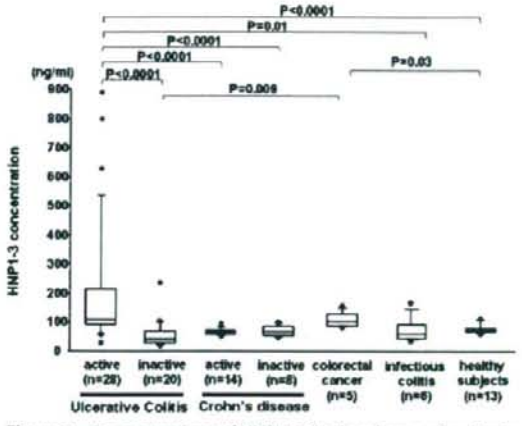

Figure 3. Concentrations of HNP 1–3 in the plasma of patients with UC, CD, colorectal cancer, infectious colitis, and healthy controls. Boxes indicate the median ± 25th percentile. The lower bar indicates the 10th percentile and the upper bar indicates the 90th percentile.

protein peaks, which were larger in the spectra for sera of UC patients, corresponded to HNP 1–3.

Concentrations of HNP 1–3 in Plasma

It was not possible to determine the individual concentrations of HNP 1, 2, or 3 using commercially available ELISA kits; therefore, we evaluated the total concentration of HNP 1, 2, and 3 in plasma. We found that there was a clear correlation between the serum HNP 1–3 peak intensities determined using the SELDI system and the plasma HNP 1–3 concentration measured using ELISAs in 11 patients with UC and 7 normal controls ($r = 0.68$, $P < 0.01$). We then determined the plasma concentrations of HNP 1–3 in 48 UC patients, 22 CD patients (Table 1), 5 CRC patients, 6 infectious colitis patients, and 13 healthy controls (Fig. 3). The plasma concentrations of HNP 1–3 were significantly higher in patients with active UC (203.1 ± 215.5 ng/mL) than in patients with inactive UC (58.3 ± 49.5 ng/mL), CD (active; 65.5 ± 11.2 ng/mL, inactive; 70.4 ± 20.0 ng/mL), infectious colitis (72.2 ± 16.5 ng/mL), or the healthy controls (77.5 ± 16.4 ng/mL). In addition, HNP 1–3 concentrations in patients with active UC tended to be higher in patients with CRC at Duke's stage A (100.8 ± 27.6 ng/mL), but not significantly. HNP 1–3 concentrations in CRC patients were also higher than those in patients with inactive UC and healthy controls.

Expression of HNP 1–3 in Intestinal Tissue

We examined the localization of HNP 1–3 in normal tissues and those from patients with active-phase CD or UC using immunohistochemistry. The colonic mucosa, lamina propria, muscle layer, and crypt abscesses of patients with active UC exhibited strong staining with anti-HNP 1–3 antibodies (Fig. 4). These sections contained a number of infiltrating neutrophils (Fig. 4B,C), which may provide a source of the secreted HNP 1–3 near the colonic epithelium. Positive staining for neutrophils, however, was seen in the blood vessels of both normal and abnormal colon tissues. In addition, small numbers of neutrophils with positive staining were seen in submucosal tissue of patients with CD (Fig. 4D). Epithelial cells in colon samples from patients with inflamed CD or from normal healthy subjects did not exhibit staining with anti-HNP 1–3 antibodies (Fig. 4D,E).

HNP 1–3 as a Biomarker in UC Patients

We investigated the association between the HNP 1–3 concentration and the clinical course of UC. We determined the HNP 1–3 concentrations in pairs of plasma samples from 15 patients with active UC obtained before and after induction therapy with corticosteroids (Table 3). Eight UC patients in the responder group were successfully treated by induction therapy. The elevated HNP 1–3 levels of UC patients in the responder group were reduced after induction therapy (Fig. 5). In contrast, 7 patients in the nonresponder group, 2 of whom had a total colectomy and 5 who quickly relapsed, were not effectively treated. The HNP 1–3 levels of patients in the nonresponder group before treatment were lower than those in the responder group and were not changed after treatment (Fig. 5). Additionally, although plasma HNP 1–3 levels (means ± SD) of responder active UC patients (273.0 ± 224.8 ng/mL) were higher than those with active CD (65.5 ± 11.2 ng/mL) ($P < 0.001$), those with nonresponder active UC (84.6 ± 26.5 ng/mL) were similar to those with active CD. These results indicate that patients with active UC and low HNP 1–3 levels do not respond well to treatment.

