Osteopontin is an extracellular matrix protein with RGD motif mainly produced in the kidney and bone [11]. Previously we found that osteopontin was expressed in activated Kupffer cells and stellate cells in injured rat liver and contributed to migration of macrophages into the necrotic areas [12,13]. Osteopontin is shown to act also as a cytokine essential for the initiation of Th1 immune response in mice [14]. It is known that genetic polymorphisms exist in osteopontin gene (*OPN*) in mice, which determine the magnitude of immune response against bacterial infection [15]. Rickettsial infection subsides in mice with *OPN* of allele A, in which up-regulation of osteopontin expression develops in macrophages, but leads to death in mice with *OPN* of alleles B and C showing no up-regulation [15]. These observations prompted us to postulate whether similar polymorphisms in human *OPN* can determine hepatitis activity through immune response against HCV infection.

In the present study, we analyzed single nucleotide polymorphisms (SNPs) in the promoter region of *OPN* in chronic hepatitis C patients and assessed the relation of identified SNPs to hepatitis activity.

Patients and methods

Patients. Patients were 176 Japanese with chronic hepatitis C who had taken medical examination in the outpatient clinic in Saitama Medical School Hospital between 1st and 31st, August 2002, except for the following patients; (1) aged less than 40 years, (2) experienced interferon therapies with or without ribavirin administration, (3) had daily drinking habit, (4) with body mass index (BMI) greater than 25 kg/m^2. All patients were positive for HCV-RNA and negative for hepatitis B virus surface (HBs) antigen in the sera. The diagnosis of chronic hepatitis was made by histological findings on liver biopsy and/or serum biochemical tests and peripheral blood cell counts. Informed consent to gene analysis was obtained from all the patients.

Serum ALT levels were measured in all the patients until 30th, June 2003 at least for 2 years at intervals of 1–3 months, and they were classified into three groups based on the maximal levels of serum ALT as follows: lower than 30 IU/L (low-activity group), between 30 and 80 IU/L with no hepatoprotective treatment (medium-activity group), and higher than 80 IU/L irrespective of hepatoprotective treatment (high-activity group). Hepatoprotective agents included glycyrrhizin, ursodeoxycholic acid, and Japanese herbal medicine, Sho-saiko-to (TJ-9). The patients given hepatoprotective agents with the maximal levels lower than 80 IU/L were excluded from the assessment.

Measurement of serum HCV-RNA. Serum HCV-RNA levels were measured using a PCR kit (Amplicore HCV Monitor; Riche Diagnostica, Tokyo, Japan). HCV genotype was determined on the basis of sequence of the core region according to the method of Okamoto et al. [16].

SNPs analysis in the promoter region of osteopontin gene. Blood was collected from the patients and genomic DNA was extracted from peripheral blood mononuclear cells. SNPs in the promoter region of *OPN* were analyzed in 20 patients randomly selected from 176 patients by direct sequencing of DNA fragments amplified by polymerase chain reaction (PCR). Then, the identified SNPs were evaluated in 156 patients by Invader assay [17].

For direct sequencing, extracted genomic DNA was amplified in a 50 μl solution with Perkin–Elmer AmpliTag DNA polymerase (Roche Molecular System, Branchburg, NJ) and the oligonucleotide primers were determined from the sequence of the promoter region of human *OPN* [18] as shown in Table 1. The reaction mixture was kept at 94 °C for 3 min for the enzyme activation, followed by 35 amplification cycles. Each cycle consisted of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 60 s. Then, the mixture was incubated at 72 °C for 7 min for final primer extension and the PCR products were checked by DNA agarose gel electrophoresis. Direct sequencing was performed with the use of Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin–Elmer Applied Biosystems) and 373A DNA sequencer (Applied Biosystems).

Invader assay was done according to the previous paper [19] with minor modifications. Primer probes and the Invader oligonucleotide for each SNP were designed with Invader Creator software to have theoretical annealing temperatures of 63 and 77 °C, respectively, shown in Table 2. The reactions were performed using 384-well plate Invader assay FRET detection plates (Third Wave Technologies, Maddison, WI), in which Cleavase XI enzyme, both F (FAM) dye and R (Redmond Red) dye (Epoch Biosciences, Redmond, WA), FRET cassettes, and reaction buffer were dried on each well. Three microliters of the mixtures consisting of appropriate primary probe, Invader oligonucleotide, and $MgCl_2$ was added to the wells, followed by addition of 3 μL of the heat-denatured genomic DNA (≥10 ng/μL) and overlaid with 6 μL of mineral oil (Sigma Chemical, St. Louis, MO). The plates were incubated at 63 °C for 4 h in the DNA thermocycler (RTC-200; MJ Research, Watertown, MA) and then kept at 4 °C until fluorescence measurements. The fluorescent intensities were measured on a Cytoflour 4000 fluorescence plate reader (Applied Biosystems, Foster City, CA) with excitation, 485/20 nm (Wavelength/Bandwidth) and emission, 530/25 nm for F dye detection; excitation, 560/20 nm and emission, 620/40 nm for R dye detection.

