Table 3. Discriminatory Peaks and Mean Values Between Groups (HCC* and Non-HCC Group)

m/z	HCC (n = 35)	Non-HCC (n = 44)	p value
Overexpressed proteins			
4067†	3.94 ± 4.56	1.92 ± 1.79	0.03
4470†	8.36 ± 4.28	6.49 ± 3.99	0.01
6433	13.61 ± 10.10	8.94 ± 8.42	0.02
6632	26.87 ± 18.11	18.20 ± 15.09	0.02
7770†	8.40 ± 5.94	5.26 ± 4.42	0.0002
8138	12.76 ± 14.78	5.86 ± 5.37	0.006
8605	4.39 ± 3.08	3.20 ± 2.45	0.02
8934	16.10 ± 10.69	10.36 ± 7.26	0.009
Downregulated proteins			
3326	1.27 ± 0.74	2.10 ± 1.21	0.003
3398	0.90 ± 0.77	2.43 ± 2.50	0.0008
3444†	2.02 ± 1.18	2.45 ± 1.50	0.2
3816	1.98 ± 1.17	3.45 ± 2.84	0.002
3826	1.65 ± 4.95	2.51 ± 3.53	0.002
3890†	3.12 ± 1.35	3.31 ± 1.41	0.2
4135	3.45 ± 2.24	5.08 ± 3.86	0.01
4175	5.49 ± 9.46	12.32 ± 14.63	0.001
4435†	1.23 ± 1.73	2.31 ± 2.63	0.006
4658	1.14 ± 0.80	1.94 ± 1.71	0.007
4791	2.42 ± 1.33	4.04 ± 3.27	0.004
6979	0.82 ± 0.52	1.19 ± 0.67	0.01

NOTE. Data are shown as the means±SD, statistical differences were determined using the Mann-Whitney U test, †Peaks selected in final classification model by decision tree analysis.

Abbreviation: *hepatocellular carcinoma.

munodepletion techniques, leading to the loss of valuable diagnostic information.[31] Therefore, we did not remove major serum proteins (albumin and IgG) from this study; analysis using the SELDI ProteinChip system can be performed without immunodepletion.

The characteristics of patients such as sex and age, sample collection method, processing and storage of samples, and data analysis methods may induce bias into proteomics-based biomarker discovery attempts. Because HCC occurs more frequently in males than females, we developed our classification model using male patients only. As a result, our study was not designed to address the benefit of our classification model for females with HCC. Villanueva et al.,[32] however, reported that gender did not appear to affect the peptide profile. We also evaluated five female patients with HCC; the peak intensity at 8136 m/z was elevated to a similar degree as that seen in male patients with HCC. Currently, a prospective study of female patients with or without HCC is underway to validate the utility of this classification model as a marker for the detection of HCC, particularly at early stages.

We demonstrated that 18 of the selected 55 protein peaks within a m/z range of 3000 to 10,500 range differed between patients with and without HCC by univariate analysis. Based on the peak intensities of the 55 peak proteins, 6 peaks were selected to construct the decision tree for the first analysis group using Biomarker Patterns Software and a 10-fold cross-validation approach. Two (3444 and 3890 m/z peaks) of those 6 peaks, however, were not significantly different between the HCC and non-HCC groups by univariate analysis (*P* values of 0.2, Table 3). The selection process to construct the decision tree was not based on univariate analysis; the presented decision tree was developed using multivariate binary logistic regression to determine the peaks best able to differentiate patients with and without HCC.[19,33] In fact, the ROC AUC of each of these 6 peaks were between 0.61 and 0.71, which tended to be more discriminatory than other serum markers. The decision tree proved to be best able to predict the presence of HCC in comparison with other serum markers. For these reasons, analysis of all 6 peaks, including the 2 peaks that were not significantly different between patients with and without HCC (peaks at m/z = 3444 and 3890), had the highest discriminatory power.

The algorithm used in this study is well established as a diagnostic tool for malignant neoplasms.[13,16,34,35] In comparison with the use of a single biomarker for the diagnosis of disease, multiple-biomarker analysis has both higher sensitivity and specificity. Indeed, our multimarker analysis was more accurate than existing tumor marker analysis methods (Table 4). Multimarker analysis is useful to predict HCC in patients with liver cirrhosis, which has high malignant potential and heterogeneous characteristics. Complex serum proteomic patterns may reflect the underlying pathological state of an organ, including

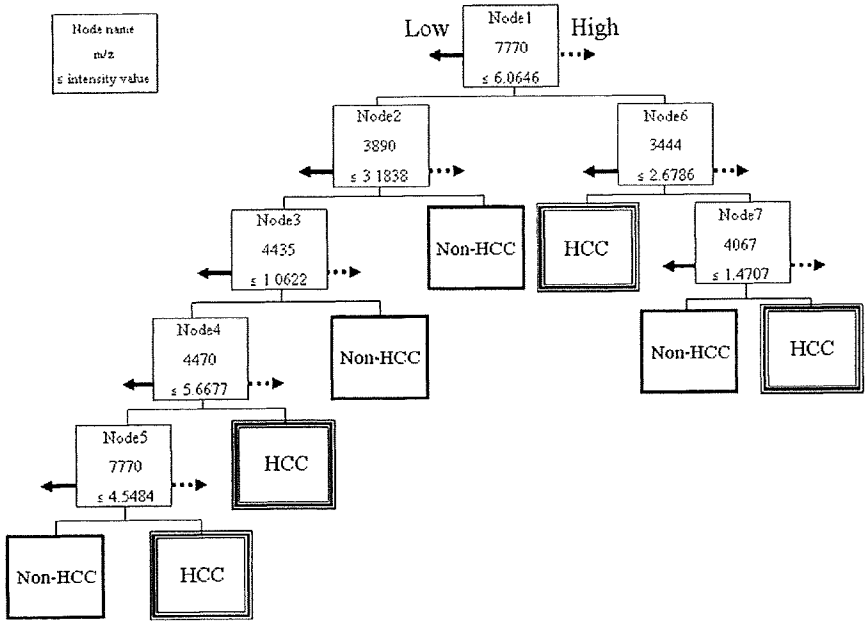

Fig. 2. Classification of HCC and non-HCC samples in the first analysis group. The decision tree was constructed using serum samples from 79 patients. The classification of a particular pattern began at the roof node, following the appropriate links based on the answer to the question at each node. If the peak 1 intensity was higher, the right node was selected. If the peak 1 intensity was lower or equal, the left node was selected. This process was repeated until a terminal node was reached. The decision tree was constructed to correctly classify 97% of the HCC samples in the first analysis group. The upper, middle, or lower lines in the box indicate the node name, molecular weight, and intensity value, respectively.

HCC. Recently, Schwegler et al.[16] reported an algorithm using the seven peaks that scored highest by SELDI TOF/MS. The determined classification tree, however, could not distinguish HCC from chronic liver disease; using 38 SELDI peaks, the sensitivity and specificity (61% and 76%) for distinguishing chronic HCV from HCV-HCC were lower than those determined for the decision tree constructed in this study. Schwegler et al. demonstrated that their sensitivity and specificity values increased to 75% and 92%, respectively, when AFP/DCP/GP73 was added to their classification model. In our model, although the sensitivity increased to 92%, specificity did not increase (52%) after the addition of AFP/AFP-L3/DCP to our classification. Serum GP73 levels, which were not available for examination in our study, or other as-yet-unknown characterizations of these patients may affect the predictive capability of this method. Although the sensitivity and specificity (92% and 90%) of another proteomics study using SELDI to distinguish chronic liver disease from HCC were higher than those determined in our study, greater than 63% of the study population examined exhibited advanced HCC (stage III and IV).[16,36] Only 14% of the HCC patients included in our study population had stage III or IV disease (Table 1), which likely accounts for the differences in the peaks used in the 2 studies. The characteristics of the patients with HCC will likely affect both the sensitivity and specificity significantly. Thus, our decision tree is more suitable for the diagnosis of early HCC than any previously reported methods.[16,36]

Although serum AFP level greater than 400 ng/ml serves as a useful method for the diagnosis of HCC,[37] this detection method is insufficiently sensitive to detect small HCCs.[38] Although the utility of several other markers has been shown to be superior to AFP in detecting early HCC,[22,39,40] these markers were determined in patients with clinically apparent HCC. Thus, the sensitivity/specificity also may not be sufficient to detect early HCC. Our classification tree was able to predict cancer occurrence before HCC was clinically apparent by US. In the third analysis group, we correctly predicted the progression of 86% of the patients to HCC from their prediagnostic

Table 4. Comparisons of Hepatocellular Carcinoma Diagnostic Rates for the Multiple Marker and Three Additional Tumor Marker Analyses in the Second Analysis Group

Markers	Sensitivity	Specificity	ROC AUC****
Multiple-marker	83% (24/29)	76% (25/33)	0.79
AFP* (>20 ng/mL)	41% (12/29)	67% (22/33)	0.57
AFP-L3** (>15%)	17% (5/29)	88% (29/33)	0.56
DCP†,*** (>40 mAU/mL)	39% (11/28)	81% (26/32)	0.64

NOTE. †excluding subject whose data could not be obtained.

Abbreviation: *alpha fetoprotein, **Lens culinaris agglutinin-reactive fraction of alpha-fetoprotein, *** des-γ-carboxy prothrombin, ****receiver operating characteristic area under the curve.

serum samples. To screen high-risk patients with chronic liver disease, such as that associated with HCV infection, our multi-marker analysis could help distinguish those patients for which the combined examination of US, CT, and arterial portography would be recommended.