We evaluated the relationship between the HNP 1–3 levels and the clinical activity of UC. There was a significant correlation between the HNP 1–3 levels and the UCDAI score or the white blood cell count (WBC) of UC patients ($r = 0.54$, $P < 0.01$; $r = 0.55$, $P < 0.01$, respectively), although no correlation between the HNP 1–3 levels and the C-reactive protein (CRP) levels was noted ($r = 0.24$). In addition, ROC analysis was performed to estimate the efficiency of induction therapy for patients with active-phase UC; we calculated the sensitivity and specificity of HNP 1–3 levels for discriminating responder UC patients from nonresponders. We obtained a sensitivity of 89% and a specificity of 80% using a cutoff value of 100 ng/mL HNP 1–3; the ROC AUC was 0.89 between the responder and nonresponder groups of UC patients. For evaluations of the activity of UC, we compared such inflammatory markers as the CRP level and the WBC to the HNP 1–3 level in patients with UC. ROC AUC of the CRP level and WBC were 0.76 and 0.56, respectively. Thus,

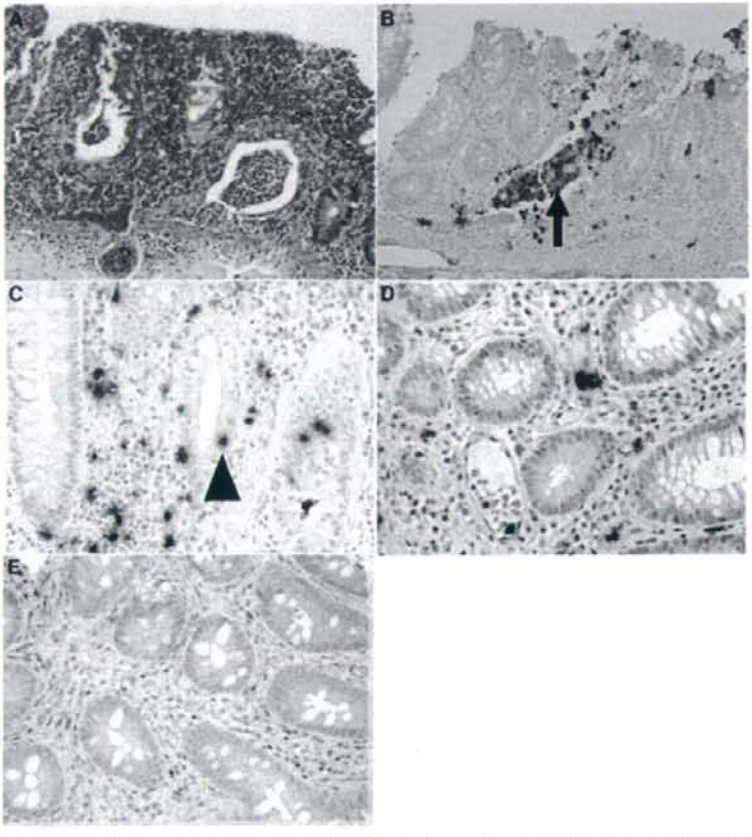

Figure 4. Expression of HNP 1–3 in the tissue of patients with active UC or CD and in normal colon tissue. (A) HE staining of colon tissues from patients with UC. (B,C) Immunohistochemical staining demonstrated extensive HNP 1–3 expression in the colon tissues of patients with UC. Many HNP 1–3-positive cells were observed in the crypt abscesses (B: arrow) and in neutrophils that had migrated into the epithelial layers (C: arrowhead). In addition, an ulcer lesion observed in the colon sample stained positive for HNP 1–3. (D,E) Although small numbers of neutrophils in the blood vessels and submucosal tissues were positive for HNP 1–3, epithelial cells in colon samples from patients with inflamed CD or normal subjects were not positive for HNP 1–3. Original magnification: 100× (A,B) and 200× (C–E).

the level of HNP 1–3 had a high discriminatory power for estimating the efficacy of treatment in patients with UC.

