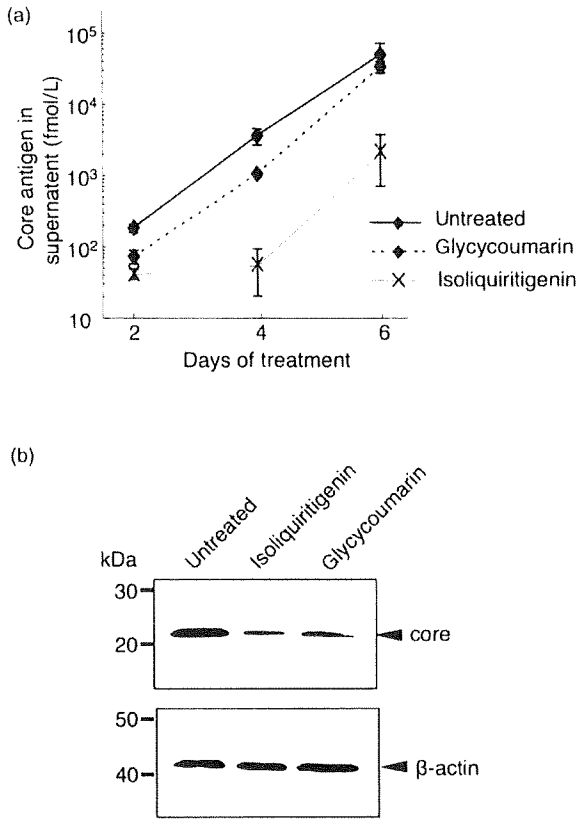

Figure 7 Suppresion of HCV-JFH1 virus expression by *isoliquiritigenin and glycycoumarin*. (a) Naïve Huh 7.5.1 cells were infected with culture supernatant of HCV-JFH1-infected cells and were subjected to culture in the presence of indicated drugs. Culture supernatants were collected at indicated days, and HCV core antigen was measured. Assays were done in triplicate and indicated as mean ± SD. (b) Cells were harvested at day 6, and Western blotting was performed using anti-core and anti-beta-actin antibodies.

Extracts of a licorice root, *Glycyrrhizae radix*, show anti-inflammatory properties in chronic and acute liver inflammation,[35] and are widely and extensively prescribed in Japan as Strong Neominophagen C (SNMC). A major ingredients of *Glyzyrrizae radix* are glycyrrhizin and liquiritin. However, glycyrrhizin and liquiritin did not suppress HCV replication, suggesting that the commercially available SNMC will not elicit antiviral effects against HCV. On the other hand, there have been reports on the pharmacological action of glycycoumarin. Glycycoumarin displays antibacterial properties in the upper respiratory tract in infections such as *Streptococcus pyogenes*, *Haemophilus infuenzae* and *Moraxella catarrhalis*,[36] and methicillin-resistant *Staphylococcus aureus*,[37] but the mechanisms of action is unclear.

To our knowledge, there have been no reports on the serum concentration of glycycoumarin and isoliquiritigenin in patients taking medicines or dietary supplements containing *Glyzyrrizae radix*. However, therapeutic doses of 3–12 g per day of powdered root have been suggested for pathological conditions including chronic hepatitis, muscle cramp, acute gastritis, and urolithiasis. Thus, further studies are required to assess the human exposure to these flavonoids, the pharmacological dose-dependent properties and the tissue distribution and drug kinetics.

Considering the current status of limited therapy options for HCV infection and their unsatisfactory outcomes, large scale screening of anti-HCV molecules for the development of novel antiviral therapies is called for. In the present study, we have screened Chinese herbal extracts for the ability to suppress HCV replication, and identified two extracts, isoliquiritigenin and glycycoumarin, which specifically suppressed HCV replication. These results suggest that these agents will be a promising for use in the stabilization of HCV replication and active liver inflammation. In addition, further investigations of the action of these drugs on the expression, processing or maturation of HCV proteins may elucidate new aspects of the viral infection and replication and may constitute novel molecular targets for anti-HCV chemotherapeutics.

ACKNOWLEDGEMENTS

WE ARE INDEBTED to Tsumura Co. Ltd for providing herbal drugs and their purified compounds. This study was supported by grants from the Japan Society for the Promotion of Science, Ministry of Health, Labour and Welfare, Miyakawa Memorial Research Foundation, and the Viral Hepatitis Research Foundation of Japan.

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Liver INTERNATIONAL
OFFICIAL JOURNAL OF THE INTERNATIONAL ASSOCIATION FOR THE STUDY OF THE LIVER

Liver International ISSN 1478-3223

CLINICAL STUDIES

Polymorphism of OAS-1 determines liver fibrosis progression in hepatitis C by reduced ability to inhibit viral replication

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Keywords
cirrhosis – fibrosis – JFH1 – replicon – SNP

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Received 29 March 2009
Accepted 5 May 2009

DOI:10.1111/j.1478-3231.2009.02061.x

Abstract

Background: Progression of disease after hepatitis C virus (HCV) infection differs among individuals, indicating a possibility of participation of host genetic factors. 2′-5′-oligoadenylate synthetase 1 (OAS-1), an important component of the innate immune system, has an antiviral function, and may therefore have a certain relationship with progression of disease. *Aim:* To evaluate single nucleotide polymorphisms (SNPs) of OAS-1 and its relationship with the disease status of HCV infection. *Methods:* Six SNPs of OAS-1 were selected and examined in 409 Japanese patients with chronic HCV infection using the TaqMan PCR genotyping method. The relationship of SNP genotypes and clinical manifestations of patients was analysed. Then, a pair of OAS-1-expression plasmids mimicking the clinical-related SNPs were created and transfected into liver cells carrying the HCV subgenomic replicon or the full-length genome, JFH1, and HCV replication after transfection was compared. *Results:* Patients with genotypes A/A, A/G and G/G of an SNP of OAS-1 at the exon 3 of its coding sequence were at gradient increased risks of suffering from higher serum alanine aminotransferase ($P < 0.001$) and aspartate aminotransferase ($P = 0.001$), higher degree of liver fibrosis ($P = 0.010$) and higher presence of liver cirrhosis ($P = 0.001$). By multivariate logistic regression analysis, genotype G/G was an independent factor associated with cirrhosis ($P = 0.013$, odds ratio 3.11, 95% confidence interval 1.27–7.63). In liver cells, OAS-1 with the G allele showed lower ability to inhibit virus replication than OAS-1 with the A allele ($P = 0.004$). *Conclusions:* The SNP of OAS-1 at the exon 3 of its coding sequence was associated with progression of disease in Japanese patients with HCV infection.

When infected by the hepatitis C virus (HCV), some patients have minimal progression of disease while some develop a severe liver disease state such as an unremittent high transaminase level, liver fibrosis and cirrhosis (1, 2). Knowledge of the risk factors of developing into severe liver disease is important for treatment of chronic HCV infection. Understanding which patients are likely to develop significant liver disease would allow more rational use of therapy, and potentially help identify new therapeutic targets.

Ageing, male sex and alcohol consumption are already been known to be risk factors associated with progression of liver disease (3–5). However, even for patients of the same age, sex and with the same alcohol consumption, there are different styles of disease progression. To determine the difference of disease progression among patients, host genetic factors, such as single nucleotide polymorphism (SNP), have been receiving the attention of many scholars (6).

It is usually accepted that inflammation-associated necrosis/apoptosis and regeneration ultimately lead to progression of liver disease (7–9). Genetic factors concerning innate immunity affect the suppression of HCV, and may therefore be related to sustained inflammation and progression of liver disease. 2′-5′-oligoadenylate synthetase 1 (OAS-1) is an important component of the innate immune system (10). After binding with double-strand RNA (dsRNA), it catalyses the formation of 2′-5′-linked oligoadenylate and activates RNaseL, which breaks down viral RNA (11, 12). An SNP of OAS-1 had been reported to be related to sustained infection of Caucasian hepatitis C patients (13). Another SNP of OAS-1 was reported in another study to be related to development of severe acute respiratory syndrome (SARS, a coronavirus

infection) (14). A nonsense single nucleotide mutation in the gene encoding OAS-1 was also reported to be associated with West Nile virus susceptibility in laboratory mice (15).

In our previous studies, we have reported the association of liver disease progression with the polymorphism of interleukin-1β, UDP-glucuronosyltransferase 1A7, MDM2, SCBY14, CRHR2 and GFRA1 (16–18). In this study, we first evaluated SNPs of OAS-1 and its relationship with the disease status of chronic hepatitis C, and then created a pair of OAS-1-expression plasmids mimicking the SNPs that are related to clinical manifestations, to investigate its function in liver cell model.

Materials and methods

Patients

We studied 409 consecutive Japanese patients with chronic HCV infection who consulted the clinic of the University of Tokyo Hospital between August 2001 and June 2003 (227 men and 182 women, 22–84 years old). The genomic DNA of these patients was made available after obtaining written informed consent for genotyping. We also obtained approval from the institutional ethics committee (no. 400), and all the procedures followed institutional guidelines (19).

