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Epidemiological survey of oral lichen planus among HCV-infected inhabitants in a town in Hiroshima Prefecture in Japan from 2000 to 2003

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Abstract. The objective of our study was to evaluate the natural history of oral lichen planus (OLP) and other extrahepatic manifestations in the inhabitants of an area in Japan that is hyperendemic for hepatitis C virus (HCV) infection. Over 4 years, 224 adult inhabitants with HCV infection were examined for OLP by a single oral surgeon. All subjects were interviewed regarding the natural history of other extrahepatic manifestations they had developed. The antibodies to HCV (anti-HCV) and serum HCV RNA were determined. Anti-HCV were detected in sera from 224 subjects (100%); HCV RNA in 210 (93.8%). Of the 224, 88 had at least 1 oral examination for OLP during the 4-year period. In 2000, 2001, 2002 and 2003, OLP was observed in 8.5 (5/59), 14.8 (8/54), 20 (11/55) and 21.4% (12/56) of subjects, respectively. OLP prevalence increased as the subjects grew older. The incidence of OLP over the 4 years among all subjects with HCV infection was 17.0% (15/88, 2 men and 13 women). None experienced natural healing or the development of malignant transformations. Between 2000 and 2003, there was an increase in the prevalence of type 2 diabetes mellitus (DM), thyroid dysfunction, skin disease, renal disease and hypertension. Screening for extrahepatic manifestations should be conducted in patients with risk factors for HCV infection.

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Abbreviations: HCV, hepatitis C virus; OLP, oral lichen planus; HCC, hepatocellular carcinoma; anti-HCV, antibodies to HCV; DM, diabetes mellitus

Key words: lichen planus, hepatitis C virus, extrahepatic manifestations

Introduction

Hepatitis C virus (HCV) infection is a major health problem in Japan. It is highly prevalent in subjects with chronic liver disease and is strongly associated with hepatocellular carcinoma (HCC). HCV-related HCC accounts in large part for the recent increase in HCC and now constitutes about 80% of all HCC cases in Japan. HCV also incites many extrahepatic manifestations (1,2) of which lichen planus is the most common (3,4). Other associated diseases include cryoglobulinaemic nephropathy and glomerulonephritis (5), thyroid dysfunction (6), porphyria cutanea tarda (7) and type 2 diabetes mellitus (DM) (8).

We previously reported that the incidence of oral lichen planus (OLP) in subjects with HCV infection was significantly higher than in those without HCV. We reached this conclusion by mass screening 685 inhabitants of a hyperendemic area, H town, located in the Fukuoka prefecture of Northern Kyushu, Japan (Fig. 1) for HCV infection (9). The prevalence of other extrahepatic manifestations in subjects with antibodies to HCV (anti-HCV) was higher than in those without HCV (10).

We also conducted an epidemiological study of another HCV hyperendemic area, O town, in the northwest of the Hiroshima prefecture in Honshu, Japan (Fig. 1). The presence of HCV-associated extrahepatic manifestations was found in 66.1% (39/59) of those screened (11). These findings suggest that the high prevalence of various extrahepatic manifestations among HCV-infected subjects is not unique to specific areas.

In the present investigation, we annually examined extrahepatic manifestations in the inhabitants of O town from 2000 to 2003. The aim of this study was to evaluate the natural history of OLP and other extrahepatic manifestations in individuals with HCV infections.

Patients and methods

Patients. From 2000 to 2003, we studied a total of 224 adult inhabitants of O town, a hyperendemic area of HCV infection. All were HCV carriers, though the causes of viral transmission were unknown. In 2000, 2001, 2002 and 2003,

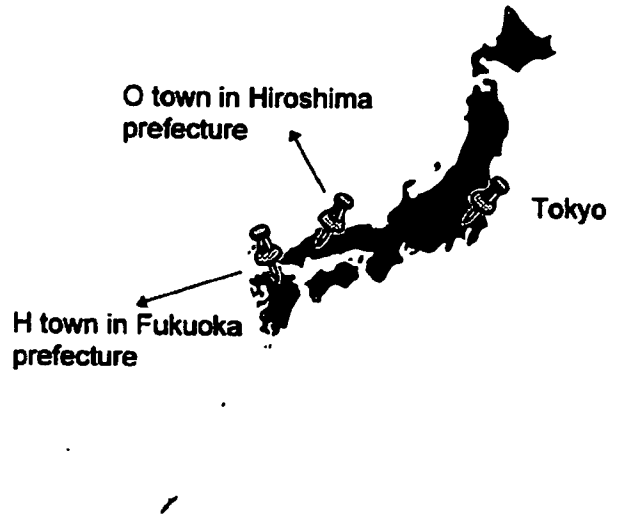

Figure 1. The research area. The location of O town in the northwest region of the Hiroshima prefecture in Honshu, Japan.

we examined 59, 54, 55 and 56 inhabitants, respectively (Table I). A single oral surgeon examined subjects for oral membrane diseases. A topographic classification of the oral mucosa, with location codes indicated, is shown in Fig. 2 (12). The diagnosis of OLP was made based on clinical and histopathological features.

All subjects were interviewed in person by 2 trained interviewers. We inquired about the following: cigarette smoking habits, present health condition and the presence of extrahepatic manifestations of HCV infection such as type 2 DM, rheumatoid arthritis, thyroid dysfunction, skin disease, renal disease, hypertension and extrahepatic malignant tumors.

Informed consent was obtained from all subjects once the purpose and methods of the study were explained.

Examination for anti-HCV and HCV RNA in serum. Sera were examined for the presence or absence of HCV. Anti-HCV were measured by a second-generation, enzyme-linked immunosorbent assay (Abbott HCV PHA 2nd Generation, Dainabot Co., Ltd., Tokyo, Japan). HCV RNA in the sera was detected using the Amplocore HCV test (Nippon Roche, Tokyo, Japan).

Examination of the prevalence of extrahepatic manifestations from 2000 to 2003. We have previously reported on the prevalence of extrahepatic manifestations in HCV infection, including OLP, for inhabitants of the same town (11). We now examined the prevalence of these extrahepatic manifestations from 2000 to 2003.

Results

Anti-HCV were detected in the sera of 224 subjects (100%) and HCV RNA in 210 subjects (93.8%), as shown in Table I. Of the 224, 88 had at least 1 oral examination over the course of the 4 years of the study (34 men and 54 women).

Table I shows the prevalence of OLP in all subjects. In 2000, 2001, 2002 and 2003 it was 8.5 (5/59), 14.8 (8/54), 20 (11/55) and 21.4% (12/56), respectively. The prevalence of OLP in HCV RNA positive subjects in 2000, 2001, 2002 and 2003 was 8.8 (5/57), 16 (8/50), 21.6 (11/51) and 23.1% (12/52), respectively. The prevalence of OLP increased with age. The incidence of OLP among all subjects with HCV infection over the 4-year period was 17.0% (15/88, 2 men and 13 women). A history of smoking was found in 1/15 OLP cases (6.7%) among inhabitants. Of the 15 cases, 2 had medical checkups once a year, 3 had them 3 times a year, 9 had them twice a year and 1 had a checkup just once in the 4-year period from 2000 to 2003 (Table II). No one had visited a clinic for the treatment of their OLP prior to our discovery of their OLP lesions. By far the most common site for OLP was the buccal mucosa. The predominant type in 53.3% of the 15 cases (8/15) was the reticular form of the disease. In 46.7% (7/15) it was the erosive form. Fig. 3 shows the erosive form (inhabitant No. 6 in Table II). Reticular lesions were generally asymptomatic. Two of the 15 cases had aggravated oral symptoms during the 4-year period. None experienced natural healing or developed malignant transformation.

From 2000 to 2003, there was an increase in the prevalence of type 2 DM, thyroid dysfunction, skin disease, renal disease and hypertension (Table I).

Discussion

HCV carriers in Japan are presumed to number 2 million (13). The growing incidence of HCC is expected to reach a plateau by around the year 2015. However, there are many people who are not aware that they are infected, some of whom will advance to liver cirrhosis or HCC (14). The incidence of HCC varies greatly among different regions. Epidemiological studies conducted by the Japanese Ministry of Health, Labour and Welfare showed that the mortality rate associated with HCC was high in several prefectures in Western Japan. Areas with high rates of anti-HCV, such as the Saga prefecture (3.9%), Hiroshima (1.8%), Fukuoka (1.7%) and Kagawa (1.7%), had high death rates for primary liver cancer of 43.1, 39.6, 39.8 and 31.9 per 100,000 people, respectively. These rates were higher than the national average (15).