DISCUSSION

We identified 27 proteins that showed significant differences in the serum protein profiles of patients with UC compared with those of healthy controls using SELDI-TOF/MS analysis. Of these proteins, 3 signals around 3400 m/z were confirmed to correspond to HNP 1, 2, and 3. In addition, we observed an increase in HNP 1–3 plasma levels in patients with active-phase UC compared with that seen in patients with remission-phase UC or CD; these levels were higher in the plasma of UC patients who showed better therapeutic outcomes than in samples from nonresponder patients.

Several studies have suggested that the development of IBD requires the interaction of genetic factors with both specific luminal bacterial antigens and environmental triggers that break the mucosal barrier.[16-18] Although the principle treatment for IBD is the suppression of inflammation, treatment strategies for the 2 diseases, UC and CD, are somewhat different. Whereas these differences may address the different biomarkers of the 2 conditions, a specific biomarker for IBD remains unknown. To discover a biomarker of UC, we

TABLE 3. Characteristics of Patients with Active UC in the Responder Group and Nonresponder Group

	Responder	Nonresponder	*P*-value
Number	8	7	
Gender (M/F)	5/3	5/2	0.7
Age (yr)	33.5 ± 13.8 [14–50]	42.3 ± 19.8 [16–68]	0.4
CRP (mg/dl)	1.7 ± 1.7	3.3 ± 4.5	0.4
WBC (cells/ul)	12714 ± 4604	7657 ± 3423	*0.04*
Platelets × 10^4/ul	40.4 ± 7.4	36.2 ± 11.1	0.3
HNP 1–3 (ng/ml)	273.0 ± 224.8	84.6 ± 26.5	*0.002*
Type of UC			
Pancolitis/Left-side colitis	7/1	5/2	0.6
UCDAI score	9.4 ± 4.6	8.6 ± 1.9	0.7
Duration	6.7 ± 6.5 [1–19]	5.7 ± 5.1 [2–16]	0.8

Data are shown as the means ± SD [ranges]. Statistical significance was determined using a Mann-Whitney *U*-test or Fisher's exact test, as appropriate. UC, ulcerative colitis; UCDAI, Ulcerative Colitis Disease Activity Index.

employed ProteinChip technology. The likelihood of finding reliable tumor markers by analyzing tissue may be higher than in analyses of serum[12]; malignant cells may produce proteins that are useful biomarkers. In nonmalignant diseases, such as UC, protein profiling of serum or plasma may be more informative than that of tissue samples. Additionally, fluid samples, such as serum, are easier to obtain than tissue samples. Thus, we used serum samples to identify new biomarkers for UC.

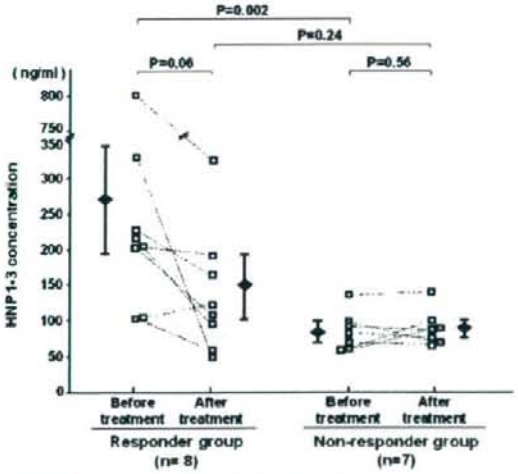

Figure 5. HNP 1–3 levels in the responder and nonresponder groups before treatment predicted therapeutic outcomes in UC patients; changes in the HNP 1–3 levels in UC patients in response to treatment are presented. The mean concentration of HNP 1–3 in the responder group before treatment was significantly higher than that seen in the nonresponder group, which indicates that HNP 1–3 levels may be an effective predictor of therapeutic outcomes. HNP 1–3 levels tended to decrease after treatment in the responder group, whereas no changes were observed for the nonresponder group. Patients whose plasma was not obtained after treatment were excluded from analysis.