Patients selected for this study were those who tested positive for HCV antibody (Ortho Diagnostics, Tokyo, Japan), and HCV RNA was measured using the Amplicor HCV assay (Roche, Tokyo, Japan). All patients were hepatitis B surface antigen (Abbott Laboratories, North Chicago, IL, USA) negative. Among 179 cirrhotic patients, 54 patients were confirmed by biopsy. In patients without biopsy specimens, the diagnosis of cirrhosis was made by the presence of clinical manifestations of portal hypertension (e.g. varices, encephalopathy or ascites), biochemical abnormalities [elevated serum bilirubin, decreased serum albumin or prolonged prothrombin time (PT)] and obvious morphological change of the liver detected by hepatic imaging (e.g. ultrasonography, computed tomography, arteriography or magnetic resonance imaging). The diagnosis of HCC was made by several imaging methods and all confirmed histologically by sonography-guided fine needle biopsy.

Polymorphism genotyping

Genomic DNA was extracted from 100 μl whole blood using the SepaGene kit (Sanko Junyaku, Tokyo, Japan) according to the manufacturer's instructions. Extracted DNA was dissolved in 20 μl of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA, and was stored at − 30 °C until use.

As shown in Table 1, six SNPs in the OAS-1 gene were selected for study. We selected two SNP sites from the promoter region, two SNP sites from the coding sequence (CDS, both of two SNPs are nonsynonymous) and two SNP sites from the 3′-untranslated region (UTR) or exon 6 (due to different transcripts). We selected these targets for the reason that different nucleic acids at the promoter region may lead to different levels of transcription, while different nucleic acids at the exon, which leads to substitution of amino acids, may eventually change the function of this protein.

Table 1. Single nucleotide polymorphisms of 2′-5′-oligoadenylate synthetase 1 selected for study

Role	Relative position	dbSNP ID	Alleles	Amino acid change
Promoter	− 1377	rs2158390	G/C	–
Promoter	− 580	rs12322047	A/G	–
CDS (exon 1)	183	rs1050994	C/T	Asp/Asn
CDS (exon 3)	4119	rs3741981	A/G	Ser/Gly
3′-UTR/exon 6	12458	rs3177979	A/G	–
3′-UTR/exon 6	12691	rs2660	A/G	–

CDS, coding sequence; UTR, untranslated region.

We performed SNP genotyping using TaqMan SNP Genotyping Assays or Custom TaqMan SNP Genotyping Assays (20), and the ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The polymerase chain reaction (PCR) mixture contained 10 ng of genomic DNA, one-fold TaqMan universal PCR master mix (Applied Biosystems), forward and reverse primers (900 μM each, Applied Biosystems) and 200 nM VIC-labelled probe and 200 nM of FAM-labelled probe (Applied Biosystems). Primers and probes were commercially available products of Applied Biosystems, but their sequence was closed under patent.

Polymerase chain reaction was carried out in 96-well plates with a reaction volume of 25 μl. Thermal cycle conditions were 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 92 °C for 15 s and 60 °C for 1 min. Completed PCR plates were read on an ABI PRISM 7000 sequence detector and analysed using ALLELIC DISCRIMINATION SEQUENCE DETECTION software (Applied Biosystems). The genotype of each patient was determined from the figure represented by this software (Fig. 1).

Construction of cell line carrying the hepatitis C virus subgenomic replicon or the full-length hepatitis C virus

Human liver tumour cell line Huh7 was maintained in Dulbecco's modified Eagle medium (GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum in an atmosphere containing 5% CO_2. *In vitro*-transcribed HCV subgenomic RNA and full-length JFH1 RNA were prepared as described previously (21, 22). An HCV subgenomic replicon plasmid expressing a chimeric protein consisting of neomycin phosphotransferase and firefly luciferase (kindly provided by Dr N. Sakamoto, Tokyo Medical and Dental University, Tokyo, Japan) (21) and a plasmid containing a full-length HCV JFH1 cDNA downstream of T7 RNA promoter (a gift from Dr T. Wakita, National Institute of Infectious Diseases, Tokyo, Japan) (22) were linearized

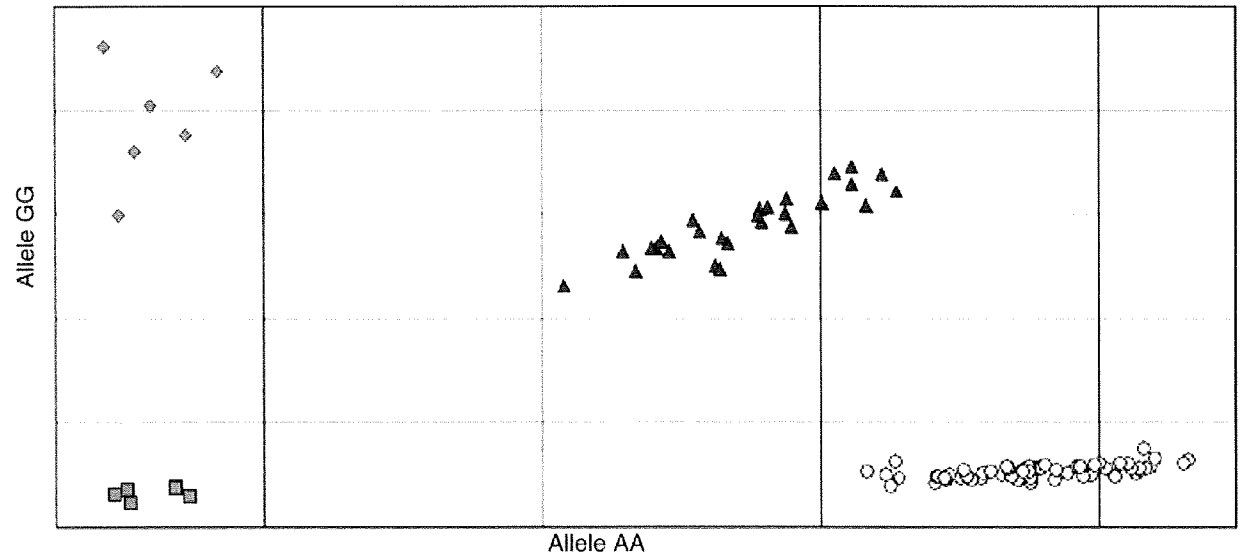

Fig. 1. An example of TaqMan allelic discrimination. The figure shows a representative result of allelic discrimination of a 96-well plate, with 91 samples and five controls. After real-time polymerase chain reaction using TaqMan primers and probes, products were divided into three groups according to their original genotype. In this example, the horizontal axis means that the sample was detected by probe connecting with the OAS-1/allele A single strain (a VIC-labelled probe), and the vertical axis means that the sample was detected by probe connecting with the OAS-1/allele G single strain (a FAM-labelled probe). Therefore, human DNA of A/A type was only detected by the first probe (round-shaped dots), the G/G type was only detected by the second probe (diamond-shaped dots) and the A/G type was detected by both probes (triangle-shaped dots). The square-shaped dots were no-template-control samples, which showed no detection by any probe. OAS-1, 2′-5′-oligoadenylate synthetase 1.

with XbaI. RNA transcripts were synthesized from 2 μg of the linearized DNA using the MEGAscript T7 system (Ambion, Austin, TX, USA) and added to 0.42 ml of 10^7/ml PBS-washed Huh7 cells. The mixture was placed in an electroporation cuvette (Bio-Rad Laboratories, Hercules, CA, USA) and pulsed with 0.27 kV for 30 μs. After 10 min of recovery at room temperature, cells were divided and plated onto five dishes for culture. Twenty-four hours later, the medium was changed to 250 μg/ml G418-containing medium, and 3 weeks later, survival colonies were picked up and passaged for further study.

These cells contained a stably harbouring HCV subgenomic replicon or full-length HCV genome. We determined the relative amount of HCV subgenomic replicon according to firefly-luciferase activity, and the relative amount of the full-length HCV by Western blotting of the HCV core protein.

Construction and transfection of plasmids expressing 2′-5′-oligoadenylate synthetase 1

A pcDNA3 plasmid containing the OAS-1 coding sequence (kindly provided by Dr Y. Li, Baylor College of Medicine, Houston, TX, USA) was sequenced to confirm the nucleotide sequence corresponding to the SNP that related to clinical manifestations. Site-directed single nucleotide mutagenesis was performed using the Site Mutation System (Stratagene, La Jolla, CA, USA) to create the second plasmid having a mutation corresponding to SNP. Two plasmids had a difference in only one nucleotide, mimicking OAS-1 having a major allele or a minor allele.

Transfection efficiency was monitored by cotransfection of pRL-TK (Toyo Ink, Tokyo, Japan), a control plasmid expressing *Renilla reniformis* (seapansy) luciferase driven by the herpes simplex virus thymidine kinase promoter.

Each of two plasmids or pcDNA3 plasmid containing no OAS-1 sequence as a negative control was transfected into Huh7 cells carrying the HCV subgenomic replicon or the full-length HCV genome using Effectene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Huh7 cells carrying the HCV subgenomic replicon or the full-length HCV were plated onto a well of a six-well tissue culture plate 24 h before transfection. A total of 0.4 μg of plasmid (OAS-1 expressing plasmid or mock plasmid plus pRL-TK) was mixed with Effectene, and was added to each well of the cells.

