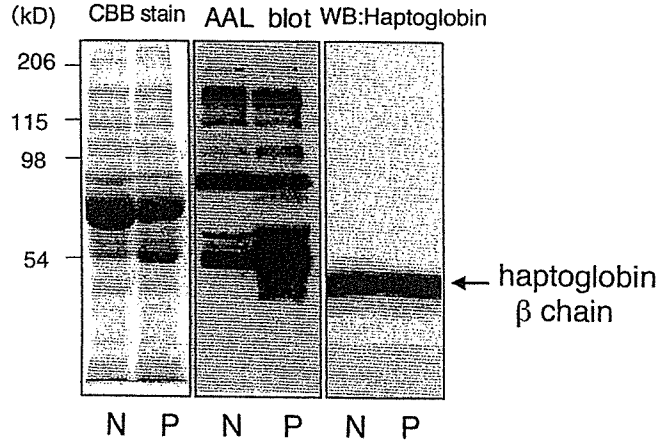

FIGURE 1 – Identification of AAL-binding proteins in serum of pancreatic cancer. 0.5 µl of sera was electrophresed on 8% acrylamide gels, and stained with CBB after blotting onto a PVDF membrane. An AAL blot analysis was performed using the same samples. An approximately 40 kD protein was excised from 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.

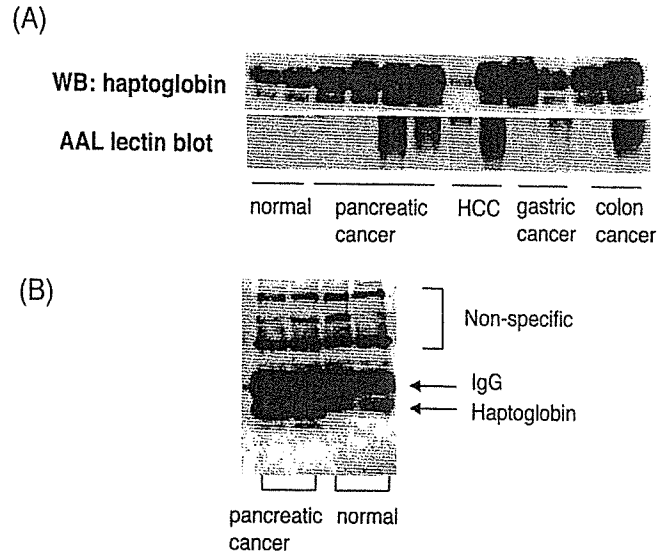

FIGURE 2 – Fucosylation of haptoglobin in the serum of patients with various cancers. (*a*) 0.5 µl of sera was electrophresed on 8% acrylamide gels, and western blot analyses were performed using anti human haptoglobin antibody and AAL lectin. (*b*) Haptoglobin was immunoprecipitated from the serum of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

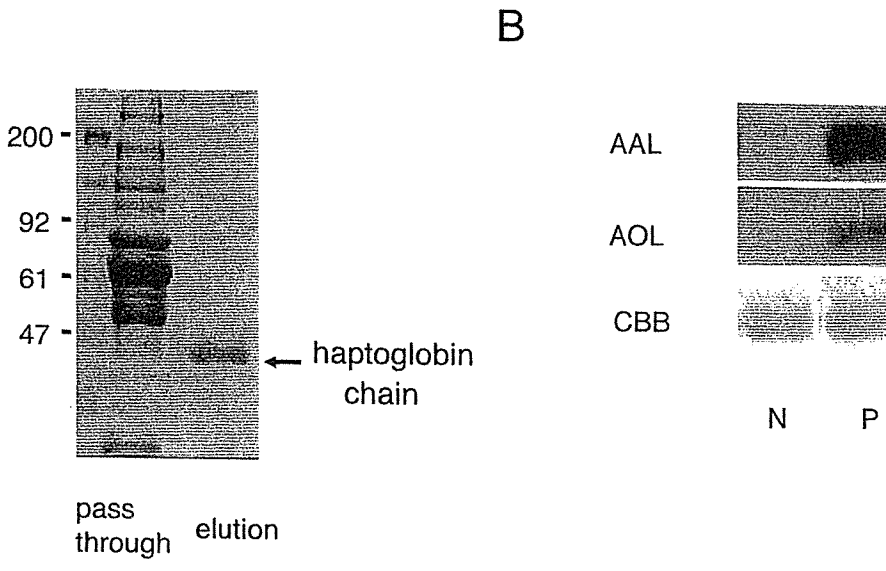

FIGURE 3 – Purification of haptoglobin β chain and analysis of its oligosaccharide structures. (*a*) Haptoglobin was purified from 80 μl of sera with pancreatic cancer and normal individuals by using an anti-haptoglobin affinity column. (*b*) Equal amounts of purified haptoglobin purified from normal 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

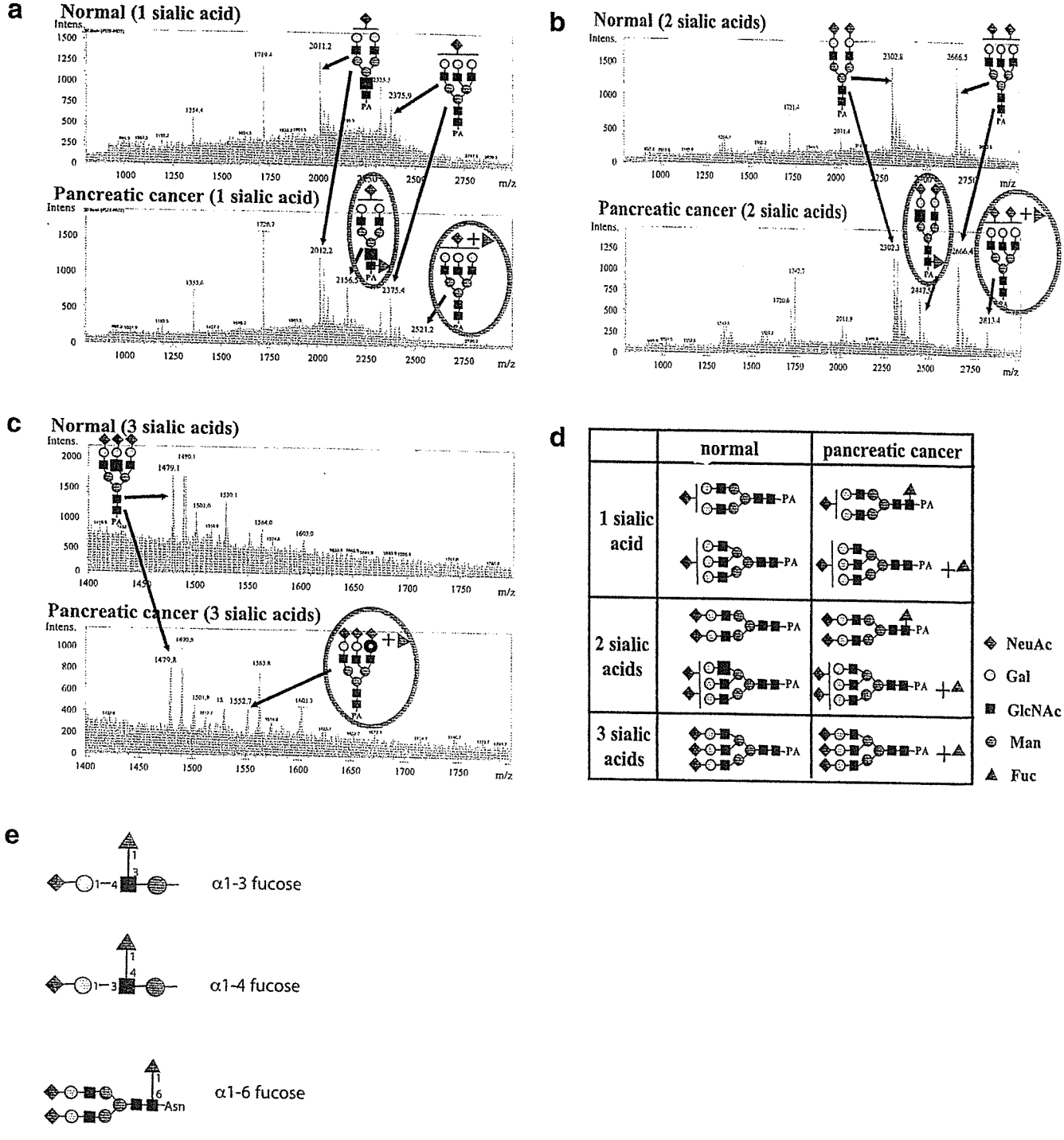

FIGURE 4 – Oligosaccharide structures of haptoglobin by mass spectrometry analysis. (*a*) Biantennary and triantennary chains with 1 sialic acid structure of haptoglobin were examined by LC-ESI-MS. (*b*) Biantennary and triantennary chains with disialic acid structures of haptoglobin were examined by 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