Defensins are one of the most extensive peptide families of naturally occurring antibiotics. These peptides exhibit microbicidal activities against Gram-positive and Gram-negative bacteria, mycobacteria, fungi, and certain enveloped viruses. HNP 1–3 are part of the α-defensin family and components of the innate immune response. HNP 1–3 are synthesized by neutrophil precursor cells and released at inflammatory sites by mature circulating neutrophils.[9,19] The expression of HNP 1–3 has been observed in epithelial cells of the ileum and colon in patients with active UC or CD.[20] Whether neutrophils within inflamed colon tissue express HNP 1–3 in IBDs, however, is not known. In this study, we demonstrated that the colon mucosal tissue of patients with active UC or CD displayed minimal immunoreactivity for HNP 1–3, whereas the infiltrating neutrophils were stained strongly. These results indicate that HNP 1–3 were secreted from neutrophils, leading to increased plasma levels in patients with UC. High concentrations of HNP 1–3 can be cytotoxic for epithelial cells due to cytolysis and can induce apical conduction in Cl^- secretory epithelia.[21,22] Thus, whereas HNP 1–3 have antibacterial activities in the early phase of UC, they also may injure the colon if they are overexpressed by infiltrating neutrophils. High concentrations of HNP 1–3 may adversely affect colon tissues in UC patients, potentially contributing to diarrhea.[23] HNP 1–3 are secreted from the azurophilic granules of neutrophils following stimulation with IL-8.[24] Epithelial-derived IL-8 is thought to mediate neutrophil migration and infiltration during the inflammatory process of UC.[25,26] IL-8 mRNA levels are

significantly higher in UC patients with crypt abscesses.[27] Although HNP 1–3 have been reported to be expressed by surface enterocytes in the mucosa of patients with active IBD,[28] we observed only minimal staining of the colonic surface mucosa from patients with active UC using anti-HNP 1–3 antibodies. Moreover, Caco-2 and HT-29 cells, 2 colon epithelial cell lines, do not express HNP 1–3 (data not shown). Therefore, we hypothesized that HNP 1–3 are expressed by neutrophils following stimulation with IL-8, which suggested a correlation between the IL-8 and HNP 1–3 levels. We did not, however, observe a correlation between the IL-8 and HNP 1–3 levels in the plasma from active UC patients, and there was no association between the disease activity score and plasma IL-8 concentrations (data not shown). These results indicate that HNP 1–3 expression may be affected by other factors and HNP 1–3 values appear to be more useful to measure clinical UC disease activity than IL-8 levels.

Neutrophils are critical cellular mediators of the inflammation observed in UC. Neutrophils increase in number and display augmented activation during active-phase UC, but not inactive-phase UC.[28] Neutrophils extensively infiltrate colon tissue in patients with UC, and can be detected in the inflamed mucosa during even the early stages of inflammation.[29,30] Platelets are also important in the pathophysiology of UC.[31] Cytapheresis therapy (including LCAP) in combination with steroid therapy can be an effective treatment option for patients with active UC.[32] LCAP may remove and modulate both leukocytes and platelets, thereby altering the expression of proinflammatory cytokines.[33,34] The effect of LCAP on HNP 1–3 levels, however, has not been examined, and further studies are needed to determine whether HNP 1–3 levels decrease in response to LCAP. In addition, we showed that HNP 1–3 levels in the plasma were higher in patients with active UC than in those with infectious colitis, and HNP 1–3 levels were similar between patients with infectious colitis and healthy controls. In contrast, it was reported that HNP 1–3 levels in patients with severe infectious diseases, such as sepsis, were higher than those in healthy controls.[35] The disease severity of the enrolled patients with infectious colitis in this study may have affected our results. Cytapheresis therapy, however, may not be effective for severe infectious diseases, including infectious colitis, and high concentrations of HNP 1–3 in patients with active UC may be associated with disease characteristics. Further examination, including cases of infectious colitis with sepsis, will be necessary.