Luciferase assay and quantification of hepatitis C virus core protein

Cells were harvested 48 h after transfection. For cells harbouring subgenomic replicon, luciferase assays were performed using the PicaGene dual seapansy system (Toyo Ink). Firefly-luciferase activity and seapansy-luciferase activity were measured as relative light units

Table 2. Frequency of 2′-5′-oligoadenylate synthetase 1polymorphisms

			Number (%)					
dbSNP ID	Position	Total number	Homozygosity		Heterozygosity		Minor alleles	
rs12322047	Promoter	92	A/A	92 (100%)	A/G	0	G/G	0
rs2158390	Promoter	409	G/G	395 (96.6%)	G/C	14 (3.4%)	C/C	0
rs1050994	CDS	409	C/C	409 (100%)	C/T	0	T/T	0
rs3741981	CDS	409	A/A	178 (43.5%)	A/G	173 (42.3%)	G/G	58 (14.2%)
rs3177979	3′UTR	409	A/A	276 (67.5%)	A/G	113 (27.6%)	G/G	20 (4.9%)
rs2660	3′UTR	409	A/A	276 (67.5%)	A/G	113 (27.6%)	G/G	20 (4.9%)

CDS, coding sequence; UTR, untranslated region.

with a luminometer (Lumat LB9507, EG&G Berthold, Bad Wildbad, Germany). The firefly-luciferase activity was then normalized for transfection efficiency based on the seapansy-luciferase activity. Data were reported as the mean value from triplicate wells. The percentage of luciferase activities relative to that of control, which reflected the percentage of residual HCV replicon after overexpression of OAS-1, was compared between the two kinds of plasmids. Besides the cotransfection of control plasmid expressing *Renilla reniformis*, Western blotting confirming OAS-1 protein expression was also performed.

For cells harbouring the full-length HCV genome, the amount of HCV core protein was determined by Western blotting 48 h after transfection. The relative level of the HCV core protein was compared among these cells transfected with two OAS-1-expressing plasmids.

Statistical analysis

Clinical parameters were evaluated using the *t*-test or ANOVA and the χ^2-test to determine their association with the genotype of OAS-1 of patients. A *P*-value of less than 0.05 was considered significant. Possible confounding effects among the variables were adjusted using a stepwise multivariate logistic regression model, and odds ratio and its 95% confidence intervals were calculated. In studies with cells, the experiment was repeated three times and the percentage of residual HCV replicon or HCV core protein after overexpression of OAS-1 was compared using a *t*-test. Data analysis was performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). The Hardy–Weinberg equilibrium (HWE) of alleles at individual loci was evaluated using HWE (ftp://linkage.rockefeller.edu/software).

Results

Frequency of 2′-5′-oligoadenylate synthetase 1 polymorphisms

Among six SNPs studied, two SNPs (rs12322047 and rs1050994), as were ever reported in the other population, were revealed to be only major allele (Table 2). This did not allow any further meaningful analyses. Genotype frequencies of rs2158390, rs3741981, rs3177979 and rs2660 are consistent with the data of the Japanese population reported before (http://snp.ims.u-tokyo.ac.jp). The genotype frequencies observed approximated those based on allele frequency calculations, and thus conformed to HWE. SNPs rs3177979 and rs2660 are of complete linkage. The frequency of each SNP in our population is listed in Table 2.

Association of genotype with patients' laboratory examinations

As shown in Table 3, for patients with the rs2158390 heterozygote, although there was a tendency towards a decrease of the platelet count (PLT), serum albumin level (ALB) and PT, and elevation of alanine aminotransferase (ALT), aspartine aminotransferase (AST), γ-glutamine phosphatase (gGTP), alkaline phosphatase (ALP) and total bilirubin (TB), there was no statistically significant difference.

For patients with rs3741981 homozygous wild type (A/A), heterozygous type (A/G) and homozygous minor allele (G/G), there was a gradient increase of ALT and AST ($P < 0.001$ and $P = 0.001$). A decrease of PLT was also found in the minor allele. There was also a tendency towards a decrease of ALB, and elevation of gGTP, ALP and TB, but not statistically significant.

In the situation of rs3177979/rs2660 (complete linkage), there was an increase of ALT and gGTP in the group of minor allele, but it did not show a gradient increase style from a homozygous wild type to a heterozygous type. The difference in the other laboratory examinations did not reach a statistically significant level.

We also tested 323 patients' peripheral serum HCV RNA level, but there was no difference in the HCV RNA level among patients with different genotypes (Table 4).

Association of genotype with liver cirrhosis and degree of liver fibrosis

A relationship was found between the rs3741981 genotype and liver cirrhosis. Patients with rs3741981 genotypes A/A, A/G and G/G were at a gradient increased risk of suffering from liver cirrhosis ($P = 0.001$, Table 5). No relationship was found between other SNPs and liver cirrhosis (Table 5). Among 95 patients with biopsy data

Table 3. Association of genotype with patients' laboratory examinations

	PLT		ALB		ALT		AST	
Genotype	Value	*P*	Value	*P*	Value	*P*	Value	*P*
rs2158390 G/G	80.4 ± 1.6	0.157	3.9 ± 0.11	0.832	76.2 ± 2.9	0.704	70.4 ± 2.4	0.841
G/C	68.9 ± 6.2		3.8 ± 0.13		82.0 ± 9.5		73.0 ± 10.2	
rs3741981 A/A	79.8 ± 1.2	0.004	3.9 ± 0.03	0.075	68.3 ± 3.5	0.000	63.1 ± 2.6	0.001
A/G	79.6 ± 1.1		3.8 ± 0.04		75.0 ± 3.9		72.0 ± 3.7	
G/G	72.6 ± 2.1		3.8 ± 0.07		105.4 ± 11.0		88.9 ± 8.8	
rs2660/A/A	80.2 ± 2.1	0.942	4.0 ± 0.16	0.854	77.9 ± 3.3	0.017	71.6 ± 2.8	0.100
rs3177979 A/G	79.2 ± 1.5		3.9 ± 0.05		67.7 ± 4.0		64.7 ± 3.2	
G/G	81.4 ± 3.3		3.9 ± 0.13		105.1 ± 24.0		87.9 ± 20.4	
	gGTP		ALP		TB		PT	
Genotype	Value	*P*	Value	*P*	Value	*P*	Value	*P*
rs2158390 G/G	69.2 ± 3.8	0.590	218 ± 4.3	0.891	0.88 ± 0.03	0.377	14.1 ± 0.5	0.529
G/C	80.2 ± 16.7		222 ± 19.6		1.01 ± 0.21		12.4 ± 1.1	
rs3741981 A/A	62.5 ± 4.2	0.051	210 ± 6.4	0.095	0.84 ± 0.03	0.098	14.0 ± 0.4	0.144
A/G	69.8 ± 6.7		222 ± 6.1		0.88 ± 0.05		13.8 ± 0.5	
G/G	90.3 ± 11.4		237 ± 12.2		1.02 ± 0.07		12.2 ± 0.8	
rs2660/A/A	71.9 ± 4.9	0.023	219 ± 5.1	0.492	0.87 ± 0.03	0.909	14.2 ± 0.7	0.782
rs3177979 A/G	57.3 ± 4.4		222 ± 8.6		0.89 ± 0.07		13.5 ± 0.6	
G/G	104.5 ± 24.5		198 ± 13.9		0.92 ± 0.10		13.6 ± 1.5	

The units are PLT 10^3/μl; ALB g/dl; ALT IU/L; AST IU/L; gGTP IU/L; ALP IU/L; TB mg/dl; and PT second respectively.
ALB, serum albumin level; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartine aminotransferase; gGTP, γ-glutamine phosphatase; PLT, platelet count; PT, prothrombin time; TB, total bilirubin.

Table 4. Association of 2′-5′-oligoadenylate synthetase 1 genotype and patients' hepatitis C virus RNA Level

dbSNP ID	Genotype	Number	HCV RNA level (ng/μl)	*P* value
rs2158390	G/G	311	468 ± 19	0.881
	G/C	12	483 ± 71	
rs3741981	A/A	139	491 ± 30	0.538
	A/G	139	457 ± 26	
	G/G	45	435 ± 49	
rs2660/3177979	A/A	218	472 ± 23	0.924
	A/G	89	464 ± 35	
	G/G	16	441 ± 71	

HCV, hepatitis C virus.

Table 5. Association of 2′-5′-oligoadenylate synthetase 1 genotype and cirrhosis

dbSNP ID	Genotype	Number	Cirrhosis (*n* = 179)	No cirrhosis (*n* = 230)	*P* value
rs2158390	G/G	395	172 (43.5%)	223 (56.5%)	0.785
	G/C	14	7 (50.0%)	7 (50.0%)	
rs3741981	A/A	178	62 (34.8%)	116 (65.2%)	0.001
	A/G	173	82 (47.4%)	91 (52.6%)	
	G/G	58	35 (60.3%)	23 (39.7%)	
rs2660/	A/A	276	122 (44.2%)	154 (55.8%)	0.930
3177979	A/G	113	49 (43.4%)	64 (56.6%)	
	G/G	20	8 (40.0%)	12 (60.0%)	

of degree of fibrosis, a relationship between the rs3741981 genotype and the degree of liver fibrosis was also found. Patients with rs3741981 genotypes A/A, A/G and G/G were at a gradient increased risk of suffering from a higher degree of liver fibrosis ($P = 0.01$, Table 6).