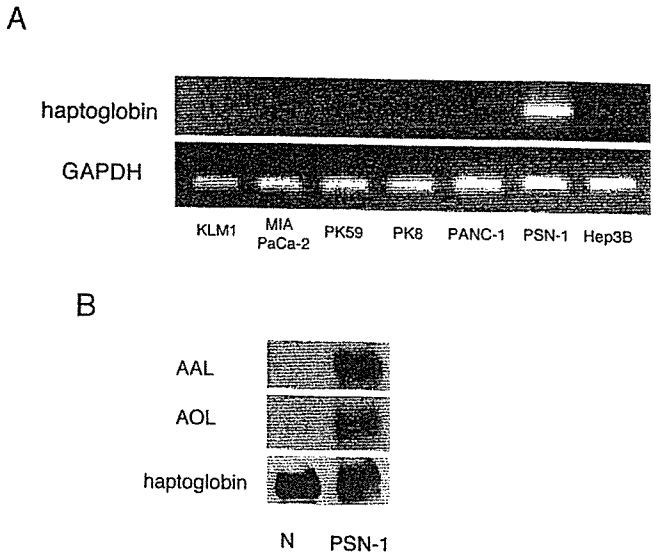

FIGURE 5 – Expression of haptoglobin mRNA and its oligosaccharide structure of pancreatic cancer cells. (*a*) The expression of haptoglobin mRNA was investigated RT-PCR. (*b*) Haptoglobin was purified from the conditioned media of PSN-1 cells and its oligosaccharide structure was analyzed by lectin blot 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*.

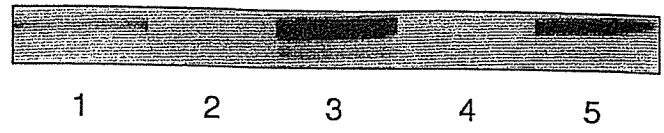

FIGURE 6 – Pancreatic cancer cells induce haptoglobin production in Hep3B cells. Hep3B, PSN-1 and MIAPaCa-2 cells were cultured in serum-free medium for 2 days. After collecting the medium, media from PSN-1 or MIAPaCa-2 cells were added to Hep3B cells and the suspension was further incubated for 2 days. 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).

References

1. Greenlee RT, Murray T, Boldon S, Wingo PA. Cancer statistics, 2000. CA Cancer J Clin 2000;50:7–33.
2. Friess H, Kleeff J, Kulli C, Wagner M, Sawhney H, Buchler MW. The impact of different types of surgery in pancreatic cancer. Eur J Surg Oncol 1999;25:124–31.
3. Rosty C, Goggins M. Early detection of pancreatic carcinoma. Hematol Oncol Clin North Am 2002;16:37–52.
4. Hakomori S. Aberrant glycosylation in tumors and tumor-associated carbohydrate antigen. Adv Cancer Res 1989;52:257–331.
5. Morinaga T, Sakai M, Wegmann TG, Tamaoki T. Primary structures of human alpha-fetoprotein and its mRNA. Proc Natl Acad Sci USA 1983;80:4604–8.
6. Sato Y, Nakata K, Kato Y, Shima M, Ishii N, Koji T, Taketa K, Endo Y, Nagataki S. Early recognition of hepatocellular carcinoma based on altered profiles of alpha-fetoprotein. N Engl J Med 1993;328:1802–6.
7. Taketa K, Endo Y, Sekiya C, Tanikawa K, Koji T, Taga H, Satomura S, Matsuura S, Kawai T, Hirai H. A collaborative study for the evaluation of lectin-reactive alpha-fetoproteins in early detection of hepatocellular carcinoma. Cancer Res 1993;53:5419–23.
8. Yamashita F, Tanaka M, Satomura S, Tanikawa K. Prognostic significance of Lens culinaris agglutinin A-reactive alpha-fetoprotein in small hepatocellular carcinomas. Gastroenterology 1996;111:996–1001.
9. Tatsumura T, Sato H, Mori A, Komori Y, Yamamoto K, Fukatani G, Kuno S. Clinical significance of fucose level in glycoprotein fraction of serum in patients with malignant tumors. Cancer Res 1977;37:4101–3.
10. Saitoh O, Wang WC, Lotan R, Fukuda M. Differential glycosylation and cell surface expression of lysosomal membrane glycoproteins in sublines of a human colon cancer exhibiting distinct metastatic potentials. J Biol Chem 1992;267:5700–11.
11. Goupille C, Hallouin F, Meflah K, Le Pendu J. Increase of rat colon carcinoma cells tumorigenicity by alpha (1–2) fucosyltransferase gene transfection. Glycobiology 1997;7:221–9.
12. Martin-Satue M, Marrugat R, Cancelas JA, Blanco J. Enhanced expression of alpha (1,3)-fucosyltransferase genes correlates with E-selectin-mediated adhesion and metastatic potential of human lung adenocarcinoma cells. Cancer Res 1998;58:1544–50.
13. Yamashita K, Kochibe N, Ohkura T, Ueda I, Kobata A. Fractionation of L-fucose-containing oligosaccharides on immobilized Aleuria aurantia lectin. J Biol Chem 1985;260:4688–93.
14. Noda K, Miyoshi E, Gao C-X, Nakahara S, Kitada T, Honke K, Suzuki K, Yoshihara H, Yoshikawa K, Kawano K, Tonetti M, Taniguchi N, et al. Relationship between elevated FX expression and increased production of GDP-L-fucose, a common donor substrate for fucosylation in human hepatocellular carcinoma and hepatoma cell lines. Cancer Res 2003;63:6282–9.
15. Ishida H, Moritani T, Hata Y, Kawato A, Suginami K, Abe Y, Imayasu S. Molecular cloning and overexpression of fleA gene encoding a fucose-specific lectin of Aspergillus oryzae. Biosci Biotechnol Biochem 2002;66:1002–8.
16. Miyoshi E, Uozumi N, Noda K, Hayashi N, Hori M, Taniguchi N. Expression of alpha 1–6 fucosyltransferase in rat tissues and human cancer cell lines. Int J Cancer 1997;72:1117–21.
17. Turner GA. Haptoglobin. A potential reporter molecule for glycosylation changes in disease. Adv Exp Med Biol 1995;376:231–8.
18. Thompson S, Turner GA. Elevated levels of abnormally-fucosylated haptoglobins in cancer sera. Br J Cancer 1987;56:605–10.
19. Thompson S, Dargan E, Griffiths ID, Kelly CA, Turner GA. The glycosylation of haptoglobin in rheumatoid arthritis. Clin Chim Acta 1993;220:107–14.
20. Thompson S, Dargan E, Turner GA. Increased fucosylation and other carbohydrate changes in haptoglobin in ovarian cancer. Cancer Lett 1992;66:43–8.
21. Goodarzi MT, Turner GA. Reproducible and sensitive determination of changed oligosaccharides from haptoglobin by PNGase F digestion and HPAEC/PAD analysis: glycan composition varies with disease. Glycoconj J 1998;15:469–75.
22. Marta Piva J. Ignacio Moreno , Kathy L. Sharpe-Timms . Glycosylation and over-expression of endometriosis-associated peritoneal haptoglobin. Glycoconj J 2002;19:33–41.
23. Mann AC, Record CO, Self CH, Turner GA. Monosaccharide composition of haptoglobin in liver diseases and alcohol abuse: large changes in glycosylation associated with alcoholic liver disease. Clin Chim Acta 1994;227:69–78.
24. Ekuni A, Miyoshi E, Ko JH, Noda K, Kitada T, Ihara S, Endo T, Hino A, Honke K, Taniguchi N. A glycomic approach to hepatic tumors in *N*-acetylglucosaminyltransferase III (GnT-III) transgenic mice induced by diethylnitorsamine (DEN): identification of haptoglobin as a target molecule of GnT-III. Free Radic Res 2002;36:827–33.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Hepatology Research 35 (2006) 185–189
HEPATOLOGY RESEARCH
http://www.elsevier.com/locate/hepres