HCV is associated with a wide range of extrahepatic manifestations. Zignego *et al* classified the extrahepatic manifestations of HCV into 4 main categories (16). The first category (A) includes extrahepatic manifestations characterised by a very strong association to HCV and supported by both epidemiological and pathogenetic evidence. Category A comprises mixed cryoglobulinaemia. The second category (B) includes disorders which are significantly associated with HCV infection, supported by adequate data. Category B comprises B-cell non-Hodgkin's lymphoma, monoclonal gammopathies, porphyria cutanea tarda and lichen planus. The third category (C) includes manifestations whose association with HCV still requires confirmation and/or a more detailed characterisation of similar pathologies of different aetiology or idiopathic nature. Finally, the fourth category (D) includes only anecdotal observations.

Lichen planus is a chronic inflammatory disease of the skin and mucous membranes that frequently involves the oral mucosa. In Japan, the age-adjusted incidence rate of OLP is 59.7 per 100,000 males and 188.0 per 100,000 females (17).

Table I. Prevalence of extrahepatic manifestations in adult inhabitants with HCV infection.

	2000	2001	2002	2003
Subjects	59	54	55	56
Age (mean years ± SD)	70.7±7.2	71.2±7.2	72.0±6.5	73.4±6.8
Sex (M/F)	21/38	22/32	23/32	24/32
% with history of smoking	18.6 (11/59)	11.1 (6/54)	12.7 (7/55)	14.3 (8/56)
% positive for anti-HCV	100 (59/59)	100 (54/54)	100 (55/55)	100 (56/56)
% positive for HCV RNA	96.6 (57/59)	92.6 (50/54)	92.7 (51/55)	92.9 (52/56)
Extrahepatic manifestations				
% positive for oral lichen planus	8.5 (5/59)	14.8 (8/54)	20.0 (11/55)	21.4 (12/56)
Age (mean years ± SD)	74.8±5.2	74.3±5.7	73.1±5.1	74.7±5.8
Sex (M/F)	1/4	2/6	2/9	2/10
% positive for anti-HCV	8.5 (5/59)	14.8 (8/54)	20.0 (11/55)	21.4 (12/56)
% positive for HCV RNA	8.8 (5/57)	16.0 (8/50)	21.6 (11/51)	23.1 (12/52)
% positive for DM	15.3 (9/59)	24.1 (13/54)	20.0 (11/55)	19.6 (11/56)
Age (mean years ± SD)	67.9±7.2	68.8±7.9	69.5±7.9	68.6±7.4
Sex (M/F)	5/4	10/3	8/3	7/4
% positive for anti-HCV	15.3 (9/59)	24.1 (13/54)	20.0 (11/55)	19.6 (11/56)
% positive for HCV RNA	14 (8/57)	20.0 (10/50)	15.7 (8/51)	15.4 (8/52)
% positive for rheumatoid arthritis	1.7 (1/59)	1.9 (1/54)	5.5 (3/55)	5.4 (3/56)
Age (mean years ± SD)	67.9±7.2	70.0±0	70.0±0.8	73.0±2.9
Sex (M/F)	5/4	0/1	1/2	1/2
% positive for Anti-HCV	15.3 (9/59)	1.9 (1/54)	5.5 (3/55)	5.4 (3/56)
% positive for HCV RNA	14 (8/57)	2.0 (1/50)	5.9 (3/51)	5.8 (3/52)
% positive for thyroid dysfunction	0	3.7 (2/54)	3.6 (2/55)	8.9 (5/56)
Age (mean years ± SD)	-	67.0±1.0	68.0±1.0	72.0±3.3
Sex (M/F)	-	1/1	1/1	1/4
% positive for anti-HCV	-	3.7 (2/54)	3.6 (2/55)	8.9 (5/56)
% positive for HCV RNA	-	4.0 (2/50)	3.9 (2/51)	9.6 (5/52)
% positive for skin disease	5.1 (3/59)	11.1 (6/54)	7.3 (4/55)	16.1 (9/56)
Age (mean years ± SD)	70.3±7.3	71.3±5.8	70.8±5.2	74.1±5.9
Sex (M/F)	0/3	1/5	1/3	4/5
% positive for anti-HCV	5.1 (3/59)	11.1 (6/54)	7.3 (4/55)	16.1 (9/56)
% positive for HCV RNA	5.3 (3/57)	12.0 (6/50)	7.8 (4/51)	15.4 (8/52)
% positive for renal disease	1.7 (1/59)	5.6 (3/54)	0	1.8 (1/56)
Age (mean years ± SD)	76.0±0	76.0±2.2	-	86.0±0
Sex (M/F)	1/0	1/2	-	1/0
% positive for anti-HCV	1.7 (1/59)	5.6 (3/54)	-	1.8 (1/56)
% positive for HCV RNA	1.8 (1/57)	6.0 (3/50)	-	1.9 (1/52)
% positive for hypertension	28.8 (17/59)	40.7 (22/54)	43.7 (24/55)	55.4 (31/56)
Age (mean years ± SD)	71.0±6.9	70.9±6.3	72.6±6.0	74.4±6.7
Sex (M/F)	6/11	7/15	10/14	13/18
% positive for anti-HCV	28.8 (17/59)	40.7 (22/54)	43.6 (24/55)	55.4 (31/56)
% positive for HCV RNA	26.3 (15/57)	42.0 (21/50)	41.8 (13/51)	57.7 (30/52)
% positive for extrahepatic malignant tumor	11.9 (7/59)	13 (7/54)	9.1 (5/55)	7.1 (4/56)
Age (mean years ± SD)	74.4±3.5	76.3±3.7	77.2±4.2	79.3±2.2
Sex (M/F)	2/5	3/4	3/2	3/1
% positive for anti-HCV	11.9 (7/59)	13 (7/54)	9.1 (5/55)	7.1 (4/56)
% positive for HCV RNA	12.3 (7/57)	14 (7/50)	9.8 (5/51)	7.7 (4/52)

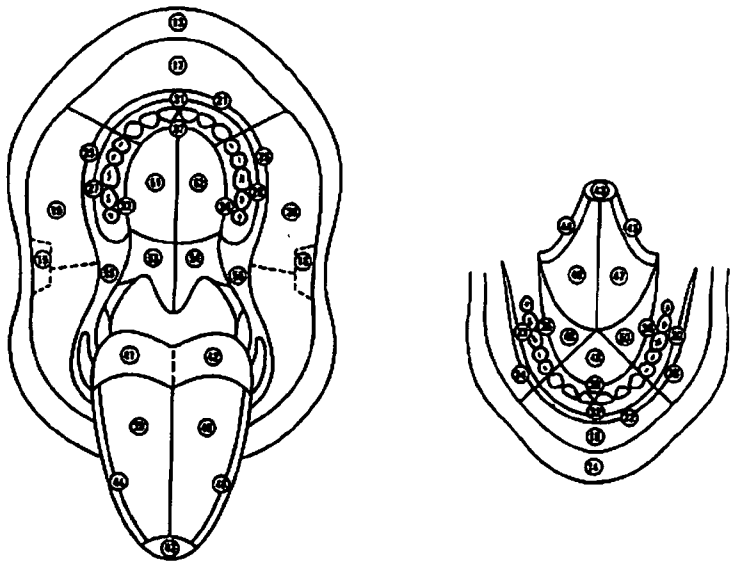

Figure 2. Topography of the oral mucosa modified from Roed-Petersen *et al* (12). The numbered locations are referred to in Table II.

Table II. Site involvement and clinical form of oral lichen planus (OLP) in subjects with HCV infection.

No.	Sex	Type	Smoking history	2000	2001	2002	2003	Course of OLP
1	F	Reticular	Negative	30	30	24, 30	24, 30	No change
2	M	Reticular	Negative	20	20	20	20	No change
3	F	Erosive	Negative	15, 16, 19-21, 23, 25, 27, 28, 31, 33, 34, 37, 39, 40, 46, 47	15, 16, 19-21, 23, 25, 27, 28, 31, 33, 34, 37, 39, 40, 46, 47	Not screened	Liver cirrhosis death	Unknown
4	F	Erosive	Negative	14, 19, 20	Not screened	Not screened	14, 19, 20	Exacerbation
5	F	Erosive	Negative	20, 45	Not screened	20, 45	Not screened	Unknown
6	F	Erosive	Negative	Not screened	19, 20, 47	Not screened	14, 19, 20, 46, 47, 51	Exacerbation
7	F	Reticular	Negative	ND	19, 55	Not screened	19, 55	No change
8	F	Reticular	Negative	Not screened	20, 26, 30	20, 26, 30	20, 26, 30	No change
9	M	Reticular	Negative	ND	46	29, 30, 35, 36, 46	29, 30, 35, 36, 46	No change
10	F	Reticular	Negative	Not screened	26, 30	26, 30	26, 30	No change
11	F	Erosive	Negative	ND	ND	14	14	No change
12	F	Erosive	Negative	Not screened	Not screened	17	17	Alleviation
13	F	Reticular	Negative	ND	Not screened	30	30	No change
14	F	Reticular	Negative	ND	ND	29	29	No change
15	F	Erosive	Positive	Not screened	Not screened	14-16, 19, 20, 24, 26, 32, 44, 45, 51, 52	Not screened	Unknown

ND, not detected. The numbers below the dates refer to locations in the oral mucosa as seen in Fig. 2.