As previously reported, we found that several inflammatory makers, including the CRP level, WBC, and platelet count, decreased after treatment. Changes in these inflammatory markers did not predict the treatment outcome of patients with UC, whereas plasma levels of HNP 1–3 correlated with UC disease activity and predicted the therapeutic outcome. There were no correlations between plasma HNP 1–3 levels and inflammatory markers, such as platelet counts and CRP levels. These results may suggest that high levels of HNP 1–3 independently indicate the activity of disease and the feasible treatment outcome in patients with UC. However, there is a limitation in the use of HNP 1–3 measurement as a biomarker; low levels of HNP 1–3 in colitis patients did not diagnose whether they had nonresponder UC or active CD. Therefore, low levels of HNP 1–3 in colitis patients should be assessed by clinical symptoms, stool for bacterial examination, and endoscopic and radiographic examination of the gastrointestinal tract for diagnosis. Other proteins and peptides that were detected by SELDI/TOF-MS in this study are now under investigation and may serve as additional biomarkers for the assessment of IBD, especially in nonresponder UC patients.

The levels of HNP 1–3 in tumor tissue and serum were reported to increase in patients with CRC.[12] It was also reported that plasma HNP 1–3 concentrations determined using ELISA increased in Duke's stages C and D, but not in A or B compared to healthy controls.[14] In contrast, we showed that HNP 1–3 concentrations in CRC patients at Duke's stage A were higher than those seen in patients with inactive UC and healthy controls. Although HNP 1–3 concentrations in CRC patients at Duke's stage A seem to be similar between our study and a previous study[14] (100.8 ± 27.6 versus 105.4± 80.6 ng/mL, respectively), the concentrations in the healthy controls were different between the 2 studies (77.5 ±16.5 versus 96.6 ± 36.2 ng/mL). In addition, Albrethsen et al[14] mentioned that in addition to Duke's C and D, HNP 1–3 expression in CRC tissues at Duke's A and B was higher than in normal tissue by SELDI ProteinChip. It is controversial whether the increased HNP 1–3 in tumors is localized to cancer cells or to neutrophilic leukocytes. There is the possibility that the plasma HNP 1–3 levels will increase in patients with CRC at Duke's stage A and that HNP 1–3 concentration is a potential marker for the assessment of CRC patients with advanced disease.[12,14] In addition, these results indicate that HNP 1–3 levels may not be able to distinguish between active UC and colon cancer. In the clinical setting, however, UC can typically be distinguished from colon cancer by various clinical features, such as diarrhea, fever, and colonoscopic findings. On the other hand, colon cancer commonly occurs in patients with UC, especially those who have suffered from the disease for a long period of time; such colon cancers are difficult to detect using colonoscopy. HNP 1–3 levels may help to signal the occurrence of colon cancer in UC patients when high concentrations of HNP 1–3 are detected in the absence of active colitis; these patients should be extensively examined, including total colonoscopy and random biopsies.

In conclusion, we used SELDI-TOF/MS to perform serum protein profiling and determined that HNP 1–3 levels increase in patients with active-phase of UC. We also con-

firmed that HNP 1–3 are predictive markers for UC treatment outcomes. Although these markers may not distinguish UC from CRC, HNP 1–3 are useful markers for the differential diagnosis of patients with IBD.