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

Naoki Hiramatsu [a,*], Tsugiko Oze [a], Natsuko Tsuda [a], Nao Kurashige [a], Keisuke Koga [a], Takashi Toyama [a], Masakazu Yasumaru [a], Tatsuya Kanto [a], Tetsuo Takehara [a], Akinori Kasahara [a], Michio Kato [b], Harumasa Yoshihara [c], Kazuhiro Katayama [d], Taizo Hijioka [e], Hideki Hagiwara [f], Shinji Kubota [g], Masahide Oshita [h], Yoshimichi Haruna [i], Eiji Mita, Kunio Suzuki [j], Kazunobu Ishibashi [k], Norio Hayashi [a]

[a] *Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka 565-0871, Japan*
[b] *National Hospital Organization Osaka National Hospital, Osaka, Japan*
[c] *Osaka Rousai Hospital, Osaka, Japan*
[d] *Osaka Kouseinenkin Hospital, Osaka, Japan*
[e] *National Hospital Organization Osaka Minami Medical Center, Osaka, Japan*
[f] *Higashiosaka City Central Hospital, Osaka, Japan*
[g] *Kansai Rousai Hospital, Hyogo, Japan*
[h] *Osaka Police Hospital, Osaka, Japan*
[i] *Osaka General Medical Center, Osaka, Japan*
[j] *Saiseikai Senri Hospital, Osaka, Japan*
[k] *Kaizuka City Hospital, Kaizuka City, Osaka, Japan*

Received 8 January 2006; received in revised form 16 March 2006; accepted 27 March 2006
Available online 4 May 2006

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

* Corresponding author. Tel.: +81 6 6879 3621; fax: +81 6 6879 3629.
E-mail address: hiramatsu@gh.med.osaka-u.ac.jp (N. Hiramatsu).

1386-6346/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.hepres.2006.03.008

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-

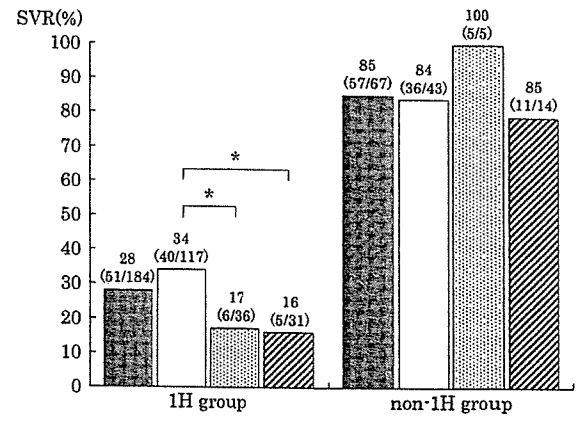

Fig. 1. Efficacy of the combination therapy according to age (PPS analysis). 1H group, patients with genotype 1 and high viral load. Non-1H group, patients not in the 1H group. (▩) all patients; (□) group A, patients under 60 years of age; (▦) group B, patients from 60 years and older but under 65 years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

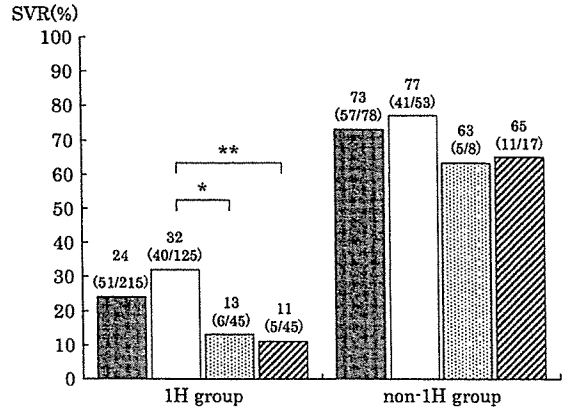

Fig. 2. Efficacy of the combination therapy according to distinction of age (ITT analysis). 1H group, patients with genotype 1 and high viral load. Non-1H group, patients not in the 1H group. (■) all patients; (□) group A, patients under 60 years of age; (▩) group B, patients from 60 years and older 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

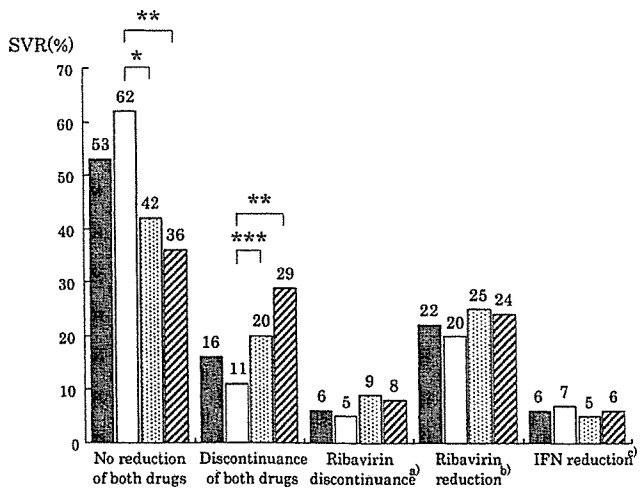

Fig. 3. Dose reduction or discontinuance of IFN and ribavirin. (a) Ribavirin discontinuance without discontinuance of IFN, (b) ribavirin reduction without discontinuance of IFN, and (c) IFN reduction regardless of discontinuance or reduction of ribavirin. (■) all patients; (□) group A, patients under 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).

That is, the SVR rates were still high for the aged patients of the non-1H group, but lower for the aged patients than the young patients in the 1H group. There are two possible reasons for this. First, the number of patients with no reduction of both drugs was significantly fewer for the patients aged 60–64 years and <60 years than for the patients aged ≥65 years, and the older patients tended to require ribavirin reduction or discontinuance (Fig. 3). Second, the liver fibrosis score tended to be higher in aged patients than in young patients, although the significant difference was not seen in this study (Table 1). These factors can decrease the SVR rates in aged patients in the 1H group, from which it is difficult to eliminate the virus, although the aged patients in the non-1H group whose viruses are easily eliminated were not affected. The results on ITT analysis account for the conclusion of the indication for IFN and ribavirin combination therapy of 24 weeks for aged patients; the patients of the 1H group do not have good application whose SVR is approximately 10%. On the other hand, patients of the non-1H group should be given the combination therapy because of the higher SVR rates of about 65%.

Better efficacy of treatments using new drugs, such as peg-IFN and ribavirin combination therapy or NS3/4 protease inhibitor, is greatly anticipated.

Acknowledgments

Other institutions and participants in the Osaka Liver Disease Study Group (Digestive Disease Study Group of Osaka Renaissance) are: National Hospital Organization Osaka National Hospital, Y. Izumi; Osaka Rousai Hospital, H. Aketa and K. Noda; Osaka Kouseinenkin Hospital, M. Kurokawa and T. Akasaka; Kansai Rousai Hospital, M. Yamamoto; Osaka General Medical Center, T. Inoue; National Hospital Organization Osaka Minami Medical Center, H. Hikita and M. Shigekawa; Osaka Police Hospital, J. Kondo; Kaizuka City Hospital, O. Nishiyama; and Osaka University Graduate School of Medicine, S. Shinzaki, M. Miyazaki, H. Miyatake, I. Itose, S. Egawa and T. Nishida.

This work was supported by a Grant-in-Aid for Research on Hepatitis and BSE from the Ministry of Health Labour and Welfare of Japan, and Scientific Research from the Ministry of Education, Science, and Culture of Japan.