Figure 3. A representative oral erosive lichen planus on the right buccal mucosa.

We conducted an epidemiological investigation to ascertain the possible correlation between OLP and HCV infection in patients living in Western Japan (9-11,18), where the prevalence of HCV infection is the highest in the country (15,19). We found the incidence of OLP in our patients to be higher than in the general population. OLP aside, the prevalence of other extrahepatic manifestations in subjects with anti-HCV was also higher than in those without HCV (10).

We previously reported a study on an HCV hyperendemic area, O town, with a population of approximately 3,900 in the northwest region of the Hiroshima prefecture in Honshu, Japan (11). The incidence there of subjects with 1 or more extrahepatic manifestations of HCV was 66.1%. In the present investigation, we examined extrahepatic manifestations in the same place over a 4-year period. Inhabitants with HCV infection had various extrahepatic manifestations, including OLP. The prevalence of OLP increased with the age of the subjects. This is consistent with an earlier study of inhabitants of the Fukuoka prefecture (20).

Patients with HCV-associated HCC in Japan are aging. People with chronic HCV infection should be monitored and followed carefully for extrahepatic manifestations. It is necessary for physicians and dentists to have an increased awareness of OLP in order for it to be detected at an early stage and treated promptly.

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HEPATOLOGY

Insulin resistance and lichen planus in patients with HCV-infectious liver diseases

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Key words
diabetes mellitus, extrahepatic manifestations, hepatitis C virus, insulin resistance, lichen planus.

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Abstract

Background and Aim: Hepatitis C virus (HCV) causes liver diseases and extrahepatic manifestations, and also contributes to insulin resistance and type 2 diabetes mellitus (DM). The aims of the present study were to examine the incidence of extrahepatic manifestations including lichen planus in HCV-infected patients and to evaluate the relationship between lichen planus and insulin resistance.

Methods: Of 9396 patients with liver diseases presenting to the study hospital, 87 patients (mean age 60.0 ± 11.5 years) with HCV-related liver diseases were identified and examined for the incidence of extrahepatic manifestations. Insulin resistance and the presence of *Helicobacter pylori* antibodies were also measured.

Results: The prevalence of DM was 21.8% (19/87), hypertension was 28.7% (25/87), thyroid dysfunction was 20.7% (18/87), and extrahepatic malignant tumor was 9.2% (8/87). The prevalence of lichen planus at oral, cutaneous, pharyngeal, and/or vulval locations was 19.5% (17/87). Characteristics of 17 patients with lichen planus (group A) were compared with 70 patients without lichen planus (group B). Prevalence of smoking history, presence of hypertension, extrahepatic malignant tumor, and insulin resistance (HOMA-IR) were significantly higher in group A than in group B. Significant differences were not observed for age, sex, body mass index, diagnosis of liver disease, alcohol consumption, presence of DM, thyroid dysfunction, liver function tests, or presence of *H. pylori* infection between the two groups.

Conclusions: Infection with HCV induces insulin resistance and may cause lichen planus. It is necessary for an HCV-infected patient to be assayed for insulin resistance, and to be checked for different extrahepatic manifestations of this infection, particularly lichen planus.

Introduction

The number of fatalities due to hepatocellular carcinoma (HCC) in Japan continues to increase, and it is estimated that this tendency will continue at least until 2015. Of the HCC cases in Japan, approximately 16% are caused by hepatitis B virus (HBV) infection and approximately 80% by hepatitis C virus (HCV) infection.[1] The average prevalence of HCV carriers in Japan is about 2%, with the absolute number estimated at 2 million.[2] The increase in HCC in Japan depends on the spread of HCV infection.[3]

Infection with HCV induces various extrahepatic manifestations as well as chronic liver diseases.[3,4] HCV infects cells or organs except hepatocytes and multiplies. Representative extrahepatic manifestations of HCV infection include lichen planus, diabetes mellitus (DM), malignant lymphoma, Sjögren's syndrome, cryoglobulinemia, and membranoproliferative glomerulonephritis. It has been reported that combined therapy using interferon and ribavirin is effective for different extrahepatic manifestations that are apt to be overlooked.[5,6]

At present, it has been shown that HCV multiplies in skin and oral mucosa leading to HCV-related lichen planus,[7,8] and that the risk of malignant transformation is higher in lichen planus with HCV infection than in lichen planus without HCV.[9] However, a mechanism for these extrahepatic manifestations has not been elucidated. Recently it was reported that there is a significant correlation between lichen planus and HCV and DM in southern Taiwan, particularly in HCV patients with elevated serum alanine aminotransferase (ALT) levels and atrophic-erosive oral lichen planus (OLP).[10] In our previous report, patients with lichen planus having DM were all found to be HCV-infected.[11]

In addition, it has been reported that DM is a risk factor for HCV-related hepatocarcinogenesis[12] and for decreased survival

among liver cirrhosis patients.[13] In addition, the incidence of diabetes in patients having HCV-related liver cirrhosis is higher than that in patients with HBV-related liver diseases.[14]

We recently showed molecular mechanisms for HCV core-induced insulin resistance.[15] HCV core up-regulates the suppressor of cytokine signaling (SOCS) 3, and inhibits insulin signaling by down-regulation of insulin receptor substrate (IRS) -1 and IRS-2 in hepatocytes. Moreover, in an epidemiological survey, we demonstrated that a significant increase in the incidence of diabetes occurs in subjects with high titers of HCV core compared to subjects who are negative for anti-HCV antibody[16] and concluded that HCV infection induces insulin resistance, which causes an increase in the incidence of extrahepatic manifestations in HCV-infected individuals.[17]

In the current study, we surveyed the incidence of abnormal glucose tolerance in patients with or without lichen planus in a study population with HCV-related chronic liver disease, and investigated the relationship between lichen planus and insulin resistance.

Methods

Patients

A total of 105 984 consecutive patients had checkups for chronic liver disease for the first time in the Digestive Disease Center at Kurume University Hospital from April 1988 to August 2005. In the Digestive Disease Center, physicians, surgeons, radiologists, and an oral surgeon hold full-time positions. One of us (M.S.) is a hepatologist and examined 9396 of these 105 984 patients. There were 522 patients who were HCV antibody positive and who thereafter continued with regular hospital visits until April 2006.

Exclusion criteria were the following: (i) other causes of chronic liver disease or disease other than chronic HCV infection; (ii) liver disease related to HBV infection; and (iii) patients treated with interferon therapy at the time of study inclusion.

We examined the presence of extrahepatic manifestations of chronic HCV infection in 87 patients. Informed consent was obtained from all patients after the purpose and methods of the study were explained. The 87 patients were 44 men and 43 women with a mean age of 60.0 ± 11.5 years.

The patients were monitored for the presence of extrahepatic manifestations of HCV infection such as lichen planus, DM, hypertension, thyroid dysfunction, and extrahepatic malignant tumor as well as liver disease. Biochemical tests were done and insulin values, blood glucose levels, and *Helicobacter pylori* antibody were measured in patient blood samples. Life histories were taken.

Clinical examinations

Patients received oral mucosa and cutaneous medical examinations by an oral surgeon and a dermatologist. The diagnosis of OLP was made on the basis of clinical and histopathological features. Diagnosis of type 2 DM was based on the American Diabetic Association (ADA) criteria of 1997.[18] Persons in whom diabetes was diagnosed before 30 years of age and who used insulin were categorized as type 1 DM and were excluded from our study.

The following definitions of cardiovascular disease were employed. Obesity was defined as a body mass index (BMI) >25 kg/m^2 or higher. Hypertension was defined as a systolic blood pressure (SBP) of 140 mmHg or higher, or a diastolic blood pressure (DBP) of 90 mmHg or higher according to the criteria of JNC-VI of the International Hypertension Society.[19] Thyroid hormones such as FT3, FT4 and thyroid stimulating hormone were measured for all patients, and thyroid echography examination was performed for some patients. Examination of the upper gastrointestinal tract or lower digestive tract was performed on patients for whom it was deemed clinically necessary.

We also took a history of smoking and alcohol consumption.

Serological assays

Serum samples from the 87 patients were collected and tested for platelets (PLT) and for the following liver function tests: serum ALT, aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (γ-GTP), lactate dehydrogenase (LDH), total bilirubin (TBil), direct bilirubin (DBil), thymol turbidity test (TTT), zinc sulfate turbidity test (ZTT), total cholesterol (TC), total protein (TP), and albumin (Alb). Sera were also examined for the presence or absence of HCV or HBV infection. Anti-HCV was measured by a chemiluminescent enzyme immunoassay kit (Lumipulse II HCV, Fujirebio, Tokyo, Japan). HCV RNA in serum was detected using the Amplicore HCV test (Roche, Tokyo, Japan). Hepatitis B virus surface antigen (HBsAg) was assayed using a chemiluminescent immunoassay kit (Architect, HBsAg QT, Dainabot, Tokyo, Japan). Ultrasonographic examination for all patients was performed in order to investigate the shape of the liver and lesions occupying the liver. Computed tomography and liver biopsy were performed in some patients. Most patients underwent endoscopy for detection of esophagogastric varices. We used other possible predictors of liver cirrhosis progression, including serum albumin, TBil, prothrombin time, and PLT.