In their investigation of differential protein expression in HBV-associated and HCV-associated HCC, Kim et al.[26] identified 60 proteins displaying significant changes in expression levels between nontumorous and tumorous tissues. Forty-six of these proteins demonstrated an association with viral infection. We analyzed the sera of patients with HBV-associated HCC; the expression of a number of protein markers differed between HCV and HBV infections (data not shown). The biological and pathogenic activities of these 2 viruses are different; the molecular mechanisms underlying the development of hepatitis and hepatocarcinogenesis also may differ between HBV and HCV infections.[26,41] Our analysis of the proteome using the SELDI technique demonstrates that this methods also may be useful for investigation of the molecular mechanisms of hepatocarcinogenesis on the background of different viral infections.

A number of the peaks may represent doubly charged peaks; for example, the peak at 4067 m/z may be the doubly charged form of the 8138-m/z peak. One of the peaks in Table 3 included in the classification model also may be a doubly charged peak (3890/7770 m/z), which could affect the independent variables. To clarify this possibility, one must identify the individual proteins. The major limitation of the SELDI technique is that identification of individual proteins is often complicated. Lee et al.,[42] however, recently isolated complement C3a as a candidate biomarker in human chronic hepatitis C and HCV-related HCC using the SELDI-TOF MS system after serum fractionation, 2-dimensional gel electrophoresis, in-gel digestion, and MS. We are now identifying the single protein represented by the 8138-m/z peak; 3 candidate proteins are known. Although we have to confirm these results by western blotting, the peak at 4067 m/z does not appear to be the doubly charged peak of the 8138-m/z peak by SELDI immunoassay. Although the serum levels of no single protein are sufficient to detect early HCC from the results of ROC AUC, identification of proteins altered in the disease may help analyze the molecular mechanisms underlying HCC development and may help identify new therapeutic targets or modalities for the treatment or prevention of HCC.

In patients with HCV infection, serum profiling using the SELDI ProteinChip system is useful both for the early detection of HCC and to distinguish HCC from chronic liver disease in the absence of HCC. Our ability to identify proteomic alterations in serum samples from HCC patients suggests that the SELDI ProteinChip system may be useful to identify proteins associated with HCC in the hopes of developing new therapeutic targets.

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Association of a genetic polymorphism in ectonucleotide pyrophosphatase/phosphodiesterase 1 with hepatitis C virus infection and hepatitis C virus core antigen levels in subjects in a hyperendemic area of Japan

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Background. The clinical course of chronic hepatitis C virus (HCV) infection is strongly associated with insulin resistance and obesity. The K121Q polymorphism in the ectonucleotide pyrophosphatase/phosphodiesterase (*ENPP*)-1 gene and the rs7566605 genotype located near insulin-induced gene 2 have been shown to be associated with insulin resistance and obesity. This study examined whether the K121Q polymorphism in *ENPP1* or the rs7566605 genotype is associated with the clinical course of HCV infection. ***Methods.*** The relationships between the clinical characteristics of 469 anti-HCV antibody-seropositive subjects (353 were positive for HCV core antigen or RNA, whereas 116 were negative for HCV RNA) and the polymorphisms were analyzed. ***Results.*** No significant differences in body mass index, plasma glucose level, serum insulin level, and other biochemical markers were observed between subgroups of subjects with different genotypes at the K121Q polymorphism or rs7566605. The frequency of the homozygous wild-type genotype at K121Q in HCV carriers, however, was significantly higher than that in subjects who were negative for HCV RNA (84.5% vs. 75.9%; $P < 0.05$). Moreover, in HCV carriers, HCV core antigen levels in subjects homozygous for the wild-type genotype at K121Q were significantly higher than in heterozygous carriers of K121Q (5358 fmol/l vs. 4002 fmol/l; $P = 0.04$). In contrast, the rs7566605 genotype was not associated with hepatitis C viremia or with the HCV core antigen level. ***Conclusions.*** The K121Q variant of *ENPP1* may be associated with hepatitis C viremia and core antigen levels in HCV carriers.

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Key words: hepatitis C virus, *ENPP1*, insulin resistance, viremia, single nucleotide polymorphism, HCV core antigen

Introduction

Hepatitis C virus (HCV) infection, a major cause of chronic hepatitis, may progress to cirrhosis or hepatocellular carcinoma (HCC). Persistent HCV infection can be detected in the sera of 50%–80% of subjects positive for anti-HCV antibodies; in contrast, 20%–50% of those subjects are consistently negative for HCV RNA, suggesting that they have successfully eliminated the HCV infection.[1] Factors such as ethnicity, icteric clinical presentation, absence of human immunodeficiency virus (HIV) infection, and specific HLA type II alleles have been shown to be associated with viral clearance.[2–4] Even in the absence of these factors, however, viral clearance may occur, suggesting the presence of other unidentified cofactors.

Being overweight or obese is an independent risk factor for hepatic steatosis, which accelerates the activity and progression of chronic hepatitis C (CHC).[5] Another risk factor for steatosis is insulin resistance, which is associated with advanced fibrosis and hyporesponsiveness to antiviral therapy.[6] Although obesity and insulin resistance are known to be caused by a combination of genetic and environmental factors, the impact of genetic factors on the clinical course of HCV infection or the severity of liver disease has not been fully elucidated.

A number of reports indicate that single nucleotide polymorphisms (SNPs) in the gene encoding the K121Q variant of ectonucleotide pyrophosphatase/phosphodi-

esterase 1 (*ENPP1*, also known as PC-1) influence insulin resistance, type 2 diabetes, and obesity.[7–11] Recently, the rs7566605 genotype, which is located near the gene encoding insulin-induced gene 2 (*INSIG2*), was also shown to be strongly associated with insulin resistance.[12] Other studies, however, have reported no significant associations between the K121Q variant and insulin resistance or type 2 diabetes,[13–15] and the association between the K121Q variant or rs7566605 genotype and the clinical features of patients with chronic HCV infection has not been fully evaluated.

We examined the natural history of HCV infections in an adult Japanese community-based population in an HCV hyperendemic area beginning in 1994.[16,17] Because movement of the residents in or out of this region is rare, this area provided an appropriate setting to investigate the effects of a genetic background on HCV infections. In this study, we sought to determine the prevalence of the rs7566605 genotype and polymorphisms of the *ENPP1* gene encoding the K121Q variant and to assess their relationship with body mass index (BMI), insulin resistance, and the clinical characteristics of subjects positive for anti-HCV antibodies in an HCV hyperendemic area in Japan.

Materials and methods

Study population

We evaluated 459 anti-HCV antibody-seropositive subjects. Among these subjects, 343 were positive for HCV RNA or HCV core antigen (HCV carrier group), and 116 were negative for both HCV RNA and HCV core antigen (HCV RNA-negative group). All the subjects were Japanese and lived in an HCV hyperendemic area (Town C).[16–18] The Town C HCV study is a cohort study examining the natural course of HCV infections in adult residents of a community in Miyazaki Prefecture, Japan. Residents who were identified as anti-HCV antibody positive at general health examinations were invited to participate in annual examinations for liver disease. No one in this study population had received interferon therapy or was positive for hepatitis B surface antigen. Informed consent was obtained from all participants at the time of enrollment. This study was approved by the human subjects committees of the University of Miyazaki (Faculty of Medicine, Japan), the Harvard School of Public Health, and the Boston University School of Public Health.

Blood tests for hepatic fibrosis markers, anti-HCV antibodies, and HCV core antigen levels

Serum anti-HCV antibodies were detected using chemiluminescence enzyme immunoassays and a third-generation kit (Lumipulse Ortho II; Ortho-Clinical Diagnostics, Tokyo, Japan) at least once for each subject between 2001 and 2003. Additionally, 301 subjects in the HCV carrier group and 100 subjects in the HCV RNA-negative group were known to be positive for anti-HCV antibodies before 1996 as a result of second-generation enzyme immunoassay testing (Immunocheck F-HCV Ab; International Reagents, Kobe, Japan).[16–19] The presence of serum HCV RNA was determined using qualitative reverse transcription-polymerase chain reaction (RT-PCR) (Amplicore HCV; Nippon Roche, Tokyo, Japan). HCV core antigen levels were measured using immunoradiometric assays and a cutoff value for a positive result of 20 fmol/l (Ortho HCV Ag IRMA test; Ortho-Clinical Diagnostic). The levels of plasma glucose (normal range, 70–109 mg/dl), serum insulin (≤17 mU/ml), aspartate aminotransferase (AST) (10–40 IU/l), alanine aminotransferase (ALT) (5–40 IU/l), γ-glutamyl transpeptidase (GTP) (female: 7–30 IU/l; male: 7–70 IU/l), ferritin (female: 7–110 mg/dl; male: 24–286 mg/dl), and the platelet count (12.0–34.0 × 10^4 cells/μl) were examined in each patient. The HCV serotype of each subject was determined before 2001. If the HCV serotype was not determined, the HCV genotype was examined (HCV Core Genotype; SRL, Tokyo, Japan). HCV genotype 1b was considered to be serotype I and genotypes 2a and 2b were considered to be serotype II. No other HCV genotype was detected in this study. Insulin resistance was assessed using a homeostasis model assessment of insulin resistance (HOMA-IR). HOMA-IR values were calculated as follows: plasma glucose (mg/dl) × serum insulin (mU/ml)/405. Hyaluronic acid and type IV collagen 7S, which are known to be hepatic fibrosis markers, were examined using a latex bead agglutination assay (LPIA-ACE HA; Mitsubishi Kagaku Iatron, Tokyo, Japan; normal range: ≤50 ng/ml) and a radioimmunoassay (Type IV collagen 7S kit; Mitsubishi Kagaku Iatron; normal range: ≤6.0 ng/ml), respectively.