References

[1] Lauer G, Walker BD. Hepatitis C virus infection. N Engl J Med 2001;345:41–52.
[2] Poynard T, Bedossa P, Opolon P, et al. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet 1997;349:825–32.
[3] Hamada H, Yatsuhashi H, Yano K, et al. Impact of aging on the development of hepatocellular carcinoma in patients with posttransfusion chronic hepatitis C. Cancer 2002;95(2):331–9.
[4] Tanaka H, Tsukuma H. Characteristics of Japanese patients with liver cancer—epidemiological study based on comparison between male and female patients. Hepatology Res 2002;24:S11–20.
[5] Yoshizawa H. Trends of hepatitis virus carriers. Hepatology Res 2002;24:S28–39.
[6] Shiratori Y, Imazeki F, Moriyama M, et al. Histologic improvement of fibrosis in patients with hepatitis C who have sustained response to interferon therapy. Ann Intern Med 2000;132:517–24.
[7] Hiramatsu N, Hayashi N, Kasahara A, et al. Improvement of liver fibrosis in chronic hepatitis C patients treated with natural interferon alpha. J Hepatol 1995;22:135–42.
[8] Yoshida H, Shiratori Y, Moriyama M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: National surveillance program of chronic and noncirrhotic patients with chronic hepatitis C in Japan. Ann Intern Med 1999;131:174–81.
[9] Kasahara A, Hayashi N, Mochizuki K, et al. Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Hepatology 1998;27:1394–402.
[10] Ikeda K, Saitoh S, Arase Y, et al. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: A long-term observation study of 1,643 patients using statistical bias correction with proportional hazard analysis. Hepatology 1999;19:1124–30.
[11] Kasahara A, Tanaka H, Okanoue T, et al. Interferon treatment improves survival in chronic hepatitis C patients showing biochemical as well as virological responses by preventing liver-related death. J Viral Hepat 2004;11(2):148–56.
[12] Yoshida H, Arakawa Y, Sata M, et al. Interferon therapy prolonged life expectancy among chronic hepatits C patients. Gastroenterology 2002;123:4483–91.
[13] Imai Y, Kasahara A, Tanaka H, et al. Interferon therapy for aged patients with chronic hepatitis C: improved survival in patients exhibiting a biochemical response. J Gastroenterol 2004;39:1069–77.
[14] McHutchison JG, Gordon SC, Schiff ER, et al. Interferon alpha 2b alone or combination with ribavirin as initial treatment for chronic hepatitis C. N Engl J Med 1998;229:1485–92.
[15] Poynard T, Marcellin P, Lee SS, et al. Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. Lancet 1998;352:1426–32.
[16] Hiramatsu N, Kasahara A, Nakanishi F, et al. The significance of interferon and ribavirin combination therapy followed by interferon monotherapy for patients with chronic hepatitis C in Japan. Hepatol Res 2004;29(3):142–7.
[17] Bodenheimer HC, Lindsay KL, Davis GL, et al. Tolerance and efficacy of oral ribavirin treatment of chronic heoatitis C: A multicenter trial. Hepatology 1997;26:437–77.
[18] Okanoue T, Sakamoto S, Itoh Y, et al. Side effects of high-dose interferon therapy for chronic hepatitis C. J Hepatol 1996;25:283–91.
[19] Yuki N, Hayashi N, Kasahara A, et al. Pretreatment viral load and response to prolonged interferon-α course for chronic hepatitis C. J Hepatol 1995;22:457–63.
[20] Lau YN, Davis G, Kniffen J, et al. Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. Lancet 1993;341:1501–4.
[21] Shiratori Y, Kato N, Yokosuka O, et al. Predictors of the efficacy of interferon therapy in chronic hepatitis C virus infection. Gastroenterology 1997;113:558–66.
[22] Tanaka T, Tsukiyama-Kohara K, Yamaguchi K, et al. Significance of specific antibody assay for genotyping of hepatitis C virus. Hepatology 1994;19:1347–53.
[23] Lindahl K, Schvarcz R, Bruchfeld A, et al. Evidence that plasma concentration rather than dose per kilogram body weight predicts ribavirin-induced anemia. J Viral Hepatitis 2004;11:84–7.
[24] Maeda Y, Kiribayashi Y, Moriya T, et al. Dosage adjustment of ribavirin based on renal function in Japanese patients with chronic hepatitis C. Ther Drug Monit 2004;26:9–15.

J Gastroenterol 2006; 41:862–872
DOI 10.1007/s00535-006-1858-2

Journal of Gastroenterology

Early decline of hemoglobin correlates with progression of ribavirin-induced hemolytic anemia during interferon plus ribavirin combination therapy in patients with chronic hepatitis C

Tsugiko Oze[1], Naoki Hiramatsu[1], Nao Kurashige[1], Natsuko Tsuda[1], Takayuki Yakushijin[1], Tatsuya Kanto[1], Tetsuo Takehara[1], Akinori Kasahara[1], Michio Kato[2], Harumasa Yoshihara[3], Kazuhiro Katayama[4], Shinji Kubota[5], Taizo Hijioka[6], Kazunobu Ishibashi[7], Masahide Oshita[8], Hideki Hagiwara[9], Yoshimichi Haruna[10], Eiji Mita[11], Shinji Tamura[1], and Norio Hayashi[1]

[1]Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita 565-0871, Japan
[2]National Hospital Organization Osaka National Hospital, Osaka, Japan
[3]Osaka Rousai Hospital, Sakai, Japan
[4]Osaka Kouseinenkin Hospital, Osaka, Japan
[5]Kansai Rousai Hospital, Amagasaki, Japan
[6]National Hospital Organization Osaka Minami Medical Center, Kawachinagano, Japan
[7]Kaizuka City Hospital, Kaizuka, Japan
[8]Osaka Police Hospital, Osaka, Japan
[9]Higashiosaka City Central Hospital, Higashiosaka, Japan
[10]Osaka General Medical Center, Osaka, Japan
[11]Saiseikai Senri Hospital, Suita, Japan

Background. The aim of this study was to examine the factors correlated with the progression of ribavirin-induced hemolytic anemia in patients with chronic hepatitis C treated by interferon and ribavirin combination therapy. ***Methods.*** This study was conducted on 505 patients by the Osaka Liver Disease Study Group. A decline of hemoglobin (Hb) concentration by 2 g/dl at the end of 2 weeks from the start of the treatment ("2 by 2" standard) was adopted as a predictive factor for progression to severe anemia. The ribavirin apparent clearance (CL/F) was also examined. ***Results.*** Of 482 patients whose Hb value was more than 12 g/dl before the treatment, 68 patients (14%) had to discontinue ribavirin owing to severe anemia. Patients in the "2 by 2"-positive group (Hb decline over 2 g/dl) and the group with lower CL/F were significantly more likely to discontinue ribavirin owing to severe anemia. Discontinuation was more common among patients aged 60 years or older than for those under 60 years old (21% vs. 9%, $P < 0.001$). Among patients aged 60 years or older, only the "2 by 2" standard was significantly associated with the discontinuance of ribavirin owing to severe anemia in a multivariate analysis (odds ratio, 4.18; $P < 0.001$). ***Conclusions.*** The "2 by 2" standard of Hb decline can be used to identify patients likely to develop severe anemia. The early reduction of ribavirin can help prevent progression to severe anemia, thus allowing ribavirin therapy to be completed even in older patients.