Plasma glucose levels were measured by a glucose oxidase method for all subjects and serum insulin levels were measured using a sandwich enzyme immunoassay kit (Eiken Chemical, Tokyo, Japan). Insulin resistance (IR) was calculated on the basis of fasting levels of plasma glucose and insulin, according to the homeostasis model assessment (HOMA-IR) method.[20] The formula for the HOMA-IR is: HOMA-IR = fasting glucose (mg/dL) × fasting insulin (μU/mL)/405.

The presence of serum IgG antibodies against *H. pylori* antibody were measured by the SRL (Tokyo) using E Plate *H. pylori* antibody produced by Eiken Chemical.

Statistical analysis

The chi-squared test and the unpaired Student *t*-test were used for statistical analyses. Differences were judged significant for $P < 0.05$ (two-tailed). This study was approved by the Institutional Review Board/Ethics Committee of our Institution.

Results

Among 87 patients with HCV-related liver diseases, the prevalence of lichen planus was 19.5% (17/87), DM was 21.8% (19/87),

Table 1 Clinical characteristics of 87 patients with HCV-related liver diseases according to presence of lichen planus (LP)

Clinical characteristic	All patients	Group A (with LP)	Group B (without LP)	*P*-value (A *vs* B)
No. subjects	87	17	70	–
Age (years)	60.0 ± 11.5	63.7 ± 10.6	59.1 ± 11.6	NS
Sex (M/F)	44/43	11/6	33/37	NS
BMI (kg/m^2)	22.8 ± 2.9	23.9 ± 2.8	22.5 ± 2.9	NS
Smoking history	32 (36.8)	10 (58.8)	22 (31.4)	0.0356
Alcohol consumption percentage	50 (57.5)	10 (58.8)	40 (57.1)	NS
Diagnosis of liver disease				
Past history of HCV infection	1	0	1	NS
Chronic hepatitis C	69	11	58	
HCV-related liver cirrhosis	9	3	6	
HCV-related HCC	8	3	5	
Comorbidities				
Diabetes mellitus	19 (21.8)	4 (23.5)	15 (21.4)	NS
Hypertension	25 (28.7)	10 (58.8)	15 (21.4)	0.0022
Thyroid dysfunction	18 (20.7)	5 (29.4)	13 (18.6)	NS
Extrahepatic malignant tumor	8 (9.2%)	5 (29.4)[†]	3 (4.3)[‡]	0.0013

Values shown as *n* (%) or mean ± SD. BMI, body mass index; F, female; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; M, male; NS, not significant.

[†]Tumors were: gastric cancer (two), tongue cancer (one), larynx cancer (one), and renal and colon cancer (one). [‡]Tumors were: gastric cancer (one), colon cancer (one), and gallbladder cancer (one).

hypertension was 28.7% (25/87), thyroid dysfunction was 20.7% (18/87), and extrahepatic malignant tumor was 9.2% (8/87).

We compared characteristics of 17 patients who had lichen planus (group A) and 70 patients who did not have lichen planus (group B). The mean age in group A was 63.7 ± 10.6 years; there were 11 men and six women. The mean age in group B was 59.1 ± 11.6 years; there were 33 men and 37 women. Table 1 shows clinical features of groups A and B. The diagnoses of liver diseases in group A were chronic hepatitis C infection (11 patients), HCV-related liver cirrhosis (three patients), and HCV-related HCC (three patients). Those of group B were chronic hepatitis C infection (58 patients), HCV-related liver cirrhosis (six patients), HCV-related HCC (five patients) and past history of HCV infection (one patient) (Table 1).

The prevalence of smoking history ($P = 0.0356$), hypertension ($P = 0.0022$), and extrahepatic malignant tumor ($P = 0.0013$) were significantly higher in group A than in group B (Table 1). Diagnoses of extrahepatic malignant tumors in group A were: tongue cancer (one squamous cell carcinoma), larynx cancer (one squamous cell carcinoma), gastric cancer (one adenocarcinoma, one signet ring cell carcinoma), renal and colon cancer (one renal cell carcinoma). Diagnoses of extrahepatic tumor in group B were: gastric cancer (one adenocarcinoma), colon cancer (one adenocarcinoma), and gallbladder cancer (one adenocarcinoma). Significant differences were not observed for age, sex, BMI, liver disease, alcohol consumption, presence of DM, or thyroid dysfunction between these two groups.

We analyzed for differences between these two groups in liver assays, blood platelets, insulin, blood glucose, HOMA-IR, and presence of *H. pylori* infection. The laboratory data of both groups are shown in Table 2. Prevalence of insulin ($P = 0.0076$) and HOMA-IR ($P = 0.0113$) were significantly higher in group A than in group B (Table 2). Significant differences were not observed for serum AST, ALT, LDH, γGTP, TP, Alb, TBil, DBil, TTT, ZTT, TC, blood platelets, blood glucose, or presence of *H. pylori* infection between these two groups.

Seventeen patients had OLP at a total of 24 sites. The site of occurrence was: buccal mucosa in 13 (76.5%), lower lip in six (35.3%), upper lip in two (11.8%), gingiva in one (5.9%), tongue in one (5.9%), and floor of mouth in one (5.9%) (Table 3). The sites of lichen planus except oral mucosa were lower leg in four (23.5%), antebrachium in one (5.9%), skin extremities in two (11.8%), hypopharynx in one (5.9%), and vulva in one (5.9%). Biopsies of hypopharyngeal lichen planus were performed by an otolaryngologist, and of vulvar lichen planus by a gynecologist. The erosive and reticular variety, respectively, was found to be the prevalent form (Table 3).

Discussion

We performed an epidemiological survey for extrahepatic manifestations and HCC in an HCV hyperendemic area in Japan.[21,22] Anti-HCV positivity among residents of this area in 1990 was 23.6%.[23] We found that the prevalence of extrahepatic manifestations among individuals with HCV infection was higher than among those without HCV,[22] and found an association between HCV core, insulin resistance, and the development of type 2 DM.[16] Recently, we reported that insulin resistance in inhabitants who have an extrahepatic manifestation including OLP with HCV infection shows significantly greater increases than for inhabitants who have neither an extrahepatic manifestation nor HCV infection.[17] By the results of these epidemiological surveys we think that insulin resistance induced by HCV infection causes an increase in the incidence of extrahepatic manifestations in HCV-infected individuals.

In this study, we did long-term follow up for insulin resistance from the standpoint of lichen planus among patients who we identified as having HCV-related chronic liver disease at our hos-

Table 2 Laboratory data of 87 patients with HCV-related liver diseases according to presence of lichen planus (LP)

Laboratory assay	All patients	Group A (with LP)	Group B (without LP)	*P*-value (A *vs* B)
AST (IU/L)	61.1 ± 38.1	60.9 ± 33.5	61.2 ± 39.3	NS
ALT (IU/L)	68.2 ± 46.7	62.4 ± 39.6	69.6 ± 48.5	NS
LDH (IU/L)	216.8 ± 62.8	205.8 ± 72.1	219.6 ± 60.6	NS
γ-GTP (IU/L)	64.1 ± 68.4	63.5 ± 50.0	64.2 ± 72.5	NS
TP (g/dL)	7.7 ± 0.5	7.7 ± 0.5	7.7 ± 0.5	NS
Alb (g/dL)	4.1 ± 0.5	3.9 ± 0.5	4.2 ± 0.5	NS
PLT (/mm^3)	13.8 ± 5.1	12.5 ± 5.0	14.1 ± 5.09	NS
TBil (mg/dL)	1.1 ± 0.6	1.2 ± 0.9	1.0 ± 0.5	NS
DBil (mg/dL)	0.2 ± 0.2	0.2 ± 0.3	0.2 ± 0.2	NS
TTT	16.2 ± 6.7	18.4 ± 4.7	15.8 ± 7.0	NS
ZTT	20.6 ± 6.9	21.8 ± 5.8	20.3 ± 7.2	NS
TC (mg/dL)	172.3 ± 35.8	164.3 ± 41.9	174.1 ± 34.4	NS
Insulin (μU/L)	23.3 ± 42.0	47.3 ± 87.8	17.4 ± 15.4	0.0076
Blood glucose (mg/dL)	97.4 ± 30.1	103 ± 33.2	96.1 ± 29.5	NS
HOMA-IR	7.1 ± 18.8	17.4 ± 40.0	4.6 ± 6.0	0.0113
Helicobacter pylori antibody (*n* (%))	58 (66.7)	10 (58.8)	48 (68.6)	NS

Values shown as mean ± SD. Alb, albumin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; DBil, direct bilirubin; γ-GTP, gamma-glutamyl transpeptidase; HOMA-IR, homeostasis model assessment; LDH, lactate dehydrogenase; NS, not significant; PLT, platelets; TBil, total bilirubin; TP, total protein; TTT, thymol turbidity test; TC, total cholesterol; ZTT, zinc sulfate turbidity test.