In order to confirm the relationship between rs3741981 genotypes with liver cirrhosis, we also performed a multivariate analysis. The factors associated with liver cirrhosis in univariate analysis included rs3741981 genotype, age, PLT, ALB, TB, α-fetoprotein level, PT and a diagnosis of hepatocellular carcinoma. Table 7 shows the factors associated with liver cirrhosis in multivariate analysis. The odds ratio of rs3741981 genotype G/G for liver cirrhosis was 3.1 ($P = 0.013$, Table 7).

Relationship of genotype and interferon treatment response

Considering that OAS-1 is an interferon (IFN)-stimulated gene, we tested the relationship of genotype and IFN treatment response. Altogether, 107 patients in our population had data of IFN treatment response, but our result showed no relationship between genotype and IFN treatment response (Table 8).

Hepatitis C virus inhibitory effect of single nucleotide polymorphism-simulating 2′-5′-oligoadenylate synthetase 1 in liver cells

A and G alleles of plasmids, mimicking the A/A and G/G genotypes of rs3741981, were confirmed by direct

Table 6. Association of 2′-5′-oligoadenylate synthetase 1 genotype and degree of fibrosis

Genotype	Genotype	Number	F1 to F2 ($n=20$)	F3 to F4 ($n=75$)	*P* value
rs2158390	G/G	93	20 (21.5%)	73 (78.5%)	0.622
	G/C	2	0 (0.0%)	2 (100.0%)	
rs3741981	A/A	41	14 (34.1%)	27 (65.9%)	0.010
	A/G	42	3 (7.1%)	39 (92.9%)	
	G/G	12	3 (25.0%)	9 (75.0%)	
rs2660/ 3177979	A/A	67	17 (25.4%)	50 (74.6%)	0.166
	A/G	25	2 (8.0%)	23 (92.0%)	
	G/G	3	1 (33.3%)	2 (66.7%)	

Table 7. Multivariate analysis of factors related to cirrhosis

Factors	*P* value	Odds ratio	95% CI
rs3741981			
A/A		1	
A/G	0.089	1.708	0.922–3.165
G/G	0.013	3.112	1.269–7.631
Age	0.015	1.043	1.008–1.079
PLT	0.000	0.851	0.794–0.912
ALB	0.018	0.426	0.210–0.866
PT	0.005	0.966	0.944–0.990
Diagnosis of HCC	0.000	4.102	2.266–7.424

ALB, serum albumin level; CI, confidence interval; HCC, hepatocellular carcinoma; PLT, platelet count; PT, prothrombin time.

Table 8. Relationship of genotype and interferon treatment response

dbSNP ID	Genotype	Number	Response ($n=18$)	No response ($n=89$)	*P* value
rs2158390	G/G	104	18 (17.3%)	86 (82.7%)	0.572
	G/C	3	0 (0.0%)	3 (100.0%)	
rs3741981	A/A	41	7 (17.1%)	34 (82.9%)	0.820
	A/G	49	9 (18.4%)	40 (81.6%)	
	G/G	17	2 (11.8%)	15 (88.2%)	
rs2660/ 3177979	A/A	71	12 (16.9%)	59 (83.1%)	0.894
	A/G	32	5 (15.6%)	27 (84.4%)	
	G/G	4	1 (25.0%)	3 (75.0%)	

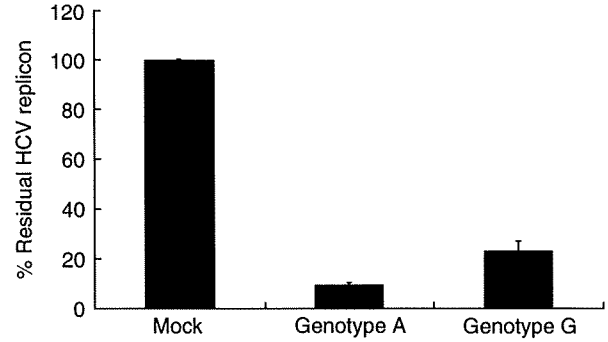

Fig. 2. Effect of different OAS-1 overexpressions on the hepatitis C virus (HCV) replicon. The A- and G-type plasmids, mimicking the A/A and G/G genotype of an SNP rs3741981, were transfected into Huh7 cells harbouring the HCV subgenomic replicon, and luciferase activity was measured 48 h later. The values were displayed as the percentage of luciferase activities relative to that of control, which reflected the percentage of the residual HCV replicon after overexpression of OAS-1. The percentage of residual HCV replicon after transfection of the G-type plasmid was significantly higher than that of the A-type plasmid ($P=0.004$). Error bars indicate standard deviation. OAS-1, 2′-5′-oligoadenylate synthetase 1; SNP, single nucleotide polymorphism.

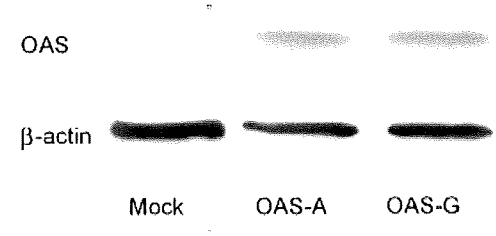

Fig. 3. Western blotting of OAS-1 after transfection of different plasmids. Each plasmid containing no OAS-1 sequence, containing the OAS-1 A-type or G-type sequence, was transfected into Huh7 cells harbouring the hepatitis C virus subgenomic replicon. Cell lysates were collected 48 h later, and Western blotting was performed. β-actin was used as a quantitative control. The figure shows no difference in OAS-1 expression between A-type and G-type plasmids. OAS-1, 2′-5′-oligoadenylate synthetase 1.

sequencing. Forty-eight hours after transfection of these plasmids into Huh7 cells harbouring the HCV subgenomic replicon, a difference in the percentage of luciferase activities relative to that of control, which reflected the percentage of residual HCV replicon after overexpression of OAS-1 with the A or the G allele, was found (Fig. 2). The percentage of residual HCV replicon after transfection of the G-type plasmid was higher than that of the A-type plasmid ($P=0.004$), indicating a weaker ability of G-type OAS-1 in inhibiting the HCV subgenomic replicon. As for Western blotting, no difference in the quantity of OAS-1 expression was found between OAS-1/allele A and OAS-1/allele G (Fig. 3), indicating that the difference of virus suppression was due to the quality of OAS-1.

While in cells harbouring the full-length HCV genome, JFH1, the relative amounts of intracellular HCV core protein determined by Western blotting showed the same tendency as the results using the HCV subgenomic replicon, the relative amounts of intracellular HCV core protein determined by Western blotting are 100% for mock-transfected, 77% for OAS-1/allele A-transfected and 84% for OAS-1 allele G-transfected.

Discussion

Our results suggested that a nonsynonymous SNP on exon 3 of OAS-1 (rs3741981) was related to a higher serum transaminase level, a higher degree of liver fibrosis and the presence of cirrhosis. In fact, OAS-1 having a G

allele in rs3741981 has a weaker ability in suppressing HCV replication compared with OAS-1 having an A allele. Thus, our results can be explained by the fact that patients with the rs3741981 G allele were weaker in suppressing HCV replication compared with patients with the rs3741981 A allele, which may lead to a higher degree of necrosis and regeneration, and ultimately fibrosis and cirrhosis of the liver.

The OAS-1 gene, located on chromosome 12, has two major transcripts that are generated by alternative splicing at the last two exons. E16 (NM_002534) is a short transcript with five exons and is translated into p40 isoform. E18 (NM_016816) is a long transcript with six exons and is translated into p46 isoform (23, 24). The A/G polymorphism in exon 3 of OAS-1 (rs3741981) causes amino acid substitution Ser162Gly in both isoforms, which is located near the dsRNA-binding domain (amino acid 104-158) of OAS-1 (25).

The OAS-1 protein belongs to the dsRNA-binding protein family, which is characterized by having one or more dsRNA-binding motif (dsRBM) and activation of enzyme activity after binding of the dsRBM with dsRNA. The A-form double helix dsRNA, compared with the double-stranded DNA, which is a B-form double helix, is known for its shallower-and-broader minor groove and narrower-and-deeper major groove. A study showed that the dsRBM uniquely contacts the dsRNA helix in two successive minor grooves and once in the intervening major groove (26). Substitution of nearly all the amino acids within the consensus dsRBM decreases dsRNA binding (27–29).

An A to G substitution in the OAS-1 gene results in a serine to glycine substitution in OAS-1 protein, which may affect the three-dimensional structure of dsRBM and thus weaken the binding of dsRNA and finally the ability to suppress virus replication. We tested this hypothesis using liver cells carrying the HCV subgenomic replicon or the full-length HCV genome. Transfection of the OAS-1 expression plasmid into cells harbouring the HCV subgenomic replicon of the full-length genome (overexpression) resulted in inhibition of HCV, which was consistent with previous reports (30). In our experiments, cells transfected with the G-type OAS-1 plasmid showed higher HCV replication compared with the A type. Therefore, patients with the rs3741981 G allele were weaker in suppressing HCV replication compared with those with the A allele. The higher transaminase level might be the clinical evidence. Increased inflammation eventually led to fibrosis and cirrhosis of the liver. The rate of fibrosis progression was believed to be higher among patients with higher serum ALT and AST levels, as reported by Ghany *et al.* (7). Thus, by *in vitro* and *in vivo* study, we ratiocinated that G allele carriers had weaker virus clearance, which resulted in more inflammation, and then more fibrosis.