Received: March 30, 2006 / Accepted: June 12, 2006
Reprint requests to: N. Hiramatsu

Key words: chronic hepatitis C, interferon and ribavirin combination therapy, progression of anemia, "2 by 2" standard

Introduction

Hepatitis C virus (HCV) is estimated to infect up to 170 million people worldwide,[1] and two million people in Japan. Long persistence of HCV infection can lead to progression of liver fibrosis, causing liver cirrhosis and ultimately hepatocellular carcinoma.[2,3] Past studies have made clear that interferon (IFN) therapy is effective for eliminating HCV,[4,5] but the sustained viral response (SVR) rate of IFN monotherapy is not sufficient. The addition of the nucleoside analog ribavirin to IFN in the treatment of patients with chronic hepatitis C can significantly improve the SVR rate, and combination therapy with IFN or pegylated-IFN (Peg-IFN) has been recommended as a standard regimen worldwide.[6–10] However, additional side effects of ribavirin have been reported, such as hemolytic anemia, which have not been found with IFN monotherapy, leading to discontinuance of the treatment.[11–14]

In previous studies, the discontinuance rate of IFN and ribavirin combination treatment due to severe side effects has been reported to be 6%–13%.[6,7] Ribavirin-induced hemolytic anemia has been suggested to depend on a high plasma concentration of ribavirin.[15] The ribavirin apparent clearance (CL/F), which reflects the plasma concentration of ribavirin at 4 weeks after the start of combination therapy, has been used as a

predictive factor for ribavirin-induced hemolytic anemia before the start of treatment.[16–18] Furthermore, in the manufacturer's drug information for ribavirin,[19] a dose reduction is recommended when hemoglobin (Hb) levels decrease to less than 10 g/dl, and discontinuance of ribavirin is recommended when Hb levels fall to less than 8.5 g/dl during combination therapy with IFN and ribavirin. However, according to this guideline, not a few patients are forced to discontinue ribavirin because the dose reduction to avoid severe anemia does not occur in time.

What is needed is a convenient guideline for avoiding ribavirin discontinuance due to severe anemia. In this study, we evaluated the correlation of Hb decline at 2 weeks after the start of combination therapy with the discontinuance of treatment due to progression of ribavirin-induced hemolytic anemia. We also assessed the utility of an early decline of Hb in comparison with the CL/F standard for predicting the progression to severe anemia.

Patients and methods

Patients

The current study was conducted at Osaka University Hospital and other institutions participating in the Osaka Liver Disease Study Group. The 505 patients with chronic hepatitis C included in this study were treated with a combination of interferon-α-2b and ribavirin between January 2001 and December 2005. All patients were anti-hepatitis C virus antibody positive, had HCV RNA detectable in their serum by the polymerase chain reaction method, and had elevated serum alanine transaminase (ALT) (above the upper limit of normal) within the 6 months prior to treatment. Excluded from this study were patients who were positive for hepatitis B surface antigen or anti-human immunodeficiency virus antibody or those with other forms of liver disease (alcoholic liver disease, hepatotoxic drugs, autoimmune hepatitis). Twenty-three patients whose Hb was under 12 g/dl before the treatment were also excluded because the aim of this study was to analyze the progression of anemia; patients with a low Hb level before treatment are known to have a tendency toward progression of anemia. The remaining 482 patients were followed in this study.

The baseline clinical features of the 482 patients are shown in Table 1. Their mean age was 55.2 ± 10.9 years, and 66% were men. Among the patients, 347 had HCV RNA with genotype 1 and high viral loads (1H group) and 130 had HCV RNA with genotype 2 or low viral loads (non-1H group). The mean ALT level was 100 ± 74 IU/l. In this study, a high viral load was defined as a serum HCV-RNA level of more than 10^6 equivalents/ml by branched DNA assay or more than 10^5 copies/ml serum by Amplicor-HCV monitor assay.

Treatment schedule

Of the 482 patients treated with a combination of interferon-α-2b and ribavirin, 273 were IFN naïve and 209 were undergoing retreatment. All patients were scheduled to receive interferon-α-2b (Intron-A, Schering-Plough, Kenilworth, NJ, USA) at a dose of 6 (n = 371) or 10 (n = 111) MU intramuscularly every day for the first 2 weeks and three times a week thereafter. Ribavirin (Rebetol; Schering-Plough) was given orally twice a day for a total dose of 800 mg (n = 261), 600 mg (n = 215), or 400 mg (n = 6) per day. The IFN dose was decreased from 10 to 6 MU or from 6 to 3 MU when the

Table 1. Baseline characteristics of patients

Number	482	
Age (y.o)	55.2 ± 10.9	(21–75)
Sex (male/female)	320/162	
Body weight (kg)	62.3 ± 9.9	(35–94)
HCV serotype (1/2/unknown)	364/111/7	
(1H/non-1H/unknown)	347/130/5	
Fibrosis (0/1/3/4/unknown)	19/192/202/13/56	
WBC (/mm^3)	5184 ± 1531	(2100–13200)
RBC (×10^4/mm^3)	449 ± 42	(329–617)
Hb (g/dl)	14.4 ± 1.2	(12.0–19.2)
Plt (×10^4/mm^3)	15.4 ± 5.4	(4.4–36.1)
ALT (IU/l)	100 ± 74	(17–736)
Serum creatinine (mg/dl)	0.8 ± 0.2	(0.3–1.7)
Ribavirin dosage/body weight (mg/kg)	11.4 ± 1.5	(4.6–17.8)

Data are shown as means ± SD
HCV, hepatitis C virus; 1H group, patients with genotype 1 and high viral load; non-1H group, patients not in the 1H group; Fibrosis, Knodell's histological score (category4); WBC, white blood cells; RBC, red blood cells; Hb, hemoglobin; Plt, platelets; ALT, alanine aminotransferase

white blood cell (WBC) count was below 1500/mm^3, the neutrocyte count below 750/mm^3, or the platelet (Plt) count below 5×10^4/mm^3. IFN was discontinued when the WBC count was below 1000/mm^3, the neutrocyte count below 500/mm^3, or the Plt count below 2.5×10^4/mm^3. The ribavirin dose of 200 mg was reduced when the Hb concentration decreased to less than 10 g/dl, and the ribavirin was discontinued when the Hb concentration decreased to less than 8.5 g/dl, in accordance with the manufacturer's drug information for ribavirin.[19] Ferric medicine or erythropoietin to prevent anemia was not administered. Ribavirin was scheduled to be administered for 24 weeks for all patients, and IFN for 24 weeks for 307 patients and for 48 weeks for 175 patients.

Patients with persistently undetectable HCV RNA 6 months after completion of treatment were considered to have achieved SVR.

Blood tests

All patients were examined for serum HCV-RNA level and underwent hematological and biochemical tests just before therapy, at the end of week 2, and every 4 weeks thereafter during treatment. When treatment was completed, the patients were assessed every 4 weeks until 24 weeks after the end of treatment.

Total ribavirin clearance

Using the method of Kamar et al.,[17] CL/F at the start of the treatment was calculated as follows:

$$\text{CL/F (l/h)} = 32.3 \times \text{BW} \times (1 - 0.0094 \times \text{Age}) \times (1 - 0.42 \times \text{Sex})/\text{Scr},$$

where BW = body weight; sex = 0 for male and 1 for female; and Scr = serum creatinine.

Definition of "severe anemia" leading to discontinuance of ribavirin

In this study, "discontinuance of ribavirin due to severe anemia" was defined as follows: discontinuance of ribavirin due to a decrease of Hb to less than 8.5 g/dl or clinical symptoms of anemia associated with a decrease of Hb of more than 3 g/dl from the start of combination therapy.

Liver histology

Hepatic fibrosis was assessed by Knodell's histological score (category 4).[20] Fibrosis stage was evaluated on a scale from 0 to 4: 0 = no fibrosis; 1 = fibrosis portal expansion; 3 = bridging fibrosis (portal–portal or portal–central linkage); 4 = cirrhosis.

Statistical analysis

Age, body weight, ribavirin dosage/body weight, WBC count, red blood cell (RBC) count, Hb concentration, Plt, serum ALT levels, and Scr are expressed as means ± SD. The SVR rate was evaluated using an intention-to-treat (ITT) analysis. The differences in proportions were tested by the χ-squared test. For univariate and multivariate analyses, a logistic regression analysis was used to predict ribavirin-induced severe anemia. A value of $P < 0.05$ (two-tailed) was considered to indicate significance.

Results

Efficacy of the combination therapy with dose reduction or discontinuance of ribavirin

The relationship between dose reduction or discontinuance of ribavirin and the SVR rate on ITT analysis is shown in Fig. 1. The SVR rate was 20% (71/347) for all 1H patients and 72% (93/130) for all non-1H patients. Among the 1H patients, SVR was achieved for 24% (45/189) without dose reduction of ribavirin and for 26% (20/76) with dose reduction. Significantly lower SVR rates were observed for patients who had to discontinue ribavirin treatment owing to adverse effects (7%, 6/82) in comparison with those with ($P < 0.01$) or without ($P < 0.01$) dose reduction. In the non-1H group, similar SVR rates were found with dose reduction of ribavirin [SVR rate without dose reduction, 83% (58/70), vs. SVR rate with dose reduction, 82% (23/28)], and the SVR rate of patients who had to discontinue ribavirin owing to adverse effects was significantly lower (38%, 12/32) than that for those with ($P < 0.001$) or without ($P < 0.0001$) dose reduction.