Table 3 Location of lichen planus in 17 patients with hepatitis C virus-related liver diseases

No	Sex	Age (years)	Liver disease	Lichen planus location			Type
				Cutaneous	Oral	Other	
1	M	71	CH	Antebrachium	–	–	–
2	M	60	CH	Extremities	–	–	
3	F	70	LC	–	Gingiva	–	Erosive
4	M	72	LC	–	Lower lip	–	Reticular
5	F	64	LC	Leg	Buccal mucosa, upper lip, lower lip	–	Erosive
6	M	66	CH	Leg	Buccal mucosa, upper lip, lower lip	–	Erosive
7	M	59	CH	–	Buccal mucosa (reticular)	Pharynx (erosive)	Erosive + reticular
8	M	66	CH	Leg	Buccal mucosa, lower lip	–	Reticular
9	M	57	CH	–	Buccal mucosa	–	Reticular
10	M	50	CH	–	Buccal mucosa, tongue, lower lip	–	Erosive
11	F	77	CH	–	Buccal mucosa	–	Atrophic
12	F	75	CH	–	Buccal mucosa	–	Reticular
13	M	62	HCC	–	Buccal mucosa, lower lip	–	Erosive
14	F	83	HCC	Leg	Buccal mucosa (atrophic)	Vulva (erosive)	Atrophic + erosive
15	M	41	CH	–	Buccal mucosa	–	Reticular
16	M	58	HCC	Extremities	Buccal mucosa, floor of mouth	–	Erosive
17	F	53	CH	–	Buccal mucosa	–	Reticular

CH, chronic hepatitis C; F, female; LC, HCV-related liver cirrhosis; HCC, HCV-related hepatocellular carcinoma; M, male.

pital. Although there was no significant difference in fasting glucose levels and BMI between patients with and without lichen planus, fasting insulin levels and HOMA-IR values, an indicator of insulin resistance, were significantly higher in patients who had lichen planus than in those who did not.

In the present study, insulin levels (17.4 ± 15.4 μU/L) and HOMA-IR values (4.6 ± 6.0) in patients having HCV infection without lichen planus (group B) were higher than the normal range. Normal values for insulin are 3.06–16.9 μU/L, and for HOMA-IR are less than 2. Therefore, the significantly higher insulinemia in patients such as those in group A (among HCV infectious patients) might cause lichen planus.

In Japan, it is known that the prevalence of HCV infection in patients with lichen planus is high;[11] therefore, interferon therapy is often administered to patients with lichen planus and a persistent HCV infection. However, it has been reported that patients cannot

complete interferon therapy because of aggravation of lichen planus.[24,25] The measurement of insulin resistance as well as a search for lichen planus may be useful before performing interferon therapy. A large series of patients with OLP was evaluated for extraoral involvement by Eisen *et al.*[26] They concluded that any patient with OLP should undergo a through history and examination as part of an investigation of potential extraoral manifestations, because a high percentage of patients with OLP develop extraoral manifestations. In our 17 cases of lichen planus, cutaneous lichen planus was diagnosed in seven (41.2%), hypopharynx in one (5.9%), and vulva in one (5.9%). The simultaneous appearance of extraoral and oral lesions was noted among six (35.3%). Because the majority of OLP patients suffer from lichen planus of the genitalia,[27] clinicians should follow OLP patients with sufficient attention to the presence of extraoral manifestations.

Sikuker *et al.* evaluated an association between HCV infection and extrahepatic malignancies. Extrahepatic malignancies were found in 14.6% of anti-HCV positive patients.[28] The incidence of extrahepatic malignant tumor in our subjects was 9.2% (8/87). The insulin-like growth factor family of proteins plays a key role in cellular metabolism, differentiation, proliferation, transformation and apoptosis, during normal development and malignant growth.[29] The hyperinsulinemia that HCV infection causes may induce an extrahepatic malignant tumor as well as HCC.

Many studies have shown that *H. pylori* is involved in the pathogenesis of gastric cancer.[30] The seroprevalence of *H. pylori* is 71% in Japanese aged 50–59 years, and is 81% in those aged 60–69 years.[31] This is almost the same as the seroprevalence of our patients, which was 66.7% (58/87) overall and 82.6% (19/23) in those aged 60–69 years. Seroprevalence of *H. pylori* in our three subjects with gastric cancer was 66.7%. In our study, we did not find an association between *H. pylori* and lichen planus in patients with HCV-infectious liver diseases.

In conclusion, we investigated the association of insulin resistance and lichen planus among patients with HCV-infected chronic liver diseases. The significant factors for development of lichen planus were smoking history, presence of hypertension, extrahepatic malignant tumor, and insulin resistance (HOMA-IR). This supports our previous conclusion that insulin resistance in patients who have an extrahepatic manifestation of HCV infection increases more than insulin resistance of patients who have neither an extrahepatic manifestation nor HCV infection. HCV-infected patients with lichen planus should pay attention to the development of an extrahepatic malignancy. Cooperation with an oral surgeon and a hepatologist is vital for early diagnosis and treatment of any extrahepatic manifestations.

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Comparative proteomic and transcriptomic profiling of the human hepatocellular carcinoma

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Abstract

Proteome analysis of human hepatocellular carcinoma (HCC) was done using two-dimensional difference gel electrophoresis. To gain an understanding of the molecular events accompanying HCC development, we compared the protein expression profiles of HCC and non-HCC tissue from 14 patients to the mRNA expression profiles of the same samples made from a cDNA microarray. A total of 125 proteins were identified, and the expression profiles of 93 proteins (149 spots) were compared to the mRNA expression profiles. The overall protein expression ratios correlated well with the mRNA ratios between HCC and non-HCC (Pearson's correlation coefficient: $r = 0.73$). Particularly, the HCC/non-HCC expression ratios of proteins involved in metabolic processes showed significant correlation to those of mRNA ($r = 0.9$). A considerable number of proteins were expressed as multiple spots. Among them, several proteins showed spot-to-spot differences in expression level and their expression ratios between HCC and non-HCC poorly correlated to mRNA ratios. Such multi-spotted proteins might arise as a consequence of post-translational modifications.

Keywords: Hepatocellular carcinoma; Proteome; Two-dimensional difference gel electrophoresis; Transcriptome; cDNA microarray

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, and a leading cause of death in Africa and Asia [1]. Although several major risks related to HCC, such as hepatitis B and/or hepatitis C virus infection, aflatoxin B1 exposure, and alcohol consumption, and genetic defects, have been revealed [2], the molecular mechanisms leading to the initiation and progression of HCC are not well known. To find the molecular basis of hepatocarcinogenesis, comprehensive gene expression analyses have been done using many systems such as hepatoma cell lines and tissue samples [3,4]. Previously, we have carried out a comprehensive mRNA expression analysis using the serial analysis of gene expression (SAGE) [5] and cDNA microarray-based comparative genomic hybridization [6] to acquire the outline of gene expression profile of HCC. Although these genomic approaches have yielded global gene expression profiles in HCC and identified a number of candidate genes as biomarkers useful for cancer staging, prediction of prognosis, and treatment selection [7], the molecular events accompanying HCC development are not yet understood. In general, proteins rather than transcripts are the major effectors of cellular and tissue function [8] and it is accepted that protein expression do not always correlate with mRNA expression [9,10]. Thus, protein expression analysis, which could complement the available mRNA data, is also important to understand the molecular mechanisms of HCC.

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The technique of two-dimensional difference gel electrophoresis (2D-DIGE), developed by Unlu et al. [11] is one of major advances in quantitative proteomics. Several groups have recently utilized 2D-DIGE to examine protein expression changes in HCC samples [12,13], whereas reports on the analysis combining both transcriptomic and proteomic approach are rare.

In the present study, we compared quantitatively protein expression profiles of HCC to non-HCC (non-cancerous liver) samples derived from 14 patients by 2D-DIGE. We also compared the protein expression profiles of the same HCC and non-HCC samples to the mRNA profiles which have been obtained using a cDNA microarray. The expression ratios of 93 proteins showed significant correlations with the mRNA ratios between HCC and non-HCC. Proteins involved in metabolic processes showed more prominent correlation. Our study describes an outline of gene and protein expression profiles in HCC, thus providing us a basis for better understanding of the disease.