Another A/G polymorphism in exon 6 (rs3177979) of OAS-1 causes the amino acid substitution Thr352Ala, which is located near the region related to the enzyme activity (amino acid 320-344) of OAS-1 (25). This could be the reason why it had a certain relationship with higher ALT.

Unfortunately, one of the shortcomings of our study was that only a part of the cases diagnosed as having cirrhosis had a pathological confirmation. We can only say that SNP of OAS-1 has a relationship with clinically diagnosed cirrhosis. However, it still provided a clue to focus more attention on patients with the rs3741981 G allele, who were also more likely to suffer from higher serum transaminase and a higher degree of fibrosis. It is reasonable to assume that this group of patients was more likely to develop significant liver disease, i.e. should be intensively cared and followed.

There was no difference in the peripheral HCV RNA level among patients with different genotypes in our study. However, serum viral loads may be affected by both viral and host factors such as viral replication ability and polymorphisms of genes related to innate immunity, viral receptors and cellular genes to help viral replication. In fact, it was previously reported that the viral sequence affects the viral load (31). Moreover, it is possible that serum viral loads do not correlate with viral loads in the liver. It is the virus in the liver that leads to inflammation and fibrosis.

Our study showed no relationship of SNPs of OAS-1 and IFN treatment response. Considering that OAS-1 is one of IFN-stimulated genes, Knapp *et al.* (13) studied the relationship of an SNP of OAS-1 (rs2660) with the result of IFN therapy, and found that the genotype of rs2660 was not associated with response to IFN therapy but was associated with persistent infection of patients. The result of IFN therapy obtained by Knapp and colleagues is consistent with our study. It is interesting that previous papers reported an increase of OAS-1 protein expression in persistent HCV infection but not in exogenous IFN treatment (32, 33). It seems that OAS-1, as a component of the innate immune system and an IFN-stimulated gene, is linked to persistent infection but not to the effect of exogenously administered IFN.

Although our result showed the relationship of the rs3741981 G allele with cirrhosis, the odds ratio of the genotype G/G was about 3.1, and for genotype A/G, the relationship was not statistically significant. Many statisticians consider that a 0.05 cut-off is inadequate for genetic association studies. They rather recommend a 0.01 cut-off to strengthen the value of the association. Therefore, our conclusion should be weakened to some extent. In fact, many genetic factors had been reported to associate with fibrosis/cirrhosis of the liver. Most reports were on cytokine polymorphisms. Polymorphisms of interleukin-10 and tumour necrosis factor-β were reported in many studies to relate to fibrosis (34, 35). The association of the polymorphism of complementary 5 and fibrosis degree was also reported recently (36). Patients with monocyte chemotactic protein-1 genotype G/G or G/A were more likely to have advanced fibrosis and severe inflammation in the liver biopsy than those

with genotype A/A (37). The polymorphism of other genes such as the angiotensinogen gene has also been reported to be associated with more advanced fibrosis (38). Patients with coagulation factor V Leiden mutation (A560G) were also more likely to progress to cirrhosis (39). In another study on different alleles for the microsomal epoxide hydrolase gene, an important antioxidant enzyme, patients with cirrhosis were more likely to have the slow metabolizer phenotype of this enzyme (40). It seems that many genetic factors can affect the progression of liver disease, involving different aspects of the liver such as immune, metabolism, circulation and so on. Therefore, in the future, many SNPs of a patient might be detected simultaneously in order to have a stronger prediction of the risk of developing significant liver disease.

In conclusion, our study once more proved the association of liver disease progression with genetic factors. The SNP of OAS-1 at the exon 3 of its coding sequence, together with other genetic factors mentioned by us (16–18) and other scholars, might be useful in the assessment and treatment of hepatitis C.

Acknowledgements

This work was supported by Global COE Program 'Center of Education and Research for Advanced Genome-Based Medicine: For personalized medicine and the control of worldwide infectious diseases', the Ministry of Education, Culture, Sports, Science and Technology of Japan, by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan.

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Cancer Science
The official journal of the Japanese Cancer Association

Report

Characteristic mutations in hepatitis C virus core gene related to the occurrence of hepatocellular carcinoma

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(Received May 06, 2009/Revised August 13, 2009/Accepted August 20, 2009)

Chronic hepatitis C virus (HCV) infection often results in hepatocellular carcinoma (HCC). Previous studies have shown that there might be some characteristic mutations in the core region of HCV related to HCC. Thus, we downloaded and analyzed HCV genotype 1b core gene sequences from HCV databases online to identify them. Based on the information of the sequences, 63 from patients with HCC and 188 from non-HCC were enrolled into our analysis. Then, the nucleotides at each position were compared by χ^2-test between the two groups, and 24 polymorphisms were found to be associated with HCC. Further analysis of these 24 polymorphisms by logistic regression indicated that eight were significantly related to the increased HCC risk: A028C, G209A, C219U/A, U264C, A271C/U, C378U/A, G435A/C, and G481A. Moreover, U303C/A was associated with the decreased HCC risk. These mutations could bring about four amino acid substitutions: K10Q, R70Q, M91L, and G161S. In conclusion, eight characteristic mutations in the *HCV-1b* core gene related to the occurrence of HCC were identified. The structural and functional alterations of core protein due to these mutations and the relationship with the occurrence of HCC need to be further studied. (*Cancer Sci* 2009; 100: 2465–2468)

Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide and a major risk factor for hepatocellular carcinoma (HCC). More than 70% of patients with HCC in Japan are infected with HCV, and HCC has become the major cause of death in patients chronically infected with HCV.[1–3] In Asia, especially in Japan, HCV-1b is the most prevalent genotype and is more likely to develop into HCC than other genotypes.[4,5] Although there are many published data about the correlation between HCV infection and HCC development, details of hepatocarcinogenesis by HCV remain unclear. HCV core protein is implicated in hepatocarcinogenesis for its ability to modulate cellular gene transcription and protein expression, intracellular signal transduction, cell proliferation and apoptosis.[6–9]

Being an RNA virus, the HCV genome exhibits a considerable degree of sequence variation. Studies have proved that certain kinds of mutations may lead to functional changes of the virus, such as the resistance to antiviral treatments and the association with HCC.[10,11] Furthermore, there are more mutations in the core region in patients with HCC than that in those without HCC. Moreover, the rate of nucleotide substitutions in the core gene is significantly greater for isolates from HCC patients compared to those from individuals with chronic hepatitis.[12] It was also found that there was a significantly higher variability within the core region of tumor tissue isolates than that of isolates from non-tumor tissue. Mutant sequence diversity ranged from silent mutations, as well as amino acid substitutions, to appearance of in-frame stop codons and deletions leading to frame-shifts. In contrast, the variability of the NS5 region sequences between isolates from tumor and non-tumor tissue was not significantly different.[13] Recently, a report indicated that amino acid substitutions in the HCV core region are important predictor of hepatocarcinogenesis.[11] Even in patients without HCC, the substitution of amino acid 70 in the hepatitis C virus core region of genotype 1b is an important predictor of elevated alpha-fetoprotein.[14]

Based on the data above, we suppose that there might be some characteristic mutations in the *HCV-1b* core gene related to the occurrence of HCC. Because we can find thousands of HCV sequences from databases online, we have tried to identify these mutations by analyzing such sequences in this study.

Materials and Methods

Collection of HCV core gene sequences. HCV core gene sequences were downloaded from the following databases: European HCV database (euHCVdb), HCV Databases from Los Alamos National Laboratory, HCV Database (HCVdb) from the Viral Bioinformatics Resource Center (VBRC), and National Center for Biotechnology Information (NCBI).

Inclusion criteria. All the sequences enrolled into our analysis needed to comply with the following inclusion criteria: (i) the diagnosis of patients could be confirmed; (ii) the genotype of HCV could be confirmed and samples were isolated from sera of patients infected with HCV-1b; and (iii) the core gene sequence was of full length.

Exclusion criteria. Sequences were excluded if: (i) the diagnosis of patients or the genotype of HCV was unknown; (ii) samples came from liver, other tissues, ascites, or pooled sera; (iii) patients were co-infected with hepatitis B virus or human immunodeficiency virus; (iv) patients had ever been treated with interferon (IFN); (v) patients had ever undergone orthotopic liver transplantation (OLT); (vi) samples came from cell culture, animal experiments, chimeric DNA, or fusion protein; (vii) sequences came from repeated samples; and (viii) existence of deletion mutations or insertion mutations.

Statistical analysis. According to the diagnosis of the cases, sequences enrolled were divided into two groups: group HCC and group non-HCC. The first base of the start codon (AUG) was designated position 1. All the 573 nucleotides of the core region were compared respectively between group HCC and non-HCC. All the 573 nucleotides of the core region were compared respectively between group HCC and non-HCC by Pearson χ^2-test to find out significantly different mutations (when

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doi: 10.1111/j.1349-7006.2009.01338.x

there were cells that the expected count was less than 5 or 1, we chose different χ^2-test methods – Continuity correction or Fisher's exact test). Then, the significant bases were further analyzed by logistic regression to identify the mutations significantly related to the occurrence of HCC. A P-value of <0.05 was considered statistically significant.