The same tendency was observed even in the 307 patients treated with IFN for 24 weeks. Among the 1H patients treated for 24 weeks, SVR was achieved for 19% (17/91) without dose reduction of ribavirin, 15% (6/41) with dose reduction, and 3% (2/75) with discontinuance. There were significant differences between the patients with discontinuance and those without ($P < 0.01$) or with ($P < 0.05$) dose reduction. Among the non-1H patients treated for 24 weeks, SVR rates were 85% (39/46) for the patients without dose reduction of ribavirin, 85% (17/20) for those with dose reduction, and 33% (10/30) for those with discontinuance. Significantly lower SVR rates were observed for patients who had to discontinue ribavirin than for those with ($P = 0.05$) or without ($P < 0.05$) dose reduction.

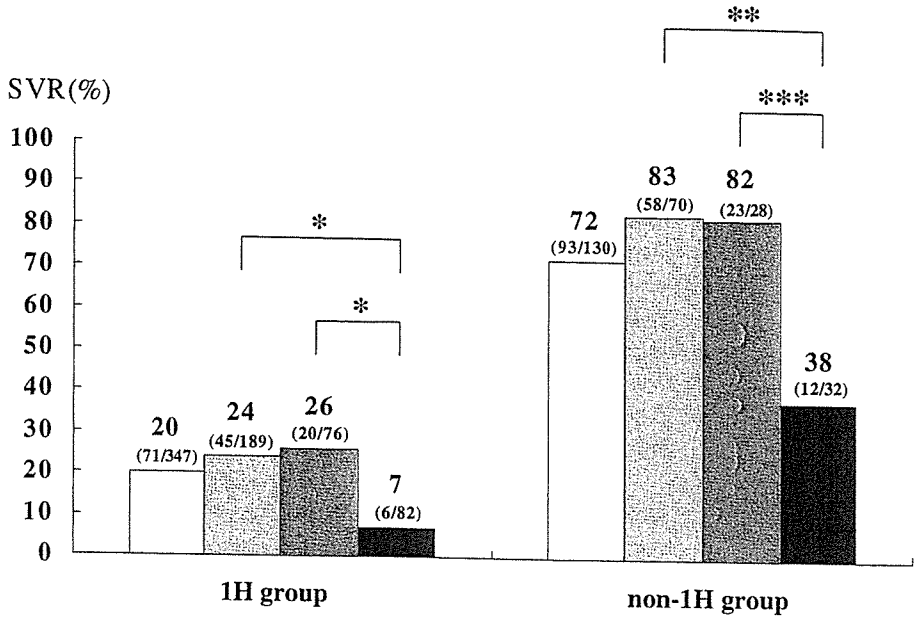

Fig. 1. Efficacy of combination therapy with dose reduction or discontinuance of ribavirin (intention-to-treat analysis). *1H group*, patients with genotype 1 and high viral load; *non-1H group*, patients not in the 1H group; *SVR*, sustained viral response. □ all patients; ▩ patients without dose reduction of ribavirin; ■ patients with dose reduction of ribavirin; ■ patients with discontinuance of ribavirin. *, $P < 0.01$; **, $P < 0.0001$; ***, $P < 0.001$

Table 2. Rate of the ribavirin reduction or discontinuance due to adverse effects with different levels of CL/F

			Discontinuance	
	No reduction	Dose reduction	All cases	Cases due to severe anemia
20 ≤ CL/F ($n = 45$)	94% (42/45)	2% (1/45)	4% (2/45)	0% (0/45)
15 ≤ CL/F < 20 ($n = 100$)	66% (66/100)	19% (19/100)	15% (15/100)	6% (6/100)
10 ≤ CL/F < 15 ($n = 179$)	54% (96/179)	24% (42/179)	23% (41/179)	14% (25/179)
CL/F < 10 ($n = 158$)	37% (58/158)	28% (44/158)	35% (56/158)	23% (37/158)

Frequency of and reasons for dose reduction or discontinuance of ribavirin during combination therapy

We examined the rate of discontinuance of therapy due to adverse effects up to the end of 24 weeks, because all cases of discontinuation occurred before the end of 24 weeks. Of the 482 patients, 401 patients completed 24 weeks of therapy, and 81 patients (17%) had to discontinue both IFN and ribavirin before the end of the 24 weeks. Of the 401 patients undergoing 24 weeks of therapy, the entire treatment schedule without reduction or discontinuance of either drug was completed by 262 patients (54%). The ribavirin dose was decreased for 106 patients (22%) and was stopped without discontinuance of IFN for 33 patients (7%). Overall, 114 patients (24%) discontinued ribavirin treatment. The reasons for dose reduction or discontinuance of ribavirin were anemia, general fatigue, digestive disorder, eczema, neutropenia, thrombocytopenia, or psychological disorder. Among the patients discontinuing ribavirin, the major reasons were anemia (14%), general fatigue (2%), or digestive disorder (2%).

CL/F and dose reduction or discontinuance of ribavirin

CL/F calculated for all patients was 4.6–32.5 l/h. The mean CL/F was 13.0 l/h, and the median was 11.9 l/h. At the start of treatment, CL/F was less than 10 l/h for 33% (158/482) of patients, 10–15 l/h for 37% (179/482), 15–20 l/h for 21% (100/482), and more 20 l/h for 9% (45/486).

Table 2 shows the rates of dose reduction or discontinuance of ribavirin in relation to different levels of CL/F. The rate of discontinuance of ribavirin among all patients was 4% (2/45) for patients with CL/F ≥ 20, 15% (15/100) for those with 15 ≤ CL/F < 20, 23% (41/179) for those with 10 ≤ CL/F < 15, and 35% (56/158) for those with CL/F < 10. The rate of discontinuance of ribavirin due to severe anemia was 14% (68/482) among all pa-

tients. There was no discontinuance of ribavirin due to severe anemia among patients with CL/F ≥ 20, but the rate of discontinuance was 6% (6/100) among those with 15 ≤ CL/F < 20, 14% (25/179) among those with 10 ≤ CL/F < 15, and 23% (37/158) among those with CL/F < 10. The rate of continuance of ribavirin without dose reduction decreased in proportion to the decline of CL/F. In this study, we adopted two categories of CL/F, below 15 l/h (CL/F < 15) and below 10 l/h (CL/F < 10), to assess CL/F as a factor for predicting anemia progression.

We also analyzed the predictive factor of anemia progression according to patient age, because CL/F varies widely with patient age and tends to be lower among older patients. Among patients under 60 years old (n = 288), 17% (48/288) had CL/F under 10 l/h, 38% (109/288) had CL/F 10–15 l/h, 30% (86/288) had CL/F 15–20 l/h, and 16% (45/288) had CL/F over 20 l/h. On the other hand, among those 60 years old or older (n = 194), 57% (110/194) had CL/F under 10 l/h, 36% (70/194) had CL/F 10–15 l/h, 7% (14/194) had CL/F 15–20 l/h, and none had CL/F over 20 l/h. Thus, the majority (93%) of the patients 60 years old or older had a low CL/F (<15), whereas only 55% of those under 60 years old had CL/F < 15.

Early decline of Hb and progression of anemia during combination therapy

Figure 2 shows the decline of Hb from the start of combination therapy. We conducted this analysis for the 433 patients: those who did not need a dose reduction of ribavirin (n = 262), those who needed a dose reduction owing to a decrease of Hb to less than 10 g/dl (n = 103), and those who discontinued ribavirin due to "severe anemia" (n = 68). We excluded 49 patients from this analysis: 46 patients stopped combination therapy for reasons other than anemia, such as general fatigue or digestive disorder, and the other three patients were not responding to antiviral treatment and stopped therapy before 24 weeks without a dose reduction of ribavirin. Following the initiation of combination therapy, Hb concentration decreased rapidly until the end of the 4th week. At the end of 2 weeks, Hb had decreased by 0.9 ± 1.2 g/dl among the patients without dose reduction of ribavirin, by 1.8 ± 1.3 g/dl among those with dose reduction, and by 2.3 ± 1.4 g/dl among those who discontinued ribavirin. At the end of 4 weeks, Hb had decreased by 2.1 ± 1.5 g/dl among the patients without dose reduction of ribavirin, by 3.2 ± 1.5 g/dl among those with dose reduction, and by 3.9 ± 1.5 g/dl among those discontinuing ribavirin.