Materials and methods

Patients. A total of 14 HCC patients who had surgical resection done in the Kanazawa University Hospital were enrolled. The clinicopathological characteristics of them are shown in Table 1. The HCC samples and adjacent non-tumor liver samples were snap frozen in liquid nitrogen, and used for cDNA microarray and 2D-DIGE analysis. All HCC and non-tumor samples were histologically diagnosed and quantitative detection of hepatitis C virus RNA by Amplicore analysis (Roche Diagnostic Systems) showed positive. The grading and staging of chronic hepatitis associated with non-tumor lesion were histologically assessed according to the method described by Desmet et al. [14] and histological typing of HCC was assessed according to Ishak et al. [15]. All strategies used for gene expression and protein expression analysis were approved by the Ethical Committee of Kanazawa University Hospital.

Preparation of cDNA microarray slides. In addition to in-house cDNA microarray slides consisting of 1080 cDNA clones as previously described [6,16–18], we made new cDNA microarray slides for detailed analysis of the signaling pathway of metabolism and enzyme function in liver disease [19]. Besides cDNA microarray analysis, a total of 256,550 tags were obtained from hepatic SAGE libraries (derived from normal liver, CH-C, CH-C related HCC, CH-B, and CH-B related HCC), including 52,149 unique tags. Among these, 16,916 tags expressing more than two hits were selected to avoid the effect of sequencing errors in the libraries. From these candidate genes, 9614 non-redundant clones were obtained from Incyte Genomics (Incyte Corporation), Clontech (Nippon Becton Dickinson), and Invitrogen (Invitrogen). Each clone was sequence validated and PCR amplified by Dragon Genomics (Takara Bio), and the cDNA microarray slides (Liver chip 10k) were constructed using SPBIO 2000 (Hitachi Software) as described previously [6,16–18].

Table 1
Characteristics of patients involved in this study

Patient No.	Age	Sex[a]	Histology of non-tumor lesion[b]	Tumor histology	Viral status
1	64	M	F4A1	Moderate	HCV
2	65	M	F4A1	Well	HCV
3	48	M	F3A1	Moderate	HCV
4	69	F	F4A2	Moderate	HCV
5	66	F	F4A2	Well	HCV
6	45	M	F4A1	Well	HCV
7	75	F	F4A1	Well	HCV
8	46	M	F4A2	Moderate	HCV
9	66	M	F2A2	Well	HCV
10	75	M	F3A1	Moderate	HCV
11	67	F	F4A2	Well	HCV
12	64	M	F4A1	Moderate	HCV
13	68	M	F4A0	Well	HCV
14	74	M	F1A0	Moderate	HCV

[a] M, male; F, female.
[b] F, fibrosis; A, activity.

RNA isolation and antisense RNA amplification. Total RNA was isolated from liver biopsy samples using an RNA extraction kit (Stratagene). Aliquots of total RNA (5 μg) were subjected to amplification with antisense RNA (aRNA) using a Message AmpTM aRNA kit (Ambion) as recommended by the manufacturer. About 25 μg of aRNA was amplified from 5 μg total RNA, assuming that 500-fold amplification of mRNA was obtained. The quality and degradation of the isolated RNA were estimated after electrophoresis using an Agilent 2001 bioanalyzer. In addition, 10 μg of aRNA was used for further labeling procedures.

Hybridization on cDNA microarray slides and image analysis. As a reference for each microarray analysis, aRNA samples prepared from the normal liver tissue from one of the patients were used. Test RNA samples fluorescently labeled with cyanine (Cy) 5 and reference RNA labeled with Cy3 were used for microarray hybridization as described previously [6,16–18]. Quantitative assessment of the signals on the slides was done by scanning on a ScanArray 5000 (General Scanning) followed by image analysis using GenePix Pro 4.1 (Axon Instruments) as described previously [6,16–18].

Protein expression analysis using 2D-DIGE. Protein samples were homogenized with lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 0.8 μM aprotinin, 15 μM pepstatin, 0.1 mM PMSF, 0.5 mM EDTA, 30 mM Tris–HCl, pH 8.5) and centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatants were used as protein samples. The protein concentrations were determined with a protein assay reagent (Bio-Rad). The non-HCC and HCC samples (50 μg each) labeled with either Cy3 or Cy5 according to the manufacture's manual were combined and separated on 2-DE gels together with the Cy2-labeled internal standard (IS), which was prepared by mixing equal amounts of all samples. Analytical 2-DE was performed as described previously [20] using Immobiline DryStrip (pH 3–10, 24 cm, GE Healthcare) in the first dimension and 12.5% SDS–polyacrylamide gels (24 × 20 cm) in the second dimension. Samples were run in triplicate to obtain statistically reasonable results. After scanning with a Typhoon 9410 scanner (GE Healthcare), gels were silver stained for protein identification. For protein identification, 400 μg of the IS sample was also separately run on a 2-DE gel and stained with SYPRO Ruby (Invitrogen). All analytical and preparative gel images were processed using ImageQuant (GE Healthcare) and the protein level analysis was done with the DeCyder software (GE Healthcare). To detect phosphoproteins, 400 μg of HCC and non-HCC samples were separately run on 2-DE gels and stained with ProQ Diamond (Invitrogen). After acquiring images, gels were counterstained with SYPRO Ruby to visualize total proteins as described above.

Protein identification. The excised protein spots were in-gel digested with porcine trypsin (Promega). For LC-ESI-IT MS/MS analysis using LCQ Deca XP (Thermo Electron), the digested and dried peptides were dissolved in 10 μl of 0.1% formic acid in 2% acetonitrile (ACN). The dissolved samples were loaded onto C18 silica gel capillary columns (Magic C18, 50 × 0.2 mm), and the elution from the column was directly connected through a sprayer to an ESI-IT MS. Mobile phase A was 2% ACN containing 0.1% formic acid, and mobile phase B was 90% ACN containing 0.1% formic acid. A linear gradient from 5% to 65% of concentration B was applied to elute peptides. The ESI-IT MS was operated in positive ion mode over the range of 350–2000 (m/z) and the database search was carried out against the IPI Human using MASCOT (Matrixscience). The following search parameters were used: the cutting enzyme, trypsin; one missed cleavage allowed, mass tolerance window, ±1 Da, the MS/MS tolerance window, ±0.8 Da; carbamidomethyl cystein and oxidized methionine as fixed and variable modifications, respectively.

Detection of phosphorylated peptide. Possible phosphorylation sites were investigated by MALDI–TOF–MS using monoammonium phosphate (MAP) added matrix mainly according to Nabetani et al. [21]. An additive of MAP was mixed with α-CHCA matrix solution (5 mg/mL, 0.1% TFA, 50% ACN aqueous) to 40 mM in final concentration. Tryptsin digests of the spots positively stained with ProQ were dissolved into 4 μL of 0.1% TFA, 50% ACN aqueous solution and 1 μL of the peptides solution was spotted on the MALDI target plate. After drying up, 1 μL of the MAP matrix was dropped on the dried peptide mixture. Voyager DE-STR (ABI) was used to obtain mass spectra both in negative and positive ion mode. MS peaks that had relatively stronger intensities in negative ion mode than in positive ion mode were selected as candidates for acidically modified peptides.

Results and discussion

We identified 195 spots representing 125 proteins (Suppl. Table 1) and obtained the corresponding mRNA expression data for a total of 93 proteins (149 spots) (Suppl. Table 2). These 93 proteins were classified according to their biological processes and subcellular localizations into categories described by the Gene Ontology Consortium (http://www.geneontology.org/index.shtml) and about a half of them were related to metabolic processes (Fig. 1A). It is a general agreement that proteins with extremely high or low p*I* as well as hydrophobic proteins are difficult to be detected by 2-DE. Being consistent with this notion, our analysis detected many cytoplasmic proteins (Fig. 1B). Therefore, the protein expression data presented here were biased in favor of cytoplasmic and soluble proteins. The protein expression abundance between non-HCC and HCC was calculated using the normalized spot volume, which was the ratio of spot volume relative to IS (Cy3:Cy2 or Cy5:Cy2) and we used the Student's paired *t*-test ($p < 0.05$) to select the protein spots which were expressed differentially between non-HCC and HCC, using 2-DE gel images run in triplicate. The spot volume of a multi-spotted protein was indicated as a total volume by integrating the intensities of multiple spots as was done by Gygi et al. [10]. Comparison of protein expression profiles revealed that several proteins were expressed differentially between HCC and non-HCC. Proteins whose abundances increased >2-fold or decreased <1/2 in HCC are listed in Table 2. While glutamine synthetase, vimentin, annexin A2 and aldo-keto reductase were up-regulated, carbonic anhydrase 2, argininosuccinate synthetase 1, carbonic anhydrase 1, fructose-1,6-bisphosphatase 1, and betaine-homocysteine methyltransferase were down-regulated in HCC. Up- or down-regulation of most of these proteins in HCC has been reported previously [22–27]. Up-regulation of vimentin and annexin A2, and reduced expression of carbonic anhydrase 1 and 2 was suspected to be associated with cellular motility and metastasis [23,24,26].