Results and Discussion

Enrollment of sequences. We downloaded sequences of HCV core region from HCV databases online, mainly from euHCVdb. A total of 2841 sequences of HCV-RNA with full-length core region were downloaded. Then, we checked the information provided by the databases and the published papers so that we could get the details about these sequences such as the diagnoses of patients, genotypes of HCV, the origin of samples, and so on. Finally, we picked out the sequences that complied with the inclusion and exclusion criteria and enrolled them into our analysis. As a result, from 2841 sequences, we found 1336 sequences of genotype 1b. Of these, 547 sequences were from identified samples and the others lacked information that identified the types of the samples. Among the 547 sequences with definite origin, 292 were from sera and the others (228 from livers, 20 from cultured cells, seven from plasmids) were ruled out. Of the 292 sequences derived from sera, seven were from animal experiments, 24 were from repeated cases at different time points, and four and six were from cases that had undergone OLT and IFN treatments, respectively. Finally, 251 sequences complied with the inclusion and exclusion criteria and were enrolled into our analysis. Sixty-three were from patients with HCC and 188 were from HCV carriers or patients with acute or chronic hepatitis without HCC. For the 251 sequences, 73.7% (185/251) were directly sequenced and 26.3% (66/251) represented one clone of available clones. Thus, it is possible that about a quarter of our data could contain mutations due to PCR error. However, the sequences were collected from 42 different studies and the statistical analysis may have overcome this problem.

Identification of the wild-type nucleotides. For the reason that there are frequent mutations of the HCV sequence, it is impossible to know the real wild-type nucleotides. It might be reasonable to identify the wild-type nucleotide based on the consensus of a large number of sequences. In our analysis, we defined the consensus of the 1336 HCV-1b sequences as the wild type, except the position of nucleotide (nt) 209. Because 209G was treated as wild type in almost all the published papers and it was also the consensus type of 2841 all genotypes sequences, we still defined "G" as the wild type even if "A" was the consensus.

Significantly different mutations between group HCC and non-HCC. By performing the χ^2-test or Fisher's exact test, we compared all the 573 nucleotides of the core gene between group HCC and non-HCC. A total of 24 nucleotide mutations were found to be associated with occurrence of HCC. Of the 24 nucleotide mutations, 21 increased the risk of HCC while three decreased the risk of HCC (Table 1).

Gene polymorphisms and amino acid substitutions associated with HCC. The 24 significant nucleotide mutations found by χ^2-tests were further analyzed by logistic regression. Eight polymorphisms were significantly related to the increased HCC risk: A028C, G209A, C219U/A, U264C, A271C/U, C378U/A, G435A/C, and G481A; and U303C/A was significantly associated with decreased HCC risk (Table 2). Furthermore, four polymorphisms brought about amino acid substitutions which might change the structure and functions of core protein and lead to HCC. For the other five synonymous mutations, it is possible that they might lead to amino acid substitutions of ARFP (alternative ribosomal frame shift protein) which has been reported to be able to affect cell proliferation and apoptosis[15] (Table 3). However, HCC is thought to be a consequence of long-term infection of HCV and chronic hepatitis. Although the gene polymorphisms and amino acid substitutions in the HCV core region identified in this analysis were proven to be significantly associated with HCC, whether they are "causes" or "effects" of HCC remains to be determined.

Recently, a similar study[16] determined four amino acid substitutions associated with increased HCC risk, and three of them were the same as our results (G209A, A271C/U, and G481A). Moreover, up to now, the G209A polymorphism has been thought to be related with both IFN/Ribavirin (RBV) treatment resistance and HCC.[10,11] The relationship between them is an interesting question. We guess that apoptosis might make an important function in this process. Apoptosis is considered to be a common pathway of virus clearance. HCV core protein could suppress apoptosis and escape this clearance mechanism.[9] On the other hand, inhibition of apoptosis might be able to make the

Table 1. Significant nucleotide mutations found by χ^2-tests

Gene position	Types of Nucleotide			Percentage of mutant			Amino acid			P-values
	Wild		Mutant	Non-HCC		HCC	Wild type		Mutant	
nt 009	G	→	A/U	20.2	→	68.3	Thr		Thr	<0.001*
nt 028	A	→	C	1.1	→	7.9	Lys	→	Gln	0.015†
nt 039	U	→	G/A	0	→	4.8	Arg		Arg	0.015‡
nt 048	C	→	G/U	0	→	4.8	Asn	→	Lys/Asn	0.015‡
nt 066	C	→	U	14.4	→	30.2	Val		Val	0.005*
nt 078	C	→	U	28.7	→	3.2 ↓	Gly		Gly	<0.001*
nt 116	G	→	C	0	→	7.9	Arg	→	Pro	0.001†
nt 209	G	→	A	28.7	→	52.4	Arg	→	Gln	0.001*
nt 219	C	→	U/A	7.4	→	23.8	Gly		Gly	<0.001*
nt 264	U	→	C	12.2	→	36.5	Asn		Asn	<0.001*
nt 270	C	→	U	8	→	33.3	Gly		Gly	<0.001*
nt 271	A	→	C/U	21.8	→	65.1	Met	→	Leu	<0.001*
nt 303	U	→	C/A	64.9	→	47.6 ↓	Arg		Arg	0.015*
nt 309	U	→	C	22.3	→	39.7	Ser		Ser	0.007*
nt 378	C	→	U/A	16.5	→	34.9	Leu		Leu	0.002*
nt 417	C	→	U	5.9	→	20.6	Leu		Leu	0.001*
nt 435	G	→	A/C	10.1	→	34.9	Gly		Gly	<0.001*
nt 446	G	→	A	0.5	→	7.9	Arg	→	Lys	0.004†
nt 456	G	→	A	24.5	→	47.6	Ala		Ala	0.001*
nt 462	C	→	U	25	→	49.2	Gly		Gly	<0.001*
nt 471	U	→	C/G	38.3	→	20.6 ↓	Val		Val	0.01*
nt 472	C	→	G/U	2.1	→	9.5	Leu	→	Val/Leu	0.026†
nt 481	G	→	A	2.7	→	20.6	Gly	→	Ser	<0.001†
nt 549	C	→	U	14.4	→	30.2	Ser		Ser	0.005*

*Pearson χ^2-tests; †continuity correction; ‡Fisher's exact test.

Table 2. Significant nucleotide mutations identified in logistic regression

Gene position	Mutation types	Odds ratio	95% CI		P-values
			Lower	Upper	
nt 028	A → C	14.65	1.31	163.51	0.029
nt 209	G → A	3.30	1.40	7.78	0.006
nt 219	C → U/A	4.37	1.49	12.77	0.007
nt 264	U → C	4.13	1.62	10.52	0.003
nt 271	A → C/U	5.50	2.26	13.37	<0.001
nt 303	U → C/A	0.42	0.18	0.98	0.045
nt 378	C → U/A	2.69	1.08	6.73	0.034
nt 435	G → A/C	4.16	1.61	10.72	0.003
nt 481	G → A	10.484	2.77	39.62	0.001

Nagelkerke R^2 = 0.572.

doi: 10.1111/j.1349-7006.2009.01338.x

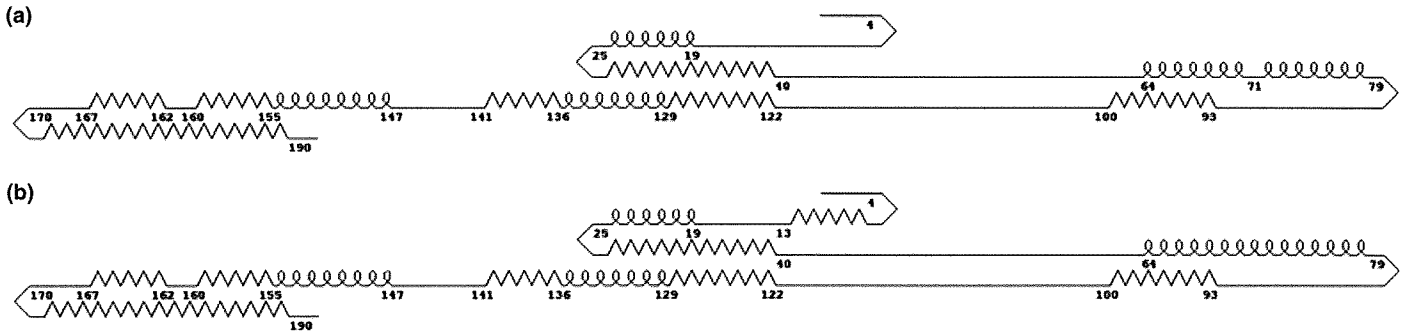

Fig. 1. Secondary structure of wild-type (a) and mutant (b) hepatitis C virus (HCV)-1b core protein predicted by Chou–Fasman analysis with Genetyx. The straight, bent, looped, and zigzag lines represent coil, β-sheet, α-helix, and turn structures, respectively. The numbers indicate the amino acid positions. A028C might turn the coil stucture of AA8-12 into β-sheet and G209A might turn the coil stucture of AA71 into α-helix. (The secondary structure of the wild type was predicted based on the following consensus amino acid sequence of 1336 HCV-1b core, except 70R: MSTNPKPQRKTKRNTNRRPQDVKFPGGGQIVGGVYLLPRRGPRLGVRATRKTSERSQPRGRRQPIPKARRPEGRAWAQPGYPWPLYGNEGMGWAGWLLSPR GSRPSWGPTDPRRRSRNLGKVIDTLTCGFADLMGYIPLVGAPLGGAARALAHGVRVLEDGVNYATGNLPGCSFSIFLLALLSCLTIPASA.)

genetically damaged hepatocytes survive and lead to neoplastic transformation. Whether the amino acid substitution of R70Q could enhance these effects is worth studying.