DNA extraction and real-time PCR allelic discrimination assays

DNA extraction and real-time PCR allelic discrimination assays were carried out as described previously.[19] Briefly, 10 μl whole blood was drawn into an ethylenediaminetetraacetic acid (EDTA)-containing Vacutainer by venipuncture. Genomic DNA was extracted from the buffy coat fraction, which was separated from the blood by centrifugation at 3000 rpm using Mag-Extractor System MFX-2000 (Toyobo, Osaka, Japan) according to the manufacturer's protocol. The *ENPP1* K121Q SNP was examined using PCR and sequence-specific primers. Real-time PCR allelic discrimination assays were designed using TaqMan SNP genotyping

assays (Applied Biosystems, Foster City, CA, USA). Assays were performed to genotype the A→C SNP corresponding to *ENPP1* K121Q using commercially available primers (dbSNP ID: rs1044498; TaqMan SNP genotyping assays ID: C_1207994_20). We also evaluated the rs7566605 genotype located near the *INSIG2* gene.[12] Genotyping of the G→C SNP (rs7566605) was performed with the primers rs7566605-F (AGTAGGGTGAGGAAACCAAATTCTC) and rs7566605-R (CATGACCCCTACCGTCTCTATTTT), and the probes rs7566605-VIC (ACAGAGATGTTA CATCAC labeled with the dye VIC) and rs7566605-FAM (CACAGAGATATTACATCAC labeled with the dye FAM) in a custom TaqMan genomic assay. Briefly, 5 ng DNA was mixed with TaqMan Universal PCR master mix (Applied Biosystems) and allelic discrimination assay mix (900 nM each primer and 200 nM each FAM or VIC-labeled probe). PCRs were carried out in a total volume of 6 or 10 µl in 96-well PCR plates. The PCR conditions were as follows: 50°C for 2 min for contamination control with AmpErase uracil-*N*-glycosylase and 95°C for 10 min to activate the AmpliTaq Gold enzyme, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min. Genotypes were assessed using the TaqMan allele-specific assay method and an ABI Prism 7000 sequence detection system according to the manufacturer's protocol (Applied Biosystems). All genotypes were scored using the allelic discrimination program from the ABI software.

Statistical evaluation

The differences in mean values were assessed using Mann–Whitney *U* tests. Fisher's exact tests and χ^2 tests were used where appropriate. Univariate and multivariate logistic regression analyses were also used to determine the factors that significantly associated with viral clearance or viral load. All statistical analyses were performed using STATVIEW 4.5 software (Abacus Concepts, Berkeley, CA, USA) or SPSS version 11.01 statistical analysis software (SPSS, Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

Results

Characteristics of the subjects

The clinical characteristics of the study population are shown in Table 1. In this study, 343 subjects were positive for anti-HCV antibodies and the presence of HCV RNA and/or HCV core antigen (HCV carrier group), whereas 116 subjects were positive for anti-HCV antibodies but were negative for both HCV RNA and HCV core antigen (HCV RNA-negative group). The mean age of the subjects was 70 years (range, 42–97 years old), and the mean BMI of the subjects positive for anti-HCV antibodies was 23 kg/m^2 (range, 15.6–33.5 kg/m^2). Although there were no differences in the distribu-

Table 1. Clinical characteristics of subjects positive for antihepatitis C virus (HCV), according to the presence of hepatitis C viremia

Characteristics	HCV carrier[a] (n = 343)	HCV RNA-negative[b] (n = 116)	P value[c]
Age (years)	70.7 ± 9.7	69.6 ± 11.2	0.67
Sex (male/female)	117/226	37/79	0.66
History of alcohol consumption (daily/occasionally/none)[d]	110/23/174	35/7/63	0.83
Past history of BT (yes/no)[d]	50/273	25/83	0.07
HCV core antigen	4871.6 ± 4869.4 (325)	–	–
HCV serotype (I/II)[e]	225/118	–	–
Body mass index	23.1/1/3.0 (286)	23.1 ± 3.3 (93)	0.73
AST (IU/l)	49.4 ± 32.9	26.4 ± 8.6	<0.001
ALT (IU/l)	44.9 ± 38.2	20 ± 10.1	<0.001
γ-GTP (IU/l)	35.0 ± 52.3 (248)	21.6 ± 26.4 (91)	<0.001
PLT (×10^4)	19.1 ± 6.2 (342)	23.8 ± 5.6	<0.001
Tryglyceride (mg/dl)	110.2 ± 57.2 (248)	123.2 ± 59.4 (93)	0.02
Total cholesterol (mg/dl)	170.3 ± 34.7 (248)	193.1 ± 30.8 (93)	<0.001
HbA1c (%)	5.3 ± 0.7 (248)	5.4 ± 1.0 (91)	0.12
Glucose (mg/dl)	97.3 ± 34.4 (273)	95.6 ± 23.6 (88)	0.86
Insulin (µU/ml)	11.4 ± 11.4 (273)	9.3 ± 13.7 (88)	<0.001

Data are shown as means ± SD (number of subjects examined)
BT, blood transfusion; AST, aspartate aminotransferase; ALT, alanine transferase; GTP, guanosine triphosphatase; PLT, platelet count
[a]Positive for HCV RNA or HCV core antigen
[b]Negative for HCV RNA and HCV core antigen
[c]Data were evaluated by χ^2 test, Fischer's exact test, or Mann–Whitney test, as appropriate
[d]Excluding subjects whose history was not available
[e]Including subjects whose HCV genotype was determined even if serotype was undetermined

Table 2. Prevalence of *ENPP1* K121Q genotype or rs7566605 genotype in subjects with positive for anti-HCV, according to the presence of hepatitis C viremia

	HCV carrier[a]	HCV RNA-negative[b]	*P* value[c]
K121Q genotype	*n* = 342	*n* = 116	
AA	289 (84.5%)	88 (75.9%)	
AC	53 (15.5%)	26 (22.4%)	
CC	0	2 (1.7%)	0.01[d]
rs 7566605 genotype	*n* = 341	*n* = 116	
GG	159 (46.6%)	52 (44.8%)	
GC	141 (41.3%)	52 (44.8%)	
CC	41 (12.0%)	12 (10.3%)	0.75

[a] Positive for HCV RNA or HCV core antigen
[b] Negative for HCV RNA and HCV core antigen
[c] Data were analyzed by χ^2 test
[d] *P* value was 0.048 evaluated by subclasses of AA or AC + CC genotype

Table 3. Prevalence of *ENPP1* K121Q genotypes or rs7566605 genotype in HCV carriers, according to the body mass index (BMI)

	Normal weight (BMI <25)	Overweight (BMI ≥25 and <30)	Obesity (BMI ≥30)	*P* value[a]
K121Q genotype	*n* = 216	*n* = 76	*n* = 4 (%)	
AA	182 (84.3%)	66 (86.8%)	3 (75.0%)	
AC	34 (15.7%)	10 (13.2%)	1 (25.0%)	0.75[b]
CC	0	0	0	
rs 7566605 genotype	*n* = 216	*n* = 75	*n* = 4	
GG	107 (49.5%)	30 (40.0%)	2 (50.0%)	
GC	83 (38.4%)	35 (46.7%)	2 (50.0%)	
CC	26 (12.0%)	10 (13.3%)	0	0.36

[a] Data were evaluated by χ^2 test
[b] Data were analyzed excluding CC genotype

tions of age, sex, history of alcohol consumption, BMI, plasma glucose levels, and HbA1c levels between the groups, AST, ALT, γ-GTP, and insulin levels were significantly higher and triglycerides, total cholesterol, and platelet counts were significantly lower in the HCV carrier group than in the HCV RNA-negative group.

Differential distributions of the ENPP1 *K121Q SNP or rs7566605 genotypes and the clinical characteristics*

We successfully genotyped 458 and 457 subjects for the *ENPP1* K121Q SNP and rs7566605, respectively. The *ENPP1* K121Q SNP was differentially distributed between the HCV carrier group and the HCV RNA-negative groups ($P < 0.01$), whereas the rs7566605 genotype was not (Table 2). In univariate analysis, the *ENPP1* K121Q genotypes AC and CC were significantly more prevalent in the HCV RNA-negative group than in the HCV carrier group [odds ratio (OR), 1.74; 95% confidence interval (CI), 1.04–2.91; $P = 0.04$]. No other factors, including age, sex, BMI, history of alcohol consumption, past history of blood transfusion, and the rs7566605 genotype, were significantly different between the groups (data not shown). In multivariate analysis using four factors (age, sex, *ENPP1* K121Q genotype, and rs7566605 genotype), only the *ENPP1* K121Q genotypes AC and CC were associated with being negative for HCV RNA (OR, 1.78; 95% CI, 1.05–2.99; $P = 0.03$).