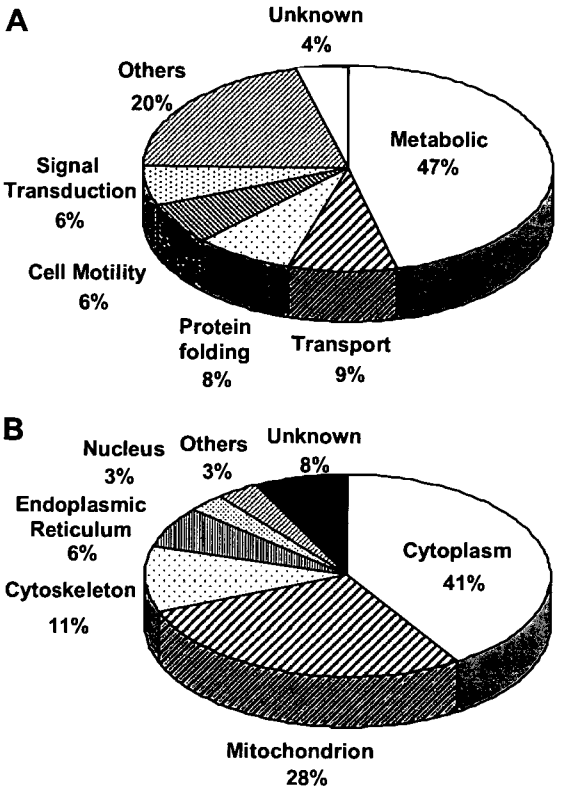

Fig. 1. Classification of identified proteins according to their cellular function (A) and subcellular localization (B).

The mRNA expression abundance was calculated from cDNA microarray data. Hierarchical clustering of

Table 2
Proteins expressed differentially between HCC and non-HCC

Spot ID	Protein name	Refseq ID	Theoretical		Fold change (HCC/non-HCC)		References
			p*I*	MW (kDa)	Protein[a]	mRNA	
1353, 1354	Glutamine synthase	NP_002056.2	6.43	42.7	2.06	3.08	[22]
1039, 1046	Vimentin	NP_003371	5.09	53.6	2.30	1.51	[23]
1716	Annexin A2	NP_001002857.1	7.57	38.8	2.57	1.82	[24]
1685, 1699	Aldo-keto reductase 1B10	NP_064695	7.12	36.2	4.29	4.73	[25]
1977	Carbonic anhydrase 2	NP_000058	6.87	29.3	0.39	0.62	[26]
1307, 1312, 1331	Argininosuccinate synthetase 1	NP_000041.2	8.08	46.8	0.41	0.30	[27]
1941	Carbonic anhydrase 1	NP_001729	6.59	28.9	0.47	1.25	[26]
1582	Fructose-1,6-bisphosphatase 1	NP_000498	6.54	37.2	0.48	0.36	
1256	Betaine-homocysteine methyltransferase	NP_001704	6.41	45.4	0.48	0.40	

[a] Integrated spot volume was used to calculate the fold change of multi-spotted proteins.

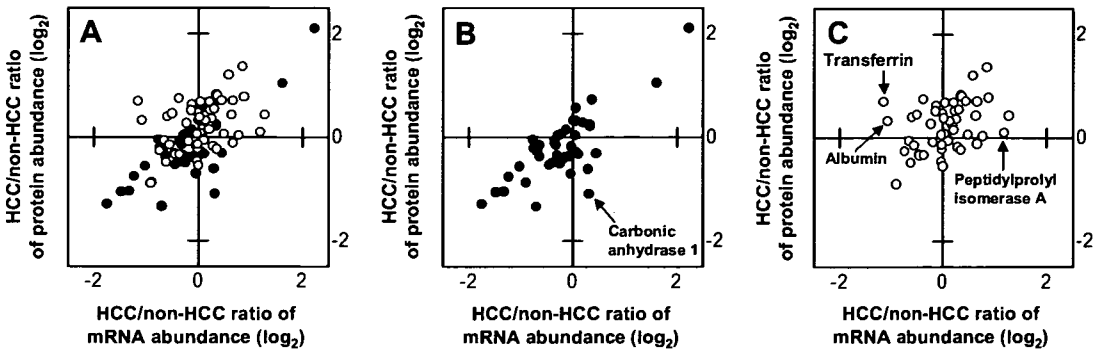

Fig. 2. Comparative analysis of protein and mRNA expression profiles between HCC and non-HCC. (A) The HCC/non-HCC ratios of averaged protein expression levels for 93 proteins were plotted against those of mRNA. Proteins related to metabolic pathways were indicated in closed circles and were shown again in (B). Proteins related to the other biochemical pathways were indicated in open circles and shown in (C). Proteins listed in Table 3 were indicated in (B) and (C). All graphs were depicted in log_2 scale.

Table 3
Proteins whose expression changes between HCC and non-HCC show poor correlation to mRNA expression changes

Spot ID	Protein name	Refseq ID	Theoretical		Spot[a] Av. Ratio	Spot *p* value	Protein ratio	Micro array Av. ratio	Micro array *p* value
			*p*I	MW (kDa)					
564	Transferrin	NP_001054	6.8	79.3	2.23	0.035	1.61	0.45	3.3E−06
565					1.87	0.079			
566					2.28	0.13			
605					0.73	0.098			
1489	Albumin	NP_000468	5.9	71.3	—	0.63	1.25	0.47	2.3E−03
1941	Carbonic anhydrase 1	NP_001729	6.6	28.9	—	3.5E−03	0.47	1.25	0.39
2290	Peptidylprolyl isomerase A	NP_066953	7.7	18.1	—	5.0E−01	1.07	2.29	1.1E−01

[a] Since transferrin was detected in multiple spots, averaged ratio and spot *p* value of each spot is shown.

Table 4
Multi-spotted proteins showing spot-to-spot differences in expression level between non-HCC and HCC

Spot ID	Spot Av. ratio	Spot *p* value	Protein name	Refseq ID	Theoretical		Protein[a] ratio
					*p*I	MW (kDa)	
436	1.92	5.3E−04	Tumor rejection antigen (gp96)	NP_003290	4.8	92.7	1.2
537	0.79	0.16					
564	2.23	0.035	Transferrin	NP_001054	6.8	79.3	1.61
565	1.87	0.079					
566	2.28	0.13					
605	0.73	0.098					
1257	1.02	0.92	Fumarate hydratase	NP_000134	8.8	54.8	0.8
1261	0.6	1.3E−03					

[a] HCC/non-HCC protein ratios were calculated using integrated spot abundances.

gene expression was done with BRB-ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.htm). The filtered data were log-transferred, normalized, centered, and applied to the average linkage clustering with centered correlation. BRB-ArrayTools contains a class comparison tool based on univariate *F* tests to find genes differentially expressed between predefined clinical groups. The permutation distribution of the *F* statistic, based on 2000 random permutations, was also used to confirm statistical significance. A *p* value of less than 0.05 for differences in HCC/non-HCC gene expression ratio was considered significant.

The average HCC/non-HCC expression ratios of the 93 proteins were plotted against the mRNA ratios in Fig. 2, where a positive value indicates increased expression in HCC and a negative ratio indicates reduced expression. The overall expression ratio of HCC/non-HCC indicated noticeable correlation between protein and mRNA

(Fig. 2A), and the Pearson's correlation coefficient for this data set (93 proteins/genes) was 0.73. Next, we divided 93 proteins into those related to metabolism and others biological processes. The HCC/non-HCC ratios of protein expression for metabolism-related proteins showed substantial correlation with those of mRNA (Fig. 2B, $r = 0.9$), whereas those of other proteins were poorly correlated (Fig. 2C, $r = 0.36$). Extreme care must be taken in a direct comparison of proteomic data with transcriptome because of multiple layers of discrepancies caused by the distinct sensitivities of cDNA array hybridization and 2-DE, the inability of a cDNA array to distinguish mRNA isoforms and post-translational modifications of proteins. Nevertheless, our results suggest that the expression of considerable portion of proteins with metabolic function listed here is regulated at transcriptional level. On the other hand, post-transcriptional and/or post-translational processes seem to be involved in the regulation of expression level for proteins with other cellular functions as a whole. Four proteins (albumin, transferrin, peptidylproryl isomerase A, and carbonic anhydrase 1) showed apparent poor correlation in protein and mRNA expression profiles (Table 3 and Fig. 2). Transcriptional control might have little effect on the expression changes of these proteins between HCC and non-HCC.

A number of proteins were expressed as multiple spots on 2-DE gels and most multi-spotted proteins showed little spot-to-spot variations in the averaged HCC/non-HCC ratio. Although we do not know how these multiple spots were generated, many of them might be due to the conformational equilibrium of proteins under electrophoresis rather than to any post-translational modifications [28]. On the other hand, the HCC/non-HCC expression ratios of several multi-spotted proteins varied from spot to spot, and three proteins (transferrin, fumarate hydratase, and tumor rejection antigen gp96) were categorized as these multi-spotted proteins (Table 4).