ACKNOWLEDGMENT

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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

Yuka Takahama[1,2], Hirofumi Uto[3], Shuji Kanmura[3], Makoto Oketani[3], Akio Ido[3], Kazunori Kusumoto[4], Satoru Hasuike[4], Kenji Nagata[4], Katsuhiro Hayashi[5], Sherri Stuver[6,7], Akihiko Okayama[2], and Hirohito Tsubouchi[3]

[1]Miyazaki Prefectural Industrial Support Foundation, Miyazaki, Japan
[2]Department of Rheumatology, Infectious Diseases and Laboratory Medicine, University of Miyazaki, Kiyotake, Japan
[3]Department of Digestive and Life-style Related Disease, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan
[4]Gastroenterology and Hematology, Faculty of Medicine, University of Miyazaki, Kiyotake, Japan
[5]Center for Medical Education, Faculty of Medicine, University of Miyazaki, Kiyotake, Japan
[6]Department of Epidemiology, Boston University School of Public Health, Boston, MA, USA
[7]Department of Epidemiology, Harvard School of Public Health, Boston, MA, USA

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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Reprint requests to: H. Uto

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 (×10⁴)	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.

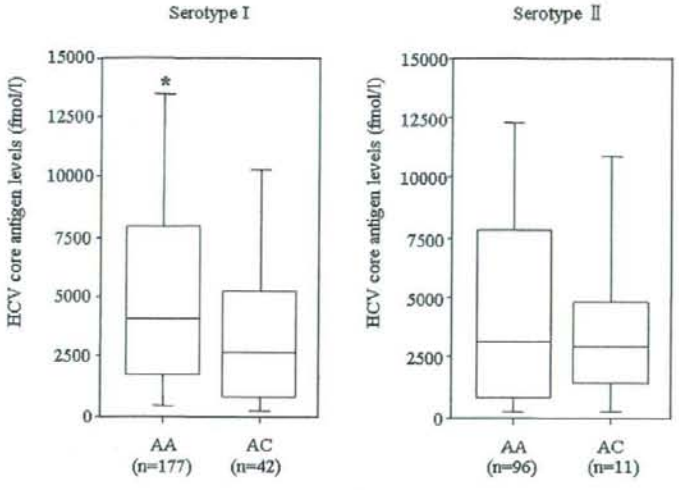

Fig. 1. The association between the K121Q genotype in *ENPP1* and the hepatitis C viral (HCV) load. The box-and-whisker plot shows the HCV core antigen level in the HCV carrier group according to the genotypes. The *boxes* indicate the 25th, 50th (median), and 75th percentiles. The *whiskers* indicate 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

Mayumi Kodama[1,2], Hirofumi Uto[3], Masatsugu Numata[4], Takeshi Hori[1], Takanobu Murayama[1], Fumisato Sasaki[3], Naoko Tsubouchi[3], Akio Ido[3], Kazuya Shimoda[2], and Hirohito Tsubouchi[3,4]

[1]Miyazaki Medical Center Hospital, Center for Digestive and Liver Diseases, Miyazaki, Japan
[2]Department of Gastroenterology and Hematology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan
[3]Department of Digestive and Life-style Related Disease, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan
[4]Department of Experimental Therapeutics, Translational Research Center, Kyoto University Hospital, Kyoto, Japan

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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Reprint requests to: H. Uto