Relationships between the ENPP1 *K121Q or rs7566605 genotypes and BMI or insulin resistance*

We examined the relationships between the SNPs and available BMI values in HCV carriers: the subjects were classified as overweight (BMI ≥25 and <30 kg/m^2), obese (BMI ≥30 kg/m^2), or normal (BMI <25 kg/m^2). The distributions of the *ENPP1* K121Q and rs7566605 genotypes were similar in all three BMI subgroups (Table 3). In addition, there was no association between these two SNPs and fasting plasma glucose levels greater than 126 mg/dl or a history of diabetes (data not shown). Then, subjects with fasting plasma glucose levels less than 126 mg/dl were selected, and the relationship between the SNPs and insulin resistance was studied after classifying the subjects as insulin resistant (HOMA-IR value ≥2) or not (HOMA-IR value <2). The distributions of the *ENPP1* K121Q and rs7566605

Table 4. Prevalence of *ENPP1* genotypes or rs7566605 genotypes in HCV carriers, according to insulin resistance

	Lower HOMA-IR index (<2)	High HOMA-IR index (≥2)	*P* value[a]
K121Q genotype	*n* = 130	*n* = 106	
AA	106 (81.5%)	94 (88.7%)	
AC	24 (18.5%)	12 (11.3%)	0.13[b]
CC	0	0	
rs 7566605 genotype	*n* = 131	*n* = 105	
GG	68 (51.9%)	48 (45.7%)	
GC	47 (35.9%)	48 (45.7%)	
CC	16 (12.2%)	9 (8.6%)	0.27

HOMA, homeostasis model assessment of insulin resistance
[a]Data were valuated by χ^2 test
[b]Data were analyzed excluding CC genotype

Table 5. Clinical and virological characteristics in individuals who are HCV carriers, according to the *ENPP1* K121Q genotype

Characteristics	*ENPP1* K121Q genotype[a]		*P* value[b]
	AA (*n* = 289)	AC (*n* = 53)	
Age (years)	70.9 ± 9.5	69.7 ± 10.5	0.43
Sex (male/female)	101/188	15/38	0.35
Body mass index	23.1 ± 3.0 (251)	22.8 ± 3.1 (45)	0.44
Alcohol consumption (daily/occasionally/none)[c]	100/22/157	18/4/30	0.98
Past history of blood transfusion (yes/no)[c]	39/234	11/38	0.15
HCV core antigen (fmol/l)[d]	5358.3 ± 4906.7 (272)	4001.8 ± 4526.4 (53)	0.04
HCV core antigen (<1000/≥1000)[e]	73/216	18/35	0.19
HCV serotype (I/II)[f]	182/107	42/11	0.02
AST (IU/l)	49.9 ± 34.4	46.7 ± 23.4	0.83
ALT (IU/l)	45.9 ± 40.5	40.2 ± 21.7	0.86
γ-GTP (IU/l)	36.2 ± 55.0 (210)	28.1 ± 32.5 (38)	0.75
PLT ($\times 10^4$)	19 ± 6.1 (288)	20.0 ± 6.7	0.30
TG (mg/dl)	110.1 ± 57.1 (210)	110.6 ± 58.6 (38)	0.92
Total cholesterol (mg/dl)	170.0 ± 35.0 (210)	172.3 ± 33.2 (38)	0.66
HbA1c (%)	5.3 ± 0.7 (210)	5.4 ± 0.9 (38)	0.67
Glucose (mg/dl)	98.0 ± 35.4 (230)	93.7 ± 28.9 (42)	0.20
Insulin (μU/ml)	11.6 ± 11.7 (230)	10.9 ± 10.2 (42)	0.59
Ferritin (mg/dl)	151.0 ± 215.5	138.5 ± 182.3	0.33
HA (ng/ml)	196.9 ± 365.9 (287)	236.4 ± 391.8	0.58
Type IV collagen 7S (ng/ml)	5.0 ± 1.8 (287)	5.0 ± 2.0	0.39

Data are shown as means ± SD (number of subjects examined)
[a]There was no subject with CC genotype in persistent HCV infection group
[b]Data were evaluated by χ^2 test, Fischer's exact test, or Mann–Whitney test, as appropriate
[c]Excluding subjects whose history was not available
[d]Excluding subjects whose HCV core antigen level was below the cutoff value
[e]Including subjects whose HCV core antigen level was below the cutoff values
[f]Including subjects whose HCV genotype was determined even if serotype was undetermined

genotypes were also similar in the HOMA-IR subgroups (Table 4).

Clinical and biochemical characteristics of the HCV carriers classified based on the ENPP1 *K121Q or rs7566605 genotype*

In the HCV carrier group, biochemical markers from the subjects with AA and AC genotypes at the *ENPP1* K121Q SNP were compared (Table 5). We did not identify any subjects in the HCV carrier group with a CC genotype at this locus. The levels of HCV core antigen in subjects with an AA genotype were higher than in subjects with an AC genotype. The frequency of serotype II was also higher in subjects with an AA genotype than in subjects with an AC genotype. No other clinical or biochemical characteristics were different between the subjects with the different K121Q genotypes.

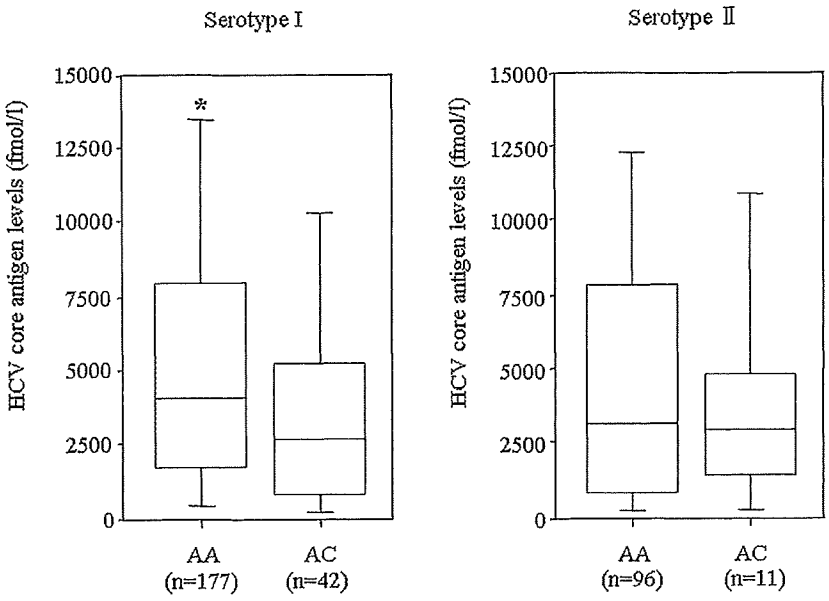

Fig. 1. The association between the K121Q genotype in *ENPP1* and the hepatitis C viral (HCV) load. The box-and-whisker plot shows the HCV core antigen level in the HCV carrier group according to the genotypes. The *boxes* indicate the 25th, 50th (median), and 75th percentiles. The *whiskers* indicate the 10th and 90th percentiles. The *asterisk* refers to a statically significant difference between the HCV core antigen levels in patients with the AA or AC genotype (Mann–Whitney *U* test, $^*P = 0.04$)

We then further analyzed the association between the *ENPP1* K121Q variant and HCV core antigen levels according to the HCV serotype (Fig. 1). In the subgroup of subjects classified as HCV serotype I, the hepatitis C viral load was significantly higher in the subjects with the AA genotype (the wild-type genotype) than in those with the AC genotype ($P = 0.04$). Five subjects with the AA genotype were not included in this comparison because their levels of HCV core antigen were below the threshold. In any case, the percentage of subjects with HCV core antigen levels below the cutoff value of 1000 fmol/l was lower in the AA genotype subgroup than in the AC genotype subgroup (23.0% vs. 61.5%, $P < 0.01$ calculated using Fisher's exact test; OR, 2.68; 95% CI, 1.30–5.54; $P < 0.01$). Although a past history of blood transfusion was also associated with HCV core antigen levels (OR, 2.75; 95% CI, 1.25–6.06; $P = 0.01$), no other factors were associated with this variable. In multivariate analysis using the *ENPP1* K121Q variant and past history of blood transfusion, these two factors were independently associated with low HCV core antigen levels (OR, 2.44; 95% CI, 1.12–5.32; $P = 0.03$ and OR, 2.56; 95% CI 1.14–5.72; $P = 0.02$, respectively). This correlation between the HCV core antigen levels and the K121Q genotype, however, was not observed in the subgroup of subjects classified as HCV serotype II (Fig. 1).

In addition, we compared the biochemical markers from the subjects with the GG, GC, and CC genotypes at rs7566605. There were no significant differences among the clinical or biochemical characteristics of the subjects from these three groups, including the viral load (data not shown).

Discussion

Obesity and insulin resistance, which are caused by a combination of genetic and environmental factors, affect the clinical course of CHC infection.[5,6] The K121Q polymorphisms in the *ENPP1* gene and the rs7566605 genotype have been shown to be significantly associated with obesity and insulin resistance.[7–12] Whether polymorphisms in genes associated with obesity or insulin resistance affect persistent HCV infection or HCV-induced liver injury, however, has yet to be determined. We sought to examine the relationship between polymorphisms in these types of genes and viremia or the clinical course of liver injury in subjects positive for anti-HCV antibodies in a community-based HCV hyperendemic area in Japan. Our study, which shows that polymorphisms associated with the K121Q variant and the rs7566605 genotype are prevalent in Japan, suggests that these genotypes are not associated with obesity or insulin resistance in the examined HCV hyperendemic area. In addition, these polymorphisms were not associated with HCV-induced liver injury. In contrast, the frequencies of the K121Q polymorphism in subjects with hepatitis C viremia and those without viremia were different. Moreover, the K121Q polymorphism was associated with HCV viral load in a subgroup of HCV carriers (serotype I).