ΔHb [ΔHb = (Hb value just before treatment) − (Hb value during treatment)] both at the end of 2 weeks and at the end of 4 weeks were significantly larger among the patients discontinuing ribavirin than among those without dose reduction of ribavirin (P < 0.0001, P < 0.0001, respectively). In this study, we adopted the category of ΔHb at the end of 2 weeks because it allowed the progression of anemia to be estimated at an earlier phase of treatment than did ΔHb at the end of 4 weeks.

To establish the cutoff value of ΔHb at the end of 2 weeks, we used two categories of ΔHb: a decrease in Hb concentration at 2 weeks to 2 g/dl below the baseline (ΔHb2.0) or to 1.5 g/dl below the baseline (ΔHb1.5). We conducted this analysis for 480 patients, because two patients stopped combination therapy before 2 weeks for reasons other than anemia. With the ΔHb2.0 standard, the rate of discontinuance of ribavirin due to severe anemia was 10% (32/338) in the ΔHb < 2.0 group and 25% (36/142) in the ΔHb ≥ 2.0 group, with the difference being significant (P < 0.0001) (Table 3). With the ΔHb1.5 standard, the rate of discontinuance of ribavirin due to severe anemia was significantly higher

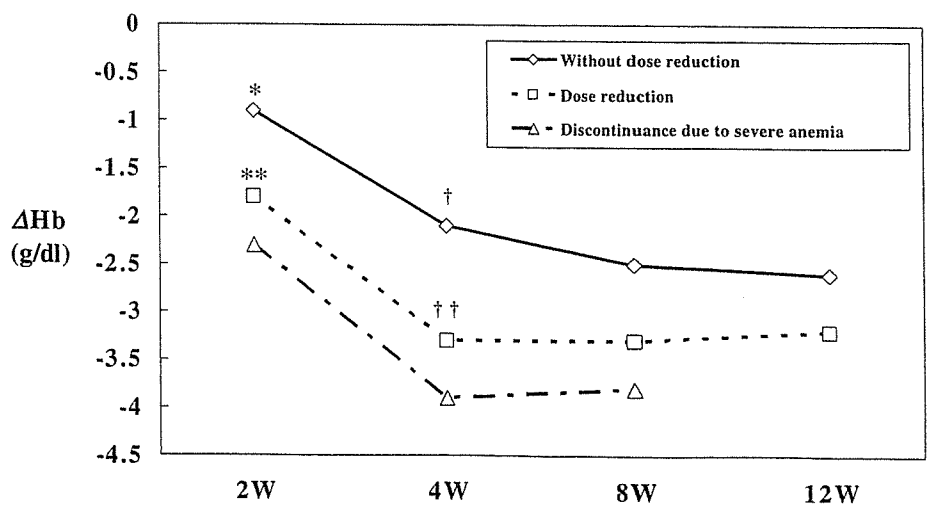

Fig. 2. Decline of hemoglobin according to dose reduction or discontinuance of ribavirin. *Significantly different from patients with dose reduction (P < 0.0001) and patients with discontinuance (P < 0.0001); **significantly different from patients with discontinuance (P < 0.02); †significantly different from patients with dose reduction (P < 0.0001) and patients with discontinuance (P < 0.0001); ††significantly different from patients with discontinuance (P < 0.01)

Table 3. Rate of the ribavirin reduction or discontinuance due to adverse effects with rate of anemia progression

	No reduction	Dose reduction	Discontinuance	
			All cases	Cases due to severe anemia
ΔHb ≥ 2.0 (n = 142)	37% (53/142)	29% (41/142)	34% (48/142)	25%* (36/142)
ΔHb < 2.0 (n = 338)	61% (209/338)	19% (65/338)	20% (64/338)	10% (32/338)

*P < 0.0001

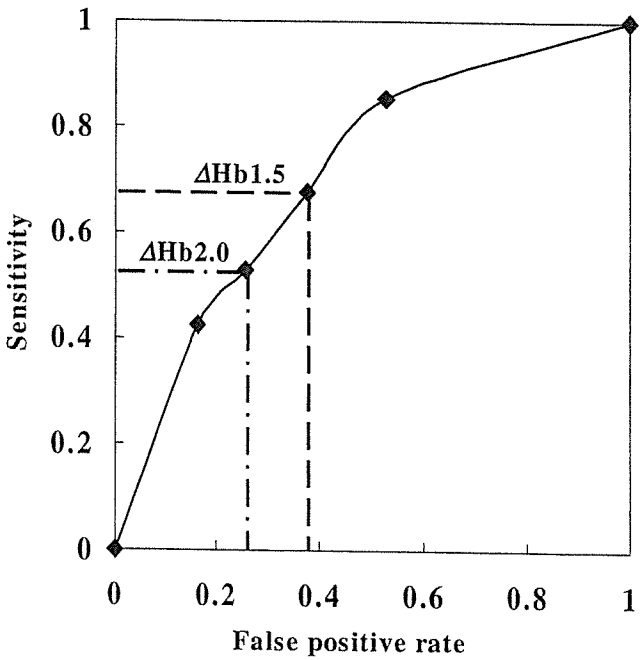

Fig. 3. Receiver-operating characteristic curve for ΔHb at the end of 2 weeks for discontinuance of ribavirin due to severe anemia

in the ΔHb ≥ 1.5 group than in the ΔHb < 1.5 group (8%, 22/279 vs. 23%, 46/201; P < 0.0001). Figure 3 shows the receiver-operating characteristic curve using ΔHb at the end of 2 weeks for the discontinuance of ribavirin due to severe anemia. Between the ΔHb2.0 and ΔHb1.5 standards, no significant difference was found in sensitivity (53%, 36/68, vs. 68%, 46/68; NS). On the other hand, the false positive rate was significantly lower with the ΔHb2.0 standard than with the ΔHb1.5 standard (26%, 93/360, vs. 38%, 136/360; P < 0.001), and accuracy was significantly higher with the ΔHb2.0 standard than with the ΔHb1.5 standard (71%, 303/428, vs. 63%, 270/428; P = 0.02). Therefore, we adopted ΔHb2.0 at the end of 2 weeks (the "2 by 2" standard) as a predictive factor for discontinuance of ribavirin due to severe anemia because of the higher specificity rate of ΔHb2.0 (lower false positive rate).

Logistic regression analysis for discontinuance of ribavirin in combination therapy

We assessed the factors correlated with the discontinuance of ribavirin due to severe anemia by logistic regression analysis. The following factors were evaluated: age, sex, body weight, ribavirin dosage/body weight, IFN dosage, Scr, Hb value at the start of the therapy, CL/F category, and early decline of Hb ("2 by 2" standard). Older age, lower body weight, lower Hb at the start of the therapy, lower CL/F (CL/F < 10 or CL/F < 15), and "2 by 2"-positive (the patients whose Hb had decreased by more than 2 g/dl at 2 weeks from the start of the treatment) were factors significantly associated with discontinuance of ribavirin due to severe anemia by univariate logistic regression analysis (Table 4). Next, we assessed the factors correlated with the discontinuance of ribavirin due to severe anemia by multivariate logistic regression analysis. Among the factors selected as significant by the univariate analysis, we omitted age and body weight from the multivariate analysis because they were included as parameters in the numerical formula for CL/F. Therefore, we evaluated the Hb value at the start of therapy, the CL/F category, and the "2 by 2" category by multivariate analysis. The CL/F borderline values of 10 l/h and 15 l/h were evaluated separately. In the multivariate logistic regression analysis, lower Hb at the start of therapy, lower CL/F (CL/F < 10 or CL/F < 15), and "2 by 2"-positive were significantly associated with discontinuance of ribavirin due to severe anemia (Table 5).