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

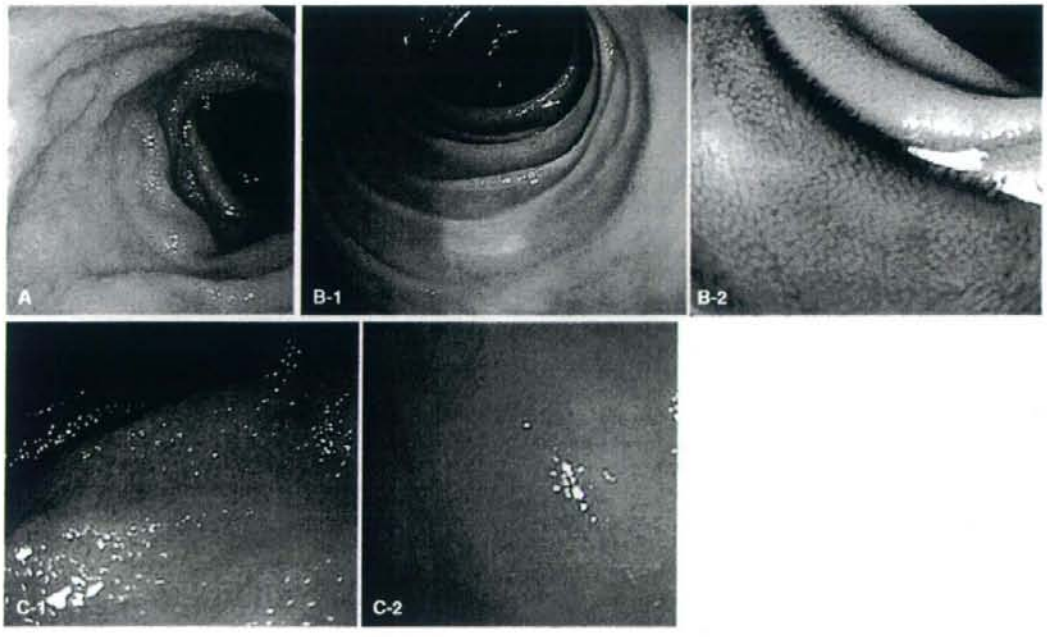

Fig. 1. Three different types of villous abnormalities were seen in the small bowel of patients with portal hypertension: edema of villi (**A**); atrophy of villi (**B-1**, **B-2**); reddening of villi (**C-1**, **C-2**)

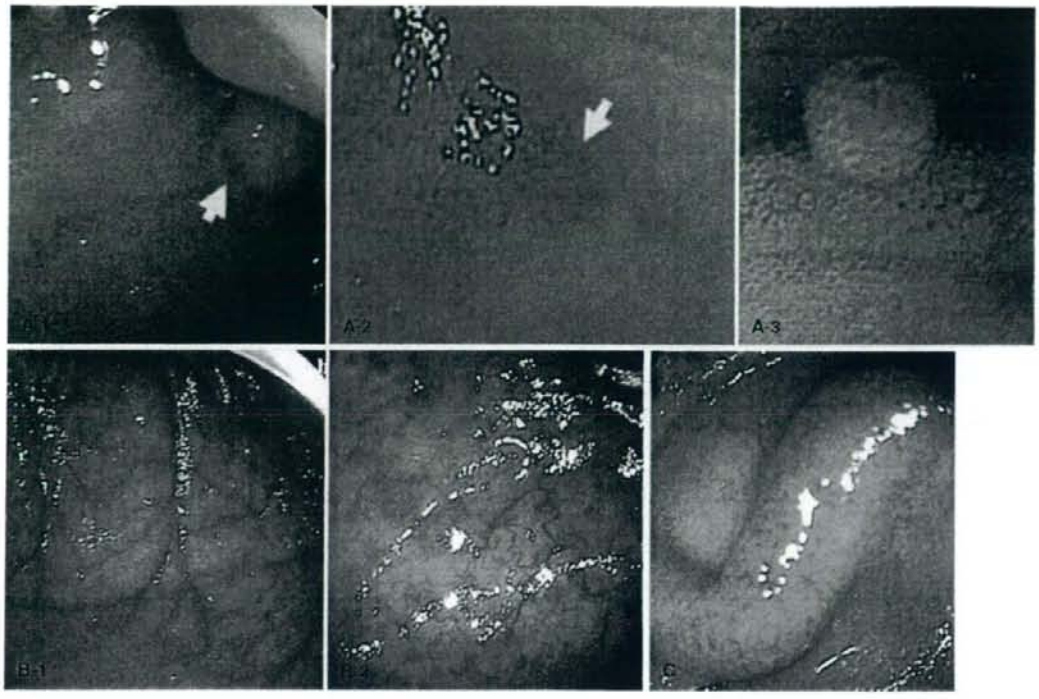

Fig. 2. Three different types of vascular lesions, including angiodysplasia-like lesions (**A**), dilated/proliferated vessels (**B**), and varices (**C**), were seen in the small bowel of patients with portal hypertension. **A-1**, red spots (*arrow*); **A-2**, vascular spiders (*arrow*); **A-3**, lymphoid 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)*	7/2/4	5/2/7	0.57
Complications	7/24 (29%)	2/90 (2%)	<0.001

*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-