ENPP1 is the best characterized of the five human ectoenzyme *ENPP* proteins. *ENPP1* is expressed in many tissues, including muscle, fat, and liver, and overexpression of *ENPP1* in various cell lines inhibits insulin receptor tyrosine kinase activity and causes insulin resistance.[20] It was also reported that the K121Q variant

of *ENPP1* is associated with insulin resistance.[21,22] Compared to the *ENPP1* K121 protein, the *ENPP1* Q121 variant interacts more strongly with the insulin receptor and more effectively inhibits insulin-stimulated insulin receptor autophosphorylation and insulin receptor substrate-1 phosphorylation in vitro.[23] In our study, however, there was no association between the *ENPP1* K121Q variant and insulin resistance in HCV carriers. Keshavarz et al. also failed to find evidence of an association between the *ENPP1* K121Q variant and type 2 diabetes in a Japanese population.[24] The overall frequency of the 121Q allele (9.1%; 83/916) in our study was similar to that in the Japanese population, as previously reported (10.5%; 375/3562).[24] These results indicate that our study population represented the rest of Japan and that the K121Q variant does not influence insulin resistance in Japanese subjects, in particular in subjects with HCV infections.

rs7566605 is upstream of the transcription start site of *INSIG2*, the protein product of which inhibits the synthesis of fatty acids and cholesterol.[25] Overexpression of *INSIG2* in the liver reduced plasma triglyceride levels in obese Zucker diabetic fatty rats, and linkage between this gene and obesity phenotypes was observed in the mice.[26,27] Association testing in nine cohorts produced evidence that individuals with the CC genotype at rs7566605 have higher BMI values and a higher risk of obesity than those with the GG or GC genotype.[28] More recently, however, no association was reported between this genotype and obesity.[29,30] In addition, the rs7566605 genotype was not associated with the clinical or biochemical characteristics of subjects positive for anti-HCV antibodies, obesity, or insulin resistance in our study. These conflicting results about the relationship between the rs7566605 genotype and BMI may have resulted from the heterogeneous population samples. Future studies should enroll a large number of patients with HCV infections and control subjects from throughout the Japanese population.

False-positive results for the HCV antibody test may have occurred in the HCV RNA-negative group in our study. Several studies have shown that samples with readings just slightly above the cutoff value of the anti-HCV test have a greater likelihood to be false-positives compared with those with higher values.[31,32] HCV-positive patients may also show reactivity to nuclear and smooth muscle antigens.[33,34] There was, however, no difference in the distributions of the *ENPP1* K121Q genotypes (AA, AC, or CC) among patients with low titers (≥1 and <5), intermediate titers (≥5 and <30), and high titers (≥30) of anti-HCV antibodies in our study (data not shown). In addition, although there was no evidence of spontaneous clearance of HCV infection in this study, Micallef et al. systematically reviewed 31 longitudinal studies with a total of 675 subjects and reported that spontaneous viral clearance occurs in approximately one in four people with acute hepatitis C, which was similar to the size of the HCV RNA-negative group (25%).[35] Although autoantibody data and evidence of spontaneous HCV clearance in the clinical courses are not available, these results indicate that many subjects in the HCV RNA-negative group in our study population may have cleared their HCV infection spontaneously without false-positive results for the HCV antibody test.

Spontaneous HCV clearance typically occurs within the first 6 months after acute infection,[36] and spontaneous elimination of HCV in subjects with chronic HCV infection is rare.[16] These results suggest that *ENPP1* may influence the spontaneous clearance of HCV during the acute phase of infection in our population. Furthermore, sex is known to be an important factor for HCV clearance,[37–39] although a sex-based difference was not observed in our study (see Table 1). Studies based on polymorphisms have been widely used to identify host genetic factors that influence disease occurrence, progression, and outcome.[40] However, it is unclear whether *ENPP1* and sex are associated in HCV clearance. Another potential confounding variable is alcohol use, which is known to be negatively associated with HCV clearance.[41] Alcohol use, however, is limited in this community, and thus was unlikely to be a confounder. Further studies are needed to clarify the associations between host factors and *ENPP1* and their roles in HCV clearance.

Analysis of the *ENPP1* gene in 6147 subjects showed an association between a three-allele risk haplotype (K121Q, IVS20delT-11, and A→G+1044TGA) and obesity and type 2 diabetes.[42] In that report, it was shown that the presence of at least one copy each of the Gln121(121Q), IVS20delT-11, and G+1044TGA variants was associated with a significant increase in serum *ENPP1* protein levels. In addition, serum levels of osteopontin were lower in *ENPP1*-deficient mice than in wild-type mice, suggesting that *ENPP1* affects osteopontin expression.[43] Osteopontin-deficient mice also suffered from prolonged rotavirus-induced diarrhea.[44] SNPs in the promoter region of the osteopontin gene have been identified as markers that predict the efficacy of interferon-based therapies in patients with CHC.[45] Although our studies do not directly identify increased serum levels of *ENPP1* or osteopontin, *ENPP1* may induce nonproductive binding of HCV to cells, blockade of HCV attachment, or inhibition of penetration into cells through osteopontin expression.

The precise roles that host factors play in HCV replication have not been well characterized. Although Woitas et al. reported that anti-HCV-antibody-seropositive patients who were homozygous for the HIV-protective CC chemokine receptor (CCR) 5-Δ32

showed a markedly increased viral load compared with CCR5 wild-type or CCR5-Δ32 heterozygous patients,[46] the authors did not show results based on the HCV genotype or serotype. Hepatitis C viral load was found to be significantly higher in patients infected with HCV genotype 1 compared to patients infected with HCV genotype 2 or 3.[47] Our study indicates that the AC genotype at the K121Q SNP of *ENPP1* is linked to lower HCV core antigen levels, which correlated with hepatitis C viral load in the HCV serotype I subgroup, but not in the serotype II subgroup. The mechanisms contributing to the relationship between the K121Q polymorphism and the hepatitis C viral load are unclear. HCV replication in the cytoplasm, however, is highly dependent on the functions of nonstructural HCV proteins together with those of host factors.[48,49] Thus, functional studies about the molecular mechanisms underlying *ENPP1* signaling in HCV replication should be conducted in the future.

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Endoscopic characterization of the small bowel in patients with portal hypertension evaluated by double balloon endoscopy

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Background. The endoscopic abnormalities present in the small bowel (SB) of patients with portal hypertension (PH) are not well understood. This study sought to evaluate endoscopic findings of the SB in patients with PH by double balloon endoscopy (DBE). ***Methods.*** We evaluated the endoscopic findings of SB in 15 patients with PH and 49 controls without liver disease or PH. A total of 24 and 90 procedures were performed for PH patients and control patients, respectively, through oral and/or anal approaches. ***Results.*** Fourteen of the 15 patients exhibited villous abnormalities, including edema (73%), atrophy (40%), and reddening (47%) of villi. Vascular lesions, such as angiodysplasia-like abnormalities (67%), dilated/proliferated vessels (93%), and varices (7%), were observed in all patients with PH. Although they were associated with ascites, these abnormalities did not correlate with any laboratory findings. None of these abnormalities was observed in controls. Definitive or suspected bleeding sources were identified in 9 of 13 patients with both PH and obscure gastrointestinal bleeding (OGIB), which was similar to the incidence in controls with OGIB. Although the frequency of postprocedure fever (>37.5°C) was higher in patients with PH in comparison to controls (29% vs. 2%, $P < 0.01$), endoscopic treatment under DBE was performed on 3 PH patients without serious complications. ***Conclusions.*** Endoscopic abnormalities of the SB may be prevalent in patients with PH. Although postprocedure fever of DBE may occur more commonly in patients with PH, DBE is useful as both a diagnostic and therapeutic tool to evaluate the SB.

Key words: portal hypertensive enteropathy, double balloon endoscopy, portal hypertension, liver cirrhosis, small bowel

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Introduction

Portal hypertension (PH) can be caused by hepatic fibrosis or obstruction of the portal vein. Hepatic fibrosis, of which liver cirrhosis is an advanced form, results from chronic liver disease. PH has numerous complications bearing high morbidity and mortality, including variceal bleeding. Splanchnic blood flow is significantly altered by PH.[1,2] Varices develop in the esophagus, stomach, duodenum, colon, or rectum. Portal hypertensive gastropathy (PHG) or colopathy follow the development of PH and can lead to gastrointestinal bleeding. There is a subset of cases, however, in which the bleeding source remains unclear following upper and lower gastrointestinal endoscopies in patients with PH.[3–6]

The changes in the gastrointestinal mucosa of the esophagus, stomach, colon, and rectum are well described in patients with PH. The majority of studies have focused on the involvement of the gastric and colonic mucosa in patients with PH; however, it is likely that the small bowel (SB), including the duodenum and ileum, would also undergo mucosal changes as a result of PH, which is defined as portal hypertensive enteropathy (PHE).[7,8] As the SB is distal to both the mouth and the anus, it is difficult to evaluate the entire SB by endoscopic diagnosis using upper and lower gastrointestinal endoscopy; regions of the small intestine will always lie beyond the limits of the endoscope. Therefore, the endoscopic abnormalities in the SB of patients with PH have not been well characterized.