Alteration of the RNA and protein secondary structures of the *HCV-1b* core gene. Gene mutations of A271C/U, G435A/C, and G481A, were found to be able to cause the changes of the RNA secondary structure of *HCV-1b* core gene using RNAdraw software[17] (Karolinska Institute, Stockholm, Sweden). Furthermore, Chou–Fasman protein secondary prediction with the gene analysis software Genetyx (Genetyx, Tokyo, Japan) showed that A028C might turn the coil structure of AA8-12 into β-sheet and G209A might turn the coil stucture of AA71 into α-helix (Fig. 1). The results indicated that the point-mutations of the core gene might change the secondary structure of not only RNA but also protein. As a result, the functions of both RNA and protein of the core region, such as interaction with other DNA/RNA or proteins (lymphotoxin B receptor, heterogeneous-nuclear ribonucleoprotein, tumor necrosis factor receptor 1, etc.),[18–20] might change and lead to HCC.

In conclusion, eight characteristic mutations in the *HCV-1b* core gene related to the occurrence of HCC were identified: A028C, G209A, C219U/A, U264C, A271C/U, C378U/A, G435A/C, and G481A. Meanwhile, U303C/A was identified to be significantly associated with the decreased HCC risk. These mutations could bring about four amino acid substitutions: K10Q, R70Q, M91L, and G161S. The structural and functional alterations of core protein due to these mutations and the relationship with the occurrence of HCC need to be further studied.

Table 3. Amino acid substitutions of core protein and ARFP due to nucleotide mutations

Codon	Gene position	Amino acid position	Nucleotide			Amino acid		
			Wild type		Mutant	Wild type		Mutant
Normal	nt 028	aa 010	AAA	→	CAA	Lys	→	Gln
	nt 209	aa 070	CGG	→	CAG	Arg	→	Gln
	nt 219	aa 073	GGC	→	GGU/A	Gly		Gly
	nt 264	aa 088	AAU	→	AAC	Asn		Asn
	nt 271	aa 091	AUG	→	C/UUG	Met	→	Leu
	nt 303	aa 101	CGU	→	CGC/A	Arg		Arg
	nt 378	aa 126	CUC	→	CUU/A	Leu		Leu
	nt 435	aa 145	GGG	→	GGA/C	Gly		Gly
	nt 481	aa 161	GGC	→	AGC	Gly	→	Ser
+1 Codon	nt 028	aa 010	GAA	→	GAC	Glu	→	Asp
	nt 209	aa 070	GGC	→	AGC	Gly	→	Ser
	nt 219	aa 073	GCA	→	GU/AA	Ala	→	Val/Glu
	nt 264	aa 088	AUG	→	ACG	Met	→	Thr
	nt 271	aa 091	GCA	→	GCC/U	Ala		Ala
	nt 303	aa 101	GUG	→	GC/AG	Val	→	Ala/Glu
	nt 378	aa 126	UCA	→	UU/AA	Ser	→	Leu/Stop
	nt 435	aa 145	GGG	→	GA/CG	Gly	→	Glu/Ala
	nt 481	aa 161	ACG	→	ACA	Thr		Thr
−1 Codon	nt 028	aa 010	AAA	→	ACA	Lys	→	Thr
	nt 209	aa 070	CCG	→	CCA	Pro		Pro
	nt 219	aa 073	CAG	→	T/AAG	Gln	→	Stop/Lys
	nt 264	aa 088	UGA	→	CGA	Stop	→	Arg
	nt 271	aa 091	CAU	→	CC/UU	His	→	Pro/Leu
	nt 303	aa 101	UGG	→	C/AGG	Trp	→	Arg
	nt 378	aa 126	CAC	→	U/AAC	His	→	Tyr/Asn
	nt 435	aa 145	GGG	→	A/CGG	Gly	→	Arg
	nt 481	aa 161	CGG	→	CAG	Arg	→	Gln

ARFP, alternative ribosomal frame shift protein.

Acknowledgments

The study was supported by the Global COE Program "Center of Education and Research for Advanced Genome-Based Medicine: For personalized medicine and the control of worldwide infectious diseases", the Ministry of Education, Culture, Sports, Science and Technology of Japan; by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by Health and Labor Sciences Research Grants for Research on Hepatitis from the Ministry of Health, Labor and Welfare, Japan; and by a Japan–China Sasakawa Medical Fellowship.

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doi: 10.1111/j.1349-7006.2009.01338.x

CHAPTER 5

HEPATOCELLULAR CARCINOMA 'EPIDEMICS' IN JAPAN

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Introduction

This chapter describes hepatitis C virus (HCV)-related hepatocellular carcinoma (HCC), which has shown an exponential increase in Japan. The epidemiology, molecular pathology, prevention and treatment of the disease are reviewed.

Epidemiology of HCC

The incidence of HCC differs widely geographically. Chronic hepatitis B and C are by far the most predominant causes of HCC. Thus, the incidence and aetiology of HCC are closely related to the prevalence of hepatitis B virus (HBV) and HCV infection in each geographical area. Since chronic HBV infection is acquired mainly through mother-to-neonate transmission during labour (vertical transmission), prevalence of chronic HBV infection varies greatly depending on the area. However, as neonate immunization has been introduced in many countries, the prevalence of HBV infection has been drastically reduced among younger generations in some of these countries. HBV infection is still highly prevalent among adults in East- and South-East Asia and sub-Saharan African countries, and remains the main cause of HCC.

In contrast to HBV, the spread of HCV infection seems to be much more recent. HBV and HCV are both transmitted as blood-borne infections but the latter is much less infectious (Table 5.1). Vertical mother-to-neonate transmission and horizontal sexual transmission are both uncommon with HCV. Instead, the rapid spread of HCV infection has resulted from changes in local circumstances. For example, the high prevalence of HCV infection in Egypt, estimated at 10% or greater, is considered to be due mainly to parenteral antischistosomal therapy commonly administered in the 1960s through to the early 1980s. This resulted in HCV genotype 4a becoming most prevalent and unique to this country. In the United States, HCV spread apparently started in the mid-1960s mainly among injecting drug users and lasted until the mid-1990s. This route of viral transmission has caused a serious problem: coinfection with human immunodeficiency virus (HIV), in which liver damage progresses more rapidly

Table 5.1 Chronic HBV and HCV infection in Japan

	HBV	HCV
Vertical transmission	Common until early 1980s	Rare
Horizontal transmission	Rare in adulthood	Common until 1990 (peaked in 1950s/1960s)
Prevalence	1.5–2.0%	1.5–2.0%
Aetiology in HCC	10–15%	75–80%

due to the immunosuppressed state of the individual. Approximately one-tenth of patients with HCV infection are also infected with HIV in the United States. As treatment for HIV has improved, HCV-related diseases are currently the major cause of mortality in patients with HIV–HCV coinfection.

Japan has had a moderate prevalence of HBV infection, about 1–2%. However, the mortality from HCC has been increasing steadily since the mid-1970s and has more than tripled in the last quarter of the twentieth century. These cases usually have underlying cirrhosis but are characteristically negative for HBV. Following the discovery of HCV, it was found that the recent increment in the HCC incidence in Japan was entirely due to HCV-related HCC. At present, approximately 80% of HCC cases in Japan are due to HCV while 10% are due to HBV.

Approximately 40% of HCV-related HCC cases in Japan have a history of blood transfusion, typically received in the 1950s or 1960s. Immediately after World War II ended in 1945, there was an outbreak of psychostimulant abuse in Japan, mainly with intravenously injected methamphetamine. HCV is thought to have spread first among these injecting drug users, but this may not fully account for the recent increase in HCC. During the same period, a public health insurance system was rapidly organized that covered practically the whole population. The opportunity to receive major surgery with blood transfusion, for conditions such as pulmonary tuberculosis and bleeding peptic ulcers, was considerably increased. However, the blood supply was not based on voluntary blood donation but mainly on paid blood donors, who were frequently also injecting drug users. This unfortunate circumstance accelerated viral spread into the general population in the 1950s and 1960s. The re-use of syringes and needles in medical practice may also have contributed to further viral spread. Commercial blood banks were entirely abolished by 1969 in Japan and replaced by the Japanese Red Cross Society which was, and is, totally based on voluntary blood donation. Syringe and needle re-use was strongly discouraged in the 1970s. Thus, although HCV transmission through blood transfusion continued until a sensitive HCV detection system was established in the early 1990s, HCV spread started to decline in the 1970s. Consequently, HCV prevalence is substantially lower in generations younger than 50 years of age today.