Useful predictive factors for discontinuance of ribavirin among older patients

Among the 288 patients under 60 years old, 50 (17%) had discontinued ribavirin by the end of 24 weeks for various reasons, including anemia, general fatigue, digestive disorder, and psychological disorders. Among the 194 patients aged 60 years and older, 64 (33%) had discontinued ribavirin, with severe anemia accounting for approximately 65% (41/64). More than twice as many patients aged 60 years and older discontinued ribavirin treatment compared with younger patients;

Table 4. Univariate analysis for the discontinuance of ribavirin due to severe anemia

Factor	Category	Odds ratio	95% CI	*P* value
Age			1.045–1.117	<0.0001
Sex	Male/Female	1/1.18	0.663–2.029	0.56
Body weight			0.928–0.981	<0.001
Serum creatinine			0.551–9.492	0.25
Ribavirin/Body weight			0.945–1.357	0.18
IFN dosage	6MU/10MU	1/1.03	0.557–1.893	0.93
Hb			0.480–0.780	<0.0001
CL/F	≥15/<15	1/5.56	0.076–0.427	0.0001
	≥10/<10	1/3.14	0.187–0.540	<0.0001
"2 by 2"	Negative/Positive	1/3.23	0.182–0.527	<0.0001

CI, confidence interval; IFN, interferon; CL/F, apparent clearance; "2 by 2", ΔHb2.0 at the end of 2 weeks; "2 by 2"-positive means ΔHb ≥ 2.0; "2 by 2"-negative means ΔHb < 2.0

Table 5. Multivariate analysis for the discontinuance of ribavirin due to severe anemia

Factor	Category	Odds ratio	95% CI	*P* value
Hb			0.446–0.785	0.0003
CL/F	≥15/<15	1/3.18	0.126–0.786	0.01
"2 by 2"	Negative/Positive	1/4.35	0.127–0.419	<0.0001
Hb			0.440–0.784	0.0003
CL/F	≥10/<10	1/1.98	0.278–0.923	0.03
"2 by 2"	Negative/Positive	1/4.63	0.119–0.393	<0.0001

this difference was significant (21%, 41/194, vs. 9%, 27/288; $P = 0.0003$) (Table 6).

We assessed the analysis for discontinuance of ribavirin due to severe anemia among the patients aged 60 years or older. Older age, lower CL/F (CL/F < 10), and "2 by 2"-positive were factors significantly associated with discontinuance of ribavirin due to severe anemia by univariate logistic regression analysis (Table 7A). Next, we assessed the factors correlated with the discontinuance of ribavirin due to severe anemia by multivariate logistic regression analysis. Among the three factors selected as significant by univariate analysis, we omitted the factor of age from the multivariate analysis as it was included as a parameter in the numerical formula for CL/F. In the multivariate logistic regression analysis of the CL/F category (CL/F < 10) and the "2 by 2" category, the latter was the only significant factor associated with the discontinuance of ribavirin due to severe anemia (Table 7B). Using the "2 by 2" standard, the rate of discontinuance of ribavirin due to severe anemia was 14% (18/133) in the "2 by 2"-negative (the patients whose Hb decreased by less than 2 g/dl from the start of treatment) group and 38% (23/60) in the "2 by 2"-positive group, with the difference being significant ($P < 0.0001$) (Table 8).

We next compared the sensitivity, specificity, and accuracy of the CL/F category with those of the "2 by 2" category as predictive factors for discontinuance of ribavirin due to severe anemia among patients aged 60 years or older. Table 9 shows the comparison between the CL/F < 15 category and the "2 by 2" category (Table 9A) and that between the CL/F < 10 category and the "2 by 2" category (Table 9B). Although sensitivity was higher for the lower CL/F category [CL/F < 15, 100% (41/41); CL/F < 10, 71% (29/41)] than for the "2 by 2" category (56%, 23/41), specificity and accuracy were significantly higher for the "2 by 2" category than for the CL/F category [specificity: "2 by 2," 77% (96/125) vs. CL/F < 15, 7% (9/125), $P < 0.0001$; "2 by 2" vs. CL/F < 10, 47% (59/125), $P < 0.0001$; accuracy: "2 by 2," 72% (119/166) vs. CL/F < 15, 30% (50/166), $P < 0.0001$; "2 by 2" vs. CL/F < 10, 53% (88/166), $P < 0.001$].

Table 6. Major causes of discontinuance of ribavirin

	Age < 60	Age ≥ 60
Severe anemia	27 (9%)	41 (21%)*
General fatigue	7	3
Digestive disorders	5	3
Neutropenia	1	1
Thrombocytopenia	2	4
Eruption with itching	2	4
Psychological disorders	3	3
Others	3	5
Total	50/288 (17%)	64/194 (33%)

*$P < 0.001$

Table 7. Univariate and multivariate analysis for the discontinuance of ribavirin due to severe anemia among the patients aged 60 years and older

A. Univariate analysis

Factor	Category	Odds ratio	95% CI	*P* value
Age			1.007–1.250	0.04
Sex	Male/Female	1/1.67	0.280–1.286	0.19
Body weight			0.947–1.021	0.37
Serum creatinine			0.865–33.586	0.07
Ribavirin/Body weight			0.775–1.205	0.76
IFN dosage	6MU/10MU	1/1.92	0.803–4.579	0.14
Hb			0.537–1.106	0.16
CL/F	≥15/<15	—	—	0.97
	≥10/<10	1/2.16	0.217–0.989	0.047
"2 by 2"	Negative/Positive	1/4.24	0.112–0.497	0.0001

B. Multivariate analysis

Factor	Category	Odds ratio	95% CI	*P* value
CL/F	≥10/<10	1/2.12	0.213–1.042	0.063
"2 by 2"	Negative/Positive	1/4.18	0.112–0.507	0.0002

Table 8. Rate of the ribavirin reduction or discontinuance due to adverse effects with the rate of anemia progression among the patients aged 60 years and older

			Discontinuance	
	No reduction	Dose reduction	All cases	Cases due to severe anemia
ΔHb ≥ 2.0	27%	23%	50%	38%*
("2 by 2"-positive) (n = 60)	(16/60)	(14/60)	(30/60)	(23/60)
ΔHb < 2.0	46%	29%	25%	14%
("2 by 2"-negative) (n = 133)	(61/133)	(39/133)	(33/133)	(18/133)

*P < 0.0001

Table 9. Comparison of "2 by 2" standard and CL/F standard for the discontinuance of ribavirin due to severe anemia among the patients aged 60 years and older

A.

	"2 by 2"-positive	CL/F < 15	*P* value
Sensitivity	56% (23/41)	100% (41/41)	<0.0001
Specificity	77% (96/125)	7% (9/125)	<0.0001
Accuracy	72% (119/166)	30% (50/166)	<0.0001

B.

	"2 by 2"-positive	CL/F < 10	*P* value
Sensitivity	56% (23/41)	71% (29/41)	0.17
Specificity	77% (96/125)	47% (59/125)	<0.0001
Accuracy	72% (119/166)	53% (88/166)	<0.001

Discussion

Ribavirin, developed in 1972, is a synthetic nucleic acid analog, which has antiviral activity in vitro against a wide variety of RNA and DNA viruses. Combination therapy of ribavirin with IFN or Peg-IFN led to remarkable progress in antiviral therapy for chronic hepatitis C. To raise the SVR rate for such combination therapy, it is very important to predict the discontinuance of the therapy due to an adverse effect and prevent it. In this study, we observed the incidence of hemolytic anemia, the major side effect of ribavirin. The factors correlated with the progression of anemia were analyzed to avert the need to discontinue ribavirin treatment of patients with chronic hepatitis C receiving combination therapy.

Several studies in the United States and European countries have reported that higher ribavirin dosage or a higher plasma concentration of ribavirin increases the SVR rate.[21,22] However, a higher ribavirin dose or higher plasma concentration of ribavirin entails the risk of having to discontinue ribavirin treatment. In Japan, analysis of the relationship between the SVR rate and a dose reduction or discontinuance of ribavirin, has shown that reducing the dose of ribavirin does not affect the SVR rate. In the present study, the SVR rate of the patients discontinuing ribavirin was also shown to be significantly lower than the patients who did not discontinue it