Recently, new endoscopic methods, video capsule endoscopy (VCE) and double balloon endoscopy (DBE), have been developed for examination of the entire SB.[9,10] VCE permits direct visualization of the SB mucosa. Although VCE is both easy and painless, this technique usually does not allow visualization in real time. In addition, the technology is limited by the inability to take biopsies for histology or perform therapeutic

endoscopic interventions using VCE. In contrast, DBE provides higher-resolution imaging with improved visualization because of the capability to insufflate air, irrigate, and suction obscuring mucus/material and the ability to perform a focused examination of any abnormality visualized. This technique also allows clinicians to obtain tissue samples, making treatment of the entire SB possible in a clinical setting.[11–13]

It has also a high diagnostic yield for occult gastrointestinal bleeding (OGIB), when the SB is suggested to be the source of bleeding by VCE or DBE.[14] However, only a few descriptions of the endoscopic findings or specific bleeding sources discovered in the SBs of patients with PH are available.[6,15] None of these studies has utilized DBE to assess the incidence and characteristics of the SB abnormalities seen in patients with PH. We sought to use DBE to define the endoscopic findings present in the SB of patients with PH and to determine if these findings are associated with specific clinical characteristics. We also evaluated the availability of DBE for endoscopic therapy and the associated complications.

Patients and methods

Patients

This study was a nonrandomized, retrospective analysis of patients with PH caused by cirrhosis or extrahepatic portal vein obstruction (EHO) who were examined by DBE at Miyazaki Medical Center Hospital between September 2004 and March 2007. We confirmed the presence of liver cirrhosis by compatible physical examination, laboratory findings, histology, or radiographic features. EHO was diagnosed in patients with PH who had normal liver function tests, no clinical signs of cirrhosis, and compatible radiographic findings. PH was diagnosed by endoscopic or radiographic evidence of esophageal, gastric, or intraabdominal varices with or without splenomegaly. The severity of cirrhosis was graded using the Child-Pugh classification.

A total of 24 procedures in 15 consecutive patients with PH (12 men, 3 women; mean age, 65.8 ± 8.7 years; age range, 48–75 years) were performed. Oral, anal, and combined approaches were performed in 2, 8, and 5 patients, respectively. One patient required 5 procedures; the anal approach had to be repeated in 1 patient. We compared these results to those for 90 DBE procedures in 49 control patients (39 men and 10 women; mean age, 48.8 ± 21.1 years; age range, 16–85 years). In 49 control patients, 14 patients underwent DBE for OGIB, 10 for abdominal pain, 8 for ileus, 7 for inflammatory bowel disease, 3 for diarrhea, 2 for suspicion of tumor, 2 for fever of unknown etiology, 2 for inability to perform an endoscopic retrograde cholangiopancreatography because of previously manipulated intestines, and 1 for suspicion of infection. Patients who did not have chronic liver disease or PH who were treated at our hospital served as controls. Oral, anal, and combined approaches were performed for 7, 22, and 20 of the control patients, respectively, which includes several who were subjected to repeated procedures. Written informed consent for examination by DBE was obtained from all patients.

Methods of double balloon endoscopy

The double balloon endoscopic system (Fujinon EN-450T5/W; Fujinon, Saitama, Japan) utilizes a video endoscope with a working length of 200 cm and a flexible single-use overtube with a length of 145 cm (including the balloon). The double balloon technique has been described previously.[10] During withdrawal, administration of hyoscine butylbromide or glucagon reduces peristalsis in the SB, optimizing visualization. Sodium picosulfate is given 1 day before examination; no other specific preparation is required for an oral approach. For retrograde enteroscopy from an anal approach, bowel cleansing was performed as for colonoscopy. Therapeutic procedures were performed through a working channel. Argon plasma beam-directed coagulation (APC; 1.2 l/min/max, 35 W; ERBE 300 series, Tubigen, Germany) was used in the subset of cases in which bleeding sources were identified.

Classification of endoscopic abnormalities in the small bowel in patients with portal hypertension

The data collected for each patient included age, gender, etiology of cirrhosis, Child-Pugh class, and gastrointestinal tract abnormalities identified by upper and lower endoscopy. We evaluated each patient for any evidence of varices in the esophagus, stomach, colon, or anorectum and for changes indicative of PHG or portal hypertensive colopathy (PHC). PHG was diagnosed following recognition of elementary lesions, such as a mosaic-like pattern, red-point lesions, cherry-red spots, or black-brown spots.[7] The colonic abnormalities seen endoscopically in PHC are similar to those seen in PHG, including diffuse hyperemia and edema resembling chronic colitis, angiodysplasia-like lesions, patchy hyperemic lesions, a severe colitis-like appearance, and spontaneous bleeding from the mucosa.[7,8] The abnormal endoscopic findings seen by DBE in patients with PH, which were definitive for PHE, were divided into two categories: villous abnormalities and vascular lesions. Villous abnormalities included edema, atrophy, and reddening

of villi. Angiodysplasia-like lesions, dilated/proliferated vessels, and varices comprised the vascular lesions. A finding of each of these lesions was scored as a point, to provide a final score with a maximum of six points. The angiodysplasia-like lesions were subclassified as red spots, vascular spiders, and lymphoid follicles with dilated vessels. Dilated/proliferated vessels were further subclassified into tree-like dilated vessels and coil-like fine vessels.

Diagnosis for source of occult gastrointestinal bleeding

Patients with positive fecal occult blood and/or iron deficiency anemia with negative upper endoscopy and colonoscopy were defined as having OGIB.[16] Before DBE, all patients with OGIB were evaluated within 1 month by upper endoscopy and colonoscopy. For patients with OGIB, endoscopic findings by DBE were classified as positive (diagnostic), suspicious, or negative (failed).[17] Findings were classified as positive if the observed findings could explain the signs/symptoms of the patient. These findings typically helped to determine further management or were confirmed by other modalities. Findings were considered suspicious if an observed finding failed to explain completely the signs/symptoms of the patient, necessitating further investigation to evaluate its clinical relevance. When no abnormality could be detected despite clinical indication of an existing lesion, findings were considered to be negative.

Clinical characteristics and endoscopic abnormalities that we defined as portal hypertensive enteropathy

We compared the clinical characteristics and prevalence of PHE-defining endoscopic abnormalities between patients with PH and those without chronic liver disease (control patients). We also calculated the number of abnormal findings in 13 patients with liver cirrhosis. We compared patients with four or more findings of PHE to those with fewer than four findings to determine if this calculated score correlated with the severity of liver disease, the presence of esophagogastric varices (EGV), PHG, PHC, or other clinical characteristics.

Statistical analysis

All statistical analyses were performed using Statview J-4.5 software (Abacus Concepts, Berkeley, CA, USA) or SPSS (Chicago, IL, USA). Data are shown as the means (± SD). Comparisons were performed using the Mann–Whitney U test, Fisher's exact test, or the χ^2 test, as appropriate. Differences were considered statistically significant when the P value was less than 0.05.

Results

Prevalence and endoscopic findings of portal hypertensive enteropathy

The characteristics of 15 patients with PH and 49 control patients evaluated by DBE are detailed in Table 1. The average age of patients and the frequency of OGIB as an indication for DBE in patients with PH were higher than those for control patients. Several laboratory values, including platelet counts, serum albumin, and bilirubin, were also significantly different between the two groups. In contrast, the levels of serum alanine transferase (ALT) did not differ between the two groups.

Fourteen of the 15 patients exhibited villous abnormalities, including edema (Fig. 1A), atrophy (Fig. 1B), or reddening (Fig. 1C) of villi. All 15 patients with PH displayed vascular lesions, including angiodysplasia-like abnormalities [Fig. 2A-(1), -(2), or -(3)], dilated/proliferating vessels [Fig. 2B-(1) or -(2)], or varices (Fig. 2C). Thus, although endoscopic abnormalities were observed in the SB of all patients with PH, there were no villous abnormalities or vascular lesions in control patients.

The association between portal hypertensive enteropathy-defining abnormal findings and clinical characteristics

The etiology of the PH was liver cirrhosis in 13 patients and EHO without cirrhosis in 2 patients (see Table 1). By DBE, 14 of the 15 patients with PH exhibited villous abnormalities, while vascular lesions were observed in all (Table 2). We sought to evaluate the correlation between these endoscopic abnormalities, which we considered to be associated with PH, and clinical parameters in the 13 patients with PH caused by cirrhosis. We compared patients with four or more positive findings of PHE to those with fewer than four positive findings to determine if PHE was associated with liver disease severity or with specific endoscopic findings of the upper or lower gastrointestinal tract (Table 3). PHE was unrelated to patient age, the presence of PHG or PHC, or severity of EGV. In addition, PHE did not correlate with any laboratory findings. The frequency of ascites in patients with high PHE scores, however, was significantly higher than that seen in those with low scores ($P = 0.02$).