For example, gp96 was detected in two spots (spot #436 and 537) with distinct molecular mass and p*I* and they showed different HCC/non-HCC expression ratio (Fig. 3A and B and Table 4). The expression of these two isoforms was observed to change in the opposite direction between non-HCC and HCC: #436 was up-regulated in HCC (HCC/non-HCC ratio: 1.96) while #537 was down-regulated (HCC/non-HCC ratio: 0.79) (Table 4 and Fig. 3C and D). Gp96 is a glycoprotein present in endoplasmic reticulum and is supposed to function as a molec-

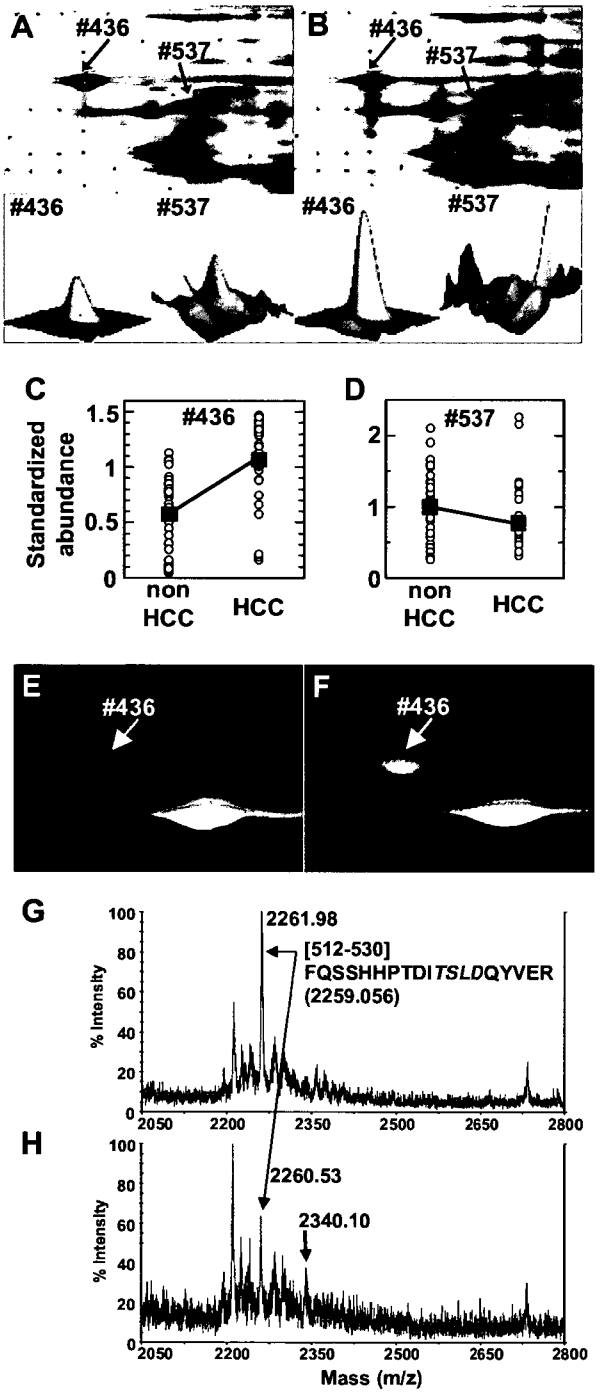

Fig. 3. Comparison of expression profiles of two gp96 spots between HCC and non-HCC. The expression profile and phosphorylation of tumor rejection antigen gp96 in HCC and non-HCC was investigated. Magnified gel images and 3D views of two gp96 spots in non-HCC (A) and HCC (B) were shown. Differences in expression level of two gp96 spots, #436 (C) and #537 (D), between non-HCC and HCC were shown. The open circle indicates the standardized abundance of the individual spot in each sample. The closed square represents the averaged abundance of each gp96 spot. Magnified gel images of non-HCC (E) and HCC (F) stained with ProQ. The #436 spot was positively stained with ProQ, while unambiguous staining of the #537 spot was not observed. Tryptic peptides prepared from the spot #436 were analyzed by MALDI–TOF mass-spectrometry in the positive ion mode (G) and the negative ion mode (H). A peak of 2261.98 detected in positive ion mode corresponds to the amino acid sequence from 512 to 530. In addition to the original peak (*m/z*: 2260.53), a peak mass shifted by +80 Da was detected in the negative ion mode. A predicted phosphorylation consensus motif for protein kinase CK2 is indicated in italics (G).

ular chaperone and intracellular Ca^{2+} regulator [29,30]. Several previous reports have shown that gp96 is glycosylated and phosphorylated, and exists as heterogeneous molecular entities with various molecular weights [31]. In order to know whether gp96 spots were phosphorylated or not, we stained the 2-DE gels with ProQ Diamond which is a dye specific to proteins phosphorylated on serine, threonine or tyrosine residues [32], and has been used successfully to visualize phosphoproteins [33]. We found that the spot #436 was positively stained with ProQ (Fig. 3E and F). We further tried to detect possible phosphorylated peptides in the tryptic digests prepared from #436 by MALDI–TOF–MS according to Nabetani et al. [21]. Searching for those peaks that had relatively stronger intensities in negative ion mode than in positive ion mode, we found two peaks as candidates for acidically modified peptides. They were assigned to the peptides SILFVPTSAPR (amino acid sequence: 385–395, data not shown) and FQSSHHPTDITSLDQYVER (aa512–530). Fig. 3G and H show the unmodified peak and the acidically modified peak (mass shifted by +80 Da in negative ion mode) of the latter peptide, respectively. This peptide contained a predicted phosphorylation consensus motif, [Ser or Thr]-X-X-[Asp or Glu], for protein kinase CK2 (Fig. 3G) which was suggested to phosphorylate gp96 [34]. These results together with ProQ staining indicated that at least one gp96 isoform was phosphorylated and was up-regulated in HCC. Over-expression of gp96 in HCC has been reported previously [35], though the reports that showed over-expression of its phosphorylated form are rare. Further investigation into biological meaning of gp96 phosphorylation may provide us important information about HCC development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.11.101.

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Research Article

Gene Systems Network Inferred from Expression Profiles in Hepatocellular Carcinogenesis by Graphical Gaussian Model

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Hepatocellular carcinoma (HCC) in a liver with advanced-stage chronic hepatitis C (CHC) is induced by hepatitis C virus, which chronically infects about 170 million people worldwide. To elucidate the associations between gene groups in hepatocellular carcinogenesis, we analyzed the profiles of the genes characteristically expressed in the CHC and HCC cell stages by a statistical method for inferring the network between gene systems based on the graphical Gaussian model. A systematic evaluation of the inferred network in terms of the biological knowledge revealed that the inferred network was strongly involved in the known gene-gene interactions with high significance ($P < 10^{-4}$), and that the clusters characterized by different cancer-related responses were associated with those of the gene groups related to metabolic pathways and morphological events. Although some relationships in the network remain to be interpreted, the analyses revealed a snapshot of the orchestrated expression of cancer-related groups and some pathways related with metabolisms and morphological events in hepatocellular carcinogenesis, and thus provide possible clues on the disease mechanism and insights that address the gap between molecular and clinical assessments.

1. INTRODUCTION

Hepatitis C virus (HCV) is the major etiologic agent of non-A non-B hepatitis, and chronically infects about 170 million people worldwide [1–3]. Many HCV carriers develop chronic hepatitis C (CHC), and finally are afflicted with hepatocellular carcinoma (HCC) in livers with advanced-stage CHC. Thus, the CHC and HCC cell stages are essential in hepatocellular carcinogenesis.

To elucidate the mechanism of hepatocellular carcinogenesis at a molecular level, many experiments have been performed from various approaches. In particular, recent advances in techniques to monitor simultaneously the expression levels of genes on a genomic scale have facilitated the identification of genes involved in the tumorigenesis [4]. Indeed, some relationships between the disease and the tumor-related genes were proposed from the gene expression analyses [5–7]. Apart from the relationship between tumor-related genes and the disease at the molecular level, the information about the pathogenesis and the clinical characteristics of hepatocellular carcinogenesis has accumulated steadily [8, 9]. However, there is a gap between the information about hepatocellular carcinogenesis at the molecular level and that at more macroscopic levels, such as the clinical level. Furthermore, the relationships between tumor-related genes and other genes also remain to be investigated. Thus, an approach to describe the perspective of carcinogenesis from measurements at the molecular level is desirable to bridge the gap between the information at the two different levels.

Recently, we have developed an approach to infer a regulatory network, which is based on graphical Gaussian modeling (GGM) [10, 11]. Graphical Gaussian modeling is one of the graphical models that includes the Boolean and Bayesian models [12, 13]. Among the graphical models, GGM has the simplest structure in a mathematical sense; only the inverse