In the United States, investigators identified HBV as a causal agent for HCC in the 1970s. The incidence of HCV-related HCC is now definitely increasing and will continue to do so despite HCV spread, particularly among injecting drug users, already declining partly due to the more rigorous precautions against blood-borne infection stemming from experience with HIV. There is an interval of at least 30 years between the spread of HCV and the increase in HCV-related HCC incidence in Japan. Because of this, HCC incidence will be seen to rise further in Japan, as it will in the USA, in the near future.

Because it takes 30, sometimes 50, years from infection to the development of HCC, tumours occur in relatively elderly people. The life expectancies of men and women in Japan are 76 and 87 years, respectively, so patients live long enough to develop HCC. However, in countries where the life expectancy is shorter, the increase of HCV-induced HCC may be mitigated.

Pathology of HCC

HCV, a positive-stranded RNA virus, is a major causative agent of HCC throughout the world. However, the molecular mechanisms of hepatocarcinogenesis by HCV are unclear. HCV is distantly related to the flaviviruses and the pestiviruses of the *Flaviviridae* family. There have been no reports that flaviviruses or pestiviruses are integrated into the human genome, thus it is unlikely that HCV exerts its oncogenicity through integration into the host genome. The HCV genome consists of approximately 10 kilobases and contains a single large open reading frame that encodes a polyprotein precursor of around 3000 amino acids. This is flanked on either side by an untranslated region (UTR) at the 5' and 3' ends of the genome (Figure 5.1). The putative organization of the HCV genome includes, in order from the 5' end, the 5'-UTR, three or four structural proteins (core, E1, E2/p7), six nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) and the 3'-UTR. It is thought that continuous inflammation, apoptosis or necrosis, and regeneration of hepatocytes caused by HCV infection may increase the probability of gene alteration and thus cause hepatocarcinogenesis. However, there is accumulation of data suggesting that HCV proteins may be directly involved in regulating

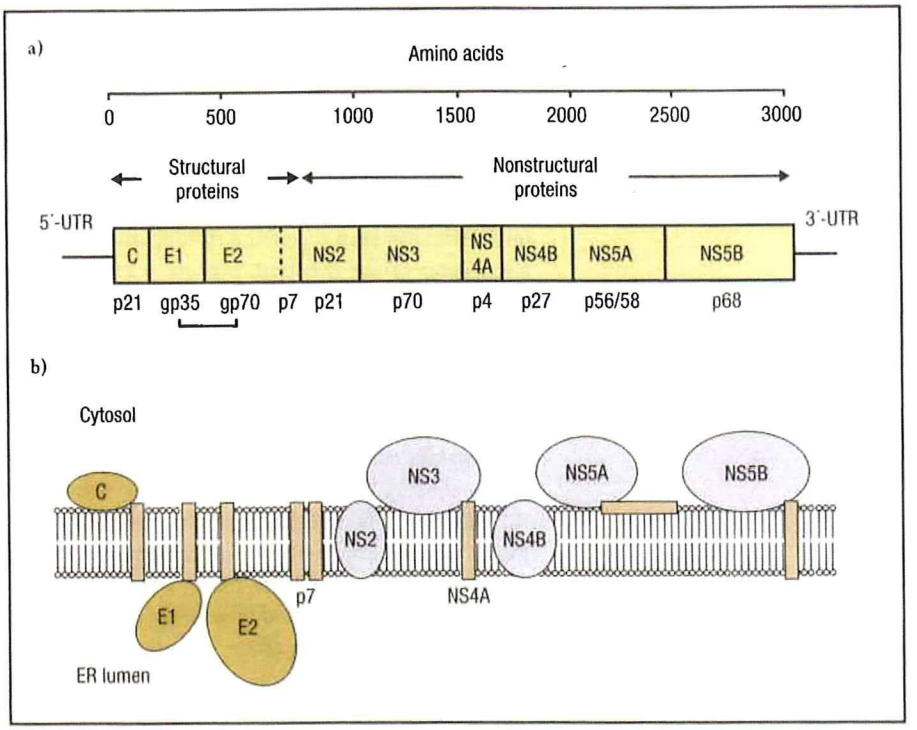

Figure 5.1 Structure of the HCV genome a) and topology of the viral proteins in relation to the endoplasmic reticulum (ER) membrane b)

hepatocyte proliferation. In fact, HCV proteins are known to have various functions other than supporting replication in the host cell, and some of their functions are possibly related to hepatocarcinogenesis, either directly or indirectly (Table 5.2).

Table 5.2 Oncogenic potential of HCV proteins and supporting evidence

Protein	Function	Oncogenic potential
Core	Nucleocapsid	Cell transformation
		Carcinogenesis in transgenic mice
		Transcriptional regulator
		Anti-apoptosis
		Activation of proto-oncogenes
		Repression of tumour suppressor genes
		Impairment of DNA repair
E1	Envelope	Unknown
E2	Envelope	Unknown
p7	Ion channel	Unknown
NS2	Metalloprotease	Unknown
NS3	Serine protease	Cell transformation
	Helicase	Anti-apoptosis
		Repression of tumour suppressor genes
NS4A	Serine protease cofactor	Unknown
NS4B	Unknown	Cell transformation
NS5A	Unknown	Cell transformation
		Anti-apoptosis
		Repression of tumour suppressor genes
		Induction of chromosome instability
NS5B	RNA-dependent RNA polymerase	Repression of tumour suppressor genes

HCV core protein

HCV core protein, forming the viral nucleocapsid, is a possible candidate for hepatocarcinogenesis. The core protein has been reported to transform primary mouse and rat fibroblasts in cooperation with the H-ras oncogene. The core protein interacts with Smad3, inhibits the transforming growth factor (TGF)-β pathway that has anti-proliferative effects, and may promote cell transformation. Moreover, two independent lines of mice transgenic for the HCV core gene developed hepatic steatosis initially, then adenoma with fat droplets, and finally HCC within the adenomas. It has been revealed that the suppressor of cytokine signalling (SOCS)-1, a negative regulator of the cytokine signalling pathway with tumour suppressor activity, is downregulated in the liver of core gene transgenic mice. Moreover, it was shown that knocking out PA28γ, a proteosome activator, disrupts development of HCC in core gene transgenic mice, suggesting that PA28γ plays a crucial role in the development of HCC. These results indicate that the core protein plays an important role in hepatocarcinogenesis.

The core protein can also act as a transcriptional regulator of cellular genes and activate several signal transduction pathways. The core protein activates the c-myc promoter, but suppresses the p21 and p53 promoters. As c-myc is a proto-oncogene and p21 and p53 are tumour suppressors, these functions of the core protein may lead to cell proliferation. It has been reported that the core protein not only decreases p21 expression but also reduces its half-life. Moreover, the core protein activates nuclear factor kappa B (NF-κB), as well as the serum response element (SRE), and activator protein-1 (AP-1)-associated pathways. The NF-κB-associated signal is recognized as a survival signal. NF-κB regulates a large number of genes involved in cell activation and growth control, and is thus thought to be responsible for cell growth and cell transformation. In fact, the core protein promotes proliferation of human hepatoma cells through enhancement of TGF-α expression via activation of NF-κB. One of the *cis*-enhancer elements, AP-1, is a heterodimeric complex containing products of the jun and fos oncogene families, and mediates signals from growth factors, inflammatory peptides, oncogenes and tumour promoters, resulting in cell proliferation. Another *cis*-enhancer element, SRE, is present in the upstream sequence of a number of immediate early genes such as c-fos and binds the complex of p67SRF and an Ets family protein such as Elk-1 to activate transcription. Phosphorylation of Elk-1 by activated mitogen-activated protein kinase (MAPK) is essential for the transcriptional activity of Elk-1. This MAPK cascade is considered to be involved in the regulation of cell proliferation. Thus, the activation of these three cascades (NF-κB-, AP-1- and SRE-associated signals) by the core protein may enhance cell proliferation. Although opposing effects of the core protein have also been reported, the ability of the core protein to activate the aforementioned signals may be advantageous for cell proliferation and transformation.

The core protein has been reported to also have anti-apoptotic effects, although these results are controversial and depend on the type of cells used or how apoptosis is induced. The core protein has been shown to inhibit cisplatin-mediated apoptosis in human cervical cancer cells, apoptosis induced by overexpression of c-myc in Chinese hamster ovarian cells and tumour necrosis factor (TNF)-α-mediated apoptosis in human breast carcinoma cells and human hepatoma cells. Moreover, the core protein inhibited Fas- and TNF-α-mediated apoptosis in human hepatoma cells and breast carcinoma cells by activating NF-κB and Fas-mediated apoptosis through enhanced Bcl-xL expression. It was also reported that the core protein interacts with p53-binding protein, 53BP2/Bbp/ASPP2, and inhibits p53-mediated apoptosis. This anti-apoptotic function of the core protein may help hepatocytes survive apoptosis and establish persistent infection. Because apoptosis is one of the important defence mechanisms against malignant transformation of cells, inhibition of apoptosis by the core protein may result in the survival of transformed cells.

HCV nonstructural proteins

In addition to the mice transgenic for core protein, liver cancer was reported in transgenic mice expressing HCV nonstructural proteins, suggesting the possibility of their involvement in oncogenesis. In fact, NS3, NS4B and NS5A have oncogenic potential. The NS3 protein encodes the viral serine protease and helicase and has been shown to transform NIH3T3 cells