Diagnostic findings in small bowel by double balloon endoscopy

We assessed the number of PHE-determining findings, diagnostic rates of small intestinal bleeding, and complications of DBE (Table 4). The frequency of

Table 1. Demographic, clinical, and endoscopic parameters of patients

Parameter	Patients with portal hypertension	Control patients	*P* value*
Patients (procedures)	15 (24)	49 (90)	–
Age (mean ± SD; years)	65.8 ± 8.7	48.8 ± 21.1	<0.01
Sex (male/female)	12/3	39/10	>0.99
Indications for double balloon endoscopy			
OGIB/ileus/other	13/1/1	14/8/27	<0.001
Etiology of portal hypertension			
Cirrhosis	13	0	–
Etiology (HBV/HCV/alcohol/unknown)	1/5/4/3	—	–
Child-Pugh class (A/B/C)	1/12/0	—	–
Extrahepatic portal vein obstruction	2	0	–
Presence of esophagogastric varices	9	0	–
Presence of portal hypertensive gastropathy	10	0	–
Presence of portal hypertensive colopathy	9	0	–
Presence of anorectal varices	8	0	–
Ascites	5	0	–
Platelet ($\times 10^4/mm^3$)	12.7 ± 11.7	24.8 ± 8.9	<0.001
Serum albumin (g/dl)	3.0 ± 0.6	3.9 ± 0.6	<0.001
Total bilirubin (mg/dl)	0.9 ± 0.4	0.6 ± 0.4	<0.01
ALT (IU/l)	28.3 ± 18.9	28.8 ± 22.1	0.91

OGIB, obscure gastrointestinal bleeding
* Comparisons were performed with the Mann–Whitney *U* test, Fisher's exact test, or the χ^2 test, as appropriate

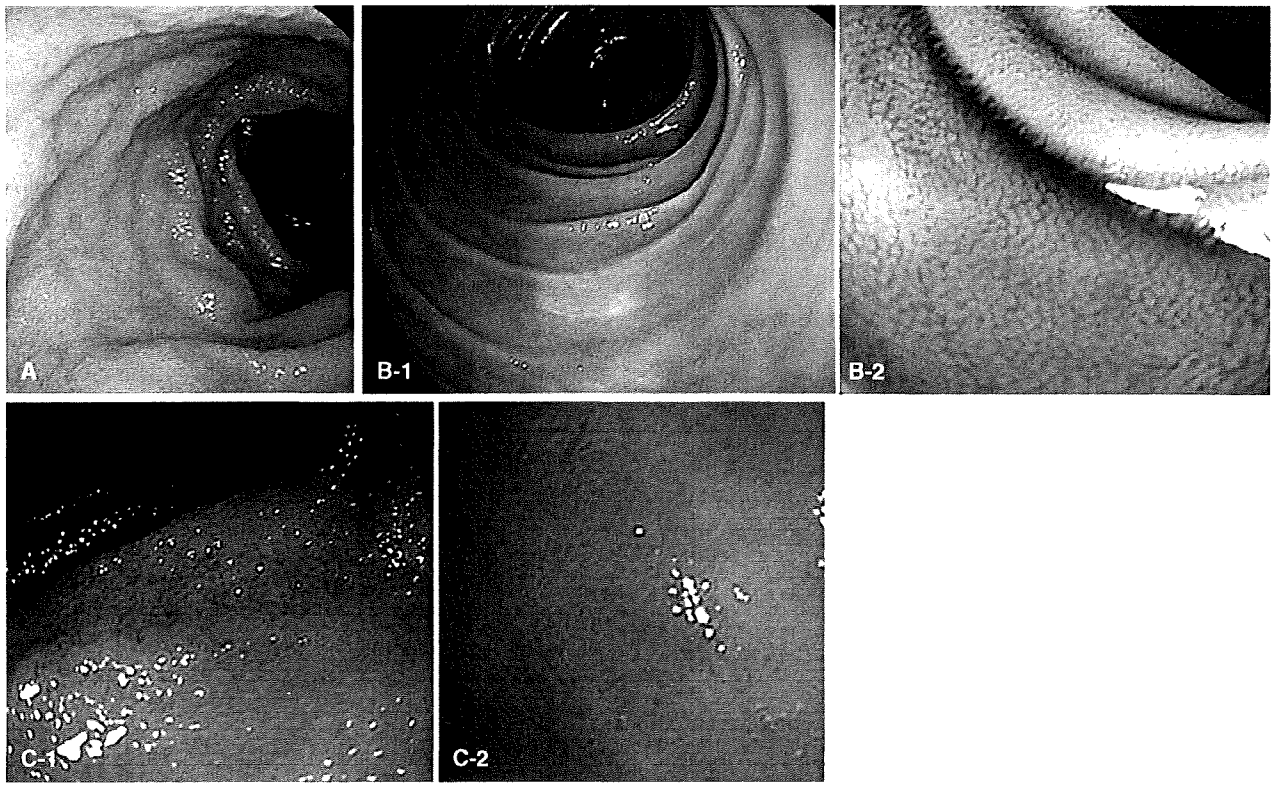

Fig. 1. Three different types of villous abnormalities were seen in the small bowel of patients with portal hypertension: edema of villi (**A**); atrophy of villi (**B-1**, **B-2**); reddening of villi (**C-1**, **C-2**)

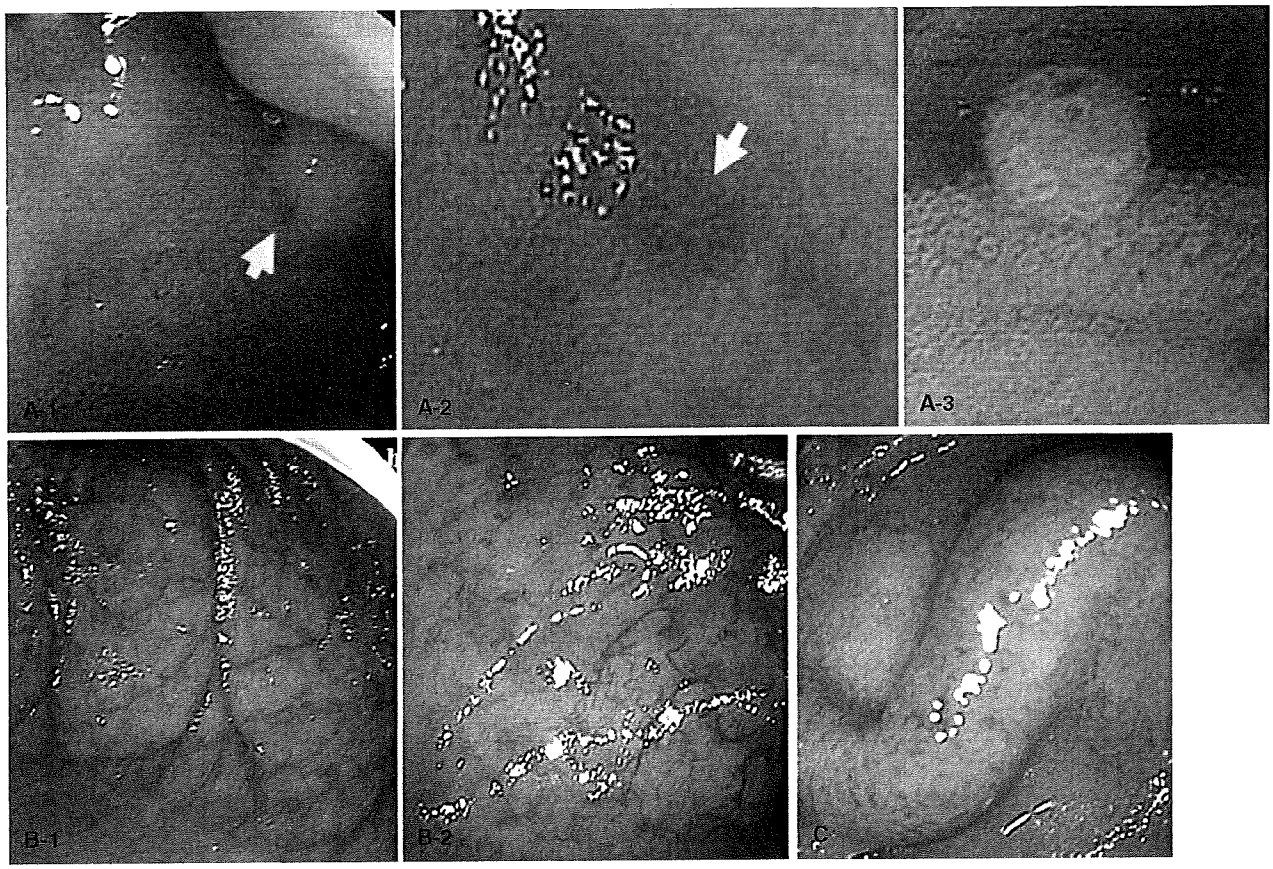

Fig. 2. Three different types of vascular lesions, including angiodysplasia-like lesions (**A**), dilated/proliferated vessels (**B**), and varices (**C**), were seen in the small bowel of patients with portal hypertension. **A-1**, red spots (*arrow*); **A-2**, vascular spiders (*arrow*); **A-3**, lymphoid follicles with dilated vessels; **B-1**, tree-like dilated vessels; **B-2**, coil-like fine vessels; **C**, varices

Table 2. Classification and frequency of the endoscopic findings of portal hypertensive enteropathy

