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.

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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 (×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.

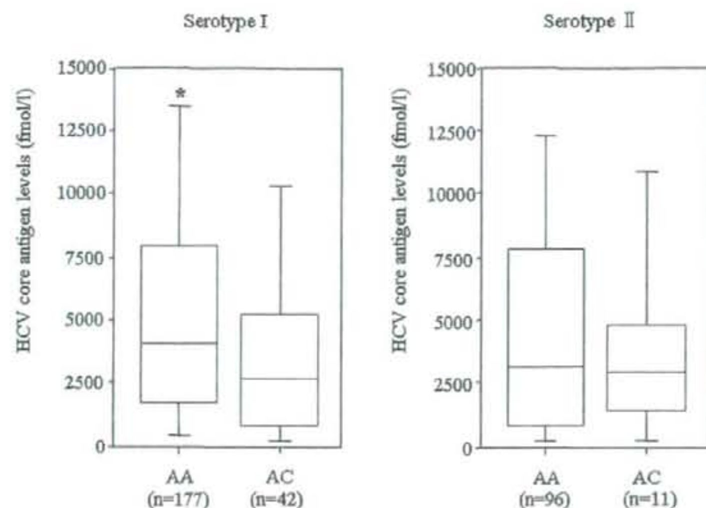

Fig. 1. The association between the K121Q genotype in *ENPP1* and the hepatitis C viral (HCV) load. The box-and-whisker plot shows the HCV core antigen level in the HCV carrier group according to the genotypes. The *boxes* indicate the 25th, 50th (median), and 75th percentiles. The *whiskers* indicate the 10th and 90th percentiles. The *asterisk* refers to a statistically 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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