Endoscopic findings	$n = 15$ (%)
1. Villous abnormalities	14 (93%)
A. Edema of villi	11 (73%)
B. Atrophy of villi	6 (40%)
C. Reddening of villi	7 (47%)
2. Vascular lesions	15 (100%)
A. Angiodysplasia-like lesions	10 (67%)
(1) Red spots	9 (60%)
(2) Vascular spiders	2 (13%)
(3) Lymphoid follicles with dilated vessels	2 (13%)
B. Dilated/proliferated vessels	14 (93%)
(1) Tree-like dilated vessels	12 (80%)
(2) Coil-like fine vessels	2 (13%)
C. Varices	1 (7%)

endoscopic abnormalities in the SB, which were diagnostic of PHE, was significantly higher in patients with PH than that seen in control patients. Definitive or suspicious bleeding sources, however, were observed in 69% (9/13) of patients with PH and 50% (7/14) of control patients; this diagnostic rate was not significantly different between the two groups of patients with OGIB. Bleeding sources identified included angiodysplasia-like lesions in the SB in 5 patients with PH, in whom 3 were definitive and 2 were suspicious. We identified

Table 3. The association of the number of positive portal hypertensive enteropathy-associated findings in patients with cirrhosis and other clinical features

	Number of positive findings of portal hypertensive enteropathy		
	4–6	0–3	*P* value*
Patients	6	7	
Age (mean ± SD; years)	71.0 ± 3.8	66.3 ± 7.1	0.28
Sex (male/female)	5/1	6/1	>0.99
Etiology (HBV/HCV/alcohol/uknown)	0/2/2/2	1/3/2/1	0.69
Child-Pugh class (A/B/C)	1/5/0	0/7/0	0.46
Presence of esophagogastric varices	2 (33%)	5 (71%)	0.29
Presence of portal hypertensive gastropathy	5 (83%)	5 (71%)	>0.99
Presence of portal hypertensive colopathy	2 (33%)	6 (86%)	0.10
Presence of anorectal varices	2 (33%)	4 (57%)	0.59
Presence of ascites	4 (67%)	0 (0%)	0.02
Prothrombin time (%)	72.7 ± 17.0	65.3 ± 15.3	0.32
Platelet ($\times 10^4/mm^3$)	11.1 ± 8.5	7.0 ± 2.7	0.32
Serum albumin (g/dl)	2.8 ± 0.5	2.8 ± 0.3	0.67
Total bilirubin (mg/dl)	0.9 ± 0.5	0.8 ± 0.4	>0.99
Alanine aminotransferase (IU/1)	34.3 ± 23.4	26.4 ± 17.2	0.62
Complication associated with double balloon endoscopy	3 (50%)	3 (43%)	>0.99

* Comparisons were performed with the Mann–Whitney *U* test, Fisher's exact test, or the χ^2 test, as appropriate

Table 4. Comparison of patients with and without portal hypertension

	Patients with portal hypertension	Control patients	*P* value*
Patients (procedures)	15 (24)	49 (90)	
Presence of portal hypertensive enteropathy	15 (100%)	0 (0%)	<0.001
Diagnostic rates of small intestinal bleeding (positive/suspicious/negative)[a]	7/2/4	5/2/7	0.57
Complications	7/24 (29%)	2/90 (2%)	<0.001

[a] In patients with obscure gastrointestinal bleeding
* Comparisons were performed with the Mann–Whitney *U* test, Fisher's exact test, or the χ^2 test, as appropriate

jejunal varices in 1 patient, a SB ulcer in 1, a SB diverticulum in 1, and duodenal varices in 1. Of these abnormalities, the varices are likely associated with PH, whereas the SB ulcer and diverticulum may not be associated. The duodenal varices were excluded from the findings of PHE in this study (see Table 2). The bleeding sources in control patients included a duodenal ulcer in 1 patient, SB ulcers in 5 patients, and SB angiodysplasia in 1 patient.

Treatment in small bowel by double balloon endoscopy or complications associated with its use

Endoscopic treatments using DBE were performed in three patients with endoscopic abnormalities in the SB. One patient received APC treatment for angiodysplasia-like lesions, one patient was treated with clipping and APC for angiodysplasia-like lesions, and a third was treated with clipping of lymphoid follicles with dilated vessels. Seven of 24 or 2 of 90 procedures in patients with or without PH, respectively, developed fevers (temperatures higher than 37.5°C) in the first 24 h after procedure (Table 4). The difference in frequency was statistically significant between the two groups ($P < 0.001$). Although aspiration pneumonia was suspected to occur in one of the patients with PH, the causes of fever in the other patients were not clear. Antibiotic therapy, however, was not necessary except for the one patient with pneumonia. There were no severe complications, excluding pneumonia, in either group with or without endoscopic treatment.

Discussion

Currently, there is no classification system with which to grade the severity of endoscopic abnormalities in cirrhotic patients with PHE. De Palma et al. proposed that PHE lesions be classified into two categories, mucosal inflammatory-like abnormalities (edema, ery-

thema, granularity, and friability) and vascular lesions (cherry-red spots, telangiectasias, angiodysplasia-like lesions, and varices).[15] Rana et al. defined the diagnosis of ileopathy as the presence of lesions similar in appearance to spider angioma, diffuse or patchy regions of hyperemia, cherry-red spots, and prominent veins.[8] Although we did not investigate the histology of mucosal lesions in this study, we classified the endoscopic findings in the SB of patients with PH into two categories, villous abnormalities and vascular lesions. We also subclassified the findings in these two categories and calculated the total number of positive findings in these six subcategories (see Table 2). Although it is unclear if these findings were specific for PHE, our observations indicated their prominence in patients with PH. Further studies are required to improve the classification and scoring system proposed in this report.

De Palma et al. reported that 68% of cirrhotic patients with PH were found to have PHE evaluated by VCE.[15] Endoscopic abnormalities in the ileum were noted in 13 of 38 patients examined (34%).[8] In contrast, we report that all patients with PH were observed to have at least one abnormal finding in the SB considered to be associated with PH. The high percentage likely correlates with the finding that 13 of the 15 patients with PH had evidence of OGIB with negative findings on upper and lower endoscopies. Previously reported prevalences of PHC vary from 37% to 70%, likely because of the heterogeneity of patients.[18–20] The limitations of studies, including our report, examining patients with PHE are the small number of patients with PH. In addition, although it would be better to compare the results of VCE and DBE in this study, we unfortunately did not have data for VCE. Further studies with larger numbers of patients will be needed to determine accurately the frequency of PHE as assessed by DBE and VCE.

One of the main causes of death for patients with PH is gastrointestinal bleeding. Portal hypertensive gastrointestinal vasculopathy, which can occur throughout the esophagus, stomach, and colon, is typically the origin of bleeding in patients with PH. PHE secondary to PH, especially the presence of varices in the SB, may also be a common source of bleeding.[15,21,22] There are no data, however, detailing that abnormal findings in the SB has any impact on the clinical treatment of PH, with the exception of cases with OGIB. Prospective observation should reveal the impact of PHE in cases without OGIB.

De Palma et al. initially demonstrated that 25 of 37 (68%) patients with cirrhosis and PH also had PHE and that the prevalence of PHE increased with worsening Child-Pugh class; 32% of patients with PHE were Child-Pugh class C, while only 9% of those without PHE were Child-Pugh class C.[15] PHE was also significantly associated with 2+ or larger esophageal varices, PHG, and PHC. Repici et al., however, found no correlation between the presence of PHE and Child-Pugh score, the size of varices, or the presence of PHG or PHC.[23] In this study, all patients with PH had evidence of PHE. We attempted to correlate the number of positive PHE-associated findings with laboratory findings. Our comparison of cirrhosis patients with at least four positive findings of PHE with those exhibiting fewer than four such findings demonstrated that laboratory findings, such as serum albumin, were not significantly different between the two groups. In addition, neither esophagogastric varices, PHG, PHC, nor anorectal varices correlated with PHE. The number of positive findings of PH, however, was associated with presence of ascites (see Table 3). The mechanism by which ascites develops in patients with cirrhosis is multifactorial, with the largest contribution from severe sinusoidal PH. Thus, the pathophysiology supports the association of PH with our PHE scoring system. Large prospective studies are required to evaluate the clinical significance of SB mucosal changes in patients with PH in the presence or absence of cirrhosis.

The complication rate of DBE is approximately 1%.[24,25] While perforation is a rare complication associated with DBE, no severe complications occurred in this study. Our study indicates, however, that postprocedure fever induced by DBE was more common in patients with PH than in those without PH. This complication may be associated with bacterial translocation, which typically occurs in patients with liver cirrhosis.[26,27] Comparison of cirrhotic patients with and without complications did not reveal any association of the incidence of complications with the severity of liver damage. In addition, the median time of the DBE procedure did not correlate with the incidence of complications (data not shown). In contrast, no complications were observed in the two EHO patients with PH. As the small number of EHO patients is insufficient to reveal any association, further examination will be required.

As DBE is contraindicated in patients with esophageal varices, because of an increased risk of rupture, VCE is the first diagnostic step for PHE in patients with PH. Performing DBE, however, is reasonable to examine the SB in patients with PH, as it provides a high diagnostic yield and the capability to perform therapeutic interventions. The small vascular lesions characteristic of PHE may only rarely be the sources of OGIB in patients with PH. After the bleeding has stopped, it is difficult to identify these sources of bleeding in patients with PHE as these vascular lesions are tiny. In cases in which bleeding is suspected in the SB, the diagnostic rate identifying the source of bleeding was higher in cases that underwent DBE within three days of the bleeding episode than in those who were evaluated after 1 to 2 weeks (unpublished data). Thus,