Statistical analysis. Univariate analysis was performed using StatView J-5.0 (SAS Institute, Cary, NC). Unpaired *t* test and Fisher's exact test were done for evaluation of the difference among three groups in age and sex of the patients, serum levels and genotypes of HCV-RNA, and the frequency of alleles in each SNP. Multivariate

Table 1
Primers for PCR amplification of the promoter region of osteopontin gene

Amplified regions		Primer sequences	nt
728 bp fragment	Forward	5′-TCA TCT CAA GAT GGC TGG GC-3′	−1880 to −1861
(−1880 to −1153)	Reverse	5′-GAA AAT TAC AGG GAA AGT CCG-3′	−1173 to −1153
609 bp fragment	Forward	5′-TCT GCT ATC CCT GAA TTC TGC-3′	−1203 to −1183
(−1203 to −595)	Reverse	5′-AAA GCA GTT TCT GAC TGA GAG-3′	−615 to −595
498 bp fragment	Forward	5′-GGG AAC AAG GAT AGG TAG GC-3′	−733 to −714
(−733 to −236)	Reverse	5′-GGC ATT CAG CAT CCA GGA AG-3′	−255 to −236
612 bp fragment	Forward	5′-AGC CCT CTC AAG CAG TCA TC-3′	−638 to −619
(−638 to −27)	Reverse	5′-ATG CTG CTG CAG ACA TCC TC-3′	−46 to −27

Table 2
Probes for invader assay of SNPs in the promoter region of osteopontin gene

SNPs	Types of probes	Probe sequences
nt −155	Primary probe 1	acggacgcggagCCAAAAACGCACACAC
	Primary probe 2	cgcgccgaggCAAAAACGCACACACAC
	Invader probe	CCACACTTCCCCCTCTGGTTTTGTGGTTAAAACAAAAAAAAAT
nt −443	Primary probe 1	cgcgccgaggAAACTTGCCTCTGTCC
	Primary probe 2	acggacgcggagGAACTTGCCTCTGTCC
	Invader probe	GAAGGCTATTGTTCAAGCCTGCAAGGAGTTCAGAT
nt −616	Primary probe 1	acggacgcggagAAGGATGACTGCTTGAG
	Primary probe 2	cgcgccgaggCAGGATGACTGCTTGAG
	Invader probe	GATGTTGCAGAAGTAAAGCAGTTTCTGACTGAGAGT
nt −1748	Primary probe 1	acggacgcggagGGACTTCCCTCCACTAA
	Primary probe 2	cgcgccgaggAGACTTCCCTCCACTAAA
	Invader probe	GGCACAGAGTAAACTACAGTAAATCCTGTGGAAATTTTGTTGTTTTTAGAATTT TCT

The small letter indicates the flap sequences of primary probes.

and univariate logistic regression analyses were performed using JMP 5.0.1a (SAS Institute) to select an independent factor that influences on serum ALT levels. Linkage disequilibrium among SNPs was calculated by D' and r^2 according to the method of Devlin and Risch [20].

Results

Demographic and clinical features of the patients

One hundred seventy-six patients were 83 men and 93 women aged 62.5 ± 10.8 (means ± SD) years. Genotypes of serum HCV-RNA were measured in 137 patients and showed 1b in 107 (78.1%), 2a in 21 (15.3%), and 2b in 9 (6.6%). Serum HCV-RNA levels analyzed in 155 patients were higher than 100 kIU/mL in 141 (91.0%).

Eighty-one patients were excluded from the analysis, because they received hepatoprotective treatment with the maximal ALT levels lower than 80 IU/L. There were 16 (16.8%), 19 (20%), and 60 patients (63.2%) in the low-, medium-, and high-activity groups, respectively. As shown in Table 3, men were included more frequently in the high-activity group than in the low-activity group. No differences were found in age of the patients, the genotypes, and serum levels of HCV-RNA among three groups.

SNPs in the promoter region of human osteopontin gene

Direct sequencing of DNA fragments between nt −1880 and −27 in 20 patients revealed 4 SNPs in the promoter region of *OPN*; locating at nt −155 [G/G homozygotes, G/– (deletion) heterozygotes, –/– homozygotes], −443 [C/C homozygotes, C/T heterozygotes, T/T homozygotes], −616 [T/T homozygotes, T/G heterozygotes, G/G homozygotes], and −1748 [A/A homozygotes, A/G heterozygotes, G/G homozygotes]. Prevalence of 4 SNPs including the data obtained from 156 patients by Invader assay is shown in Table 4.

Table 3
Demographic and clinical features of the patients with chronic hepatitis C classified on basis of serum ALT levels.

	Groups[a]		
	Low-activity	Medium-activity	High-activity
Number of patients:	16	19	60
Age	60.7 ± 12.1[d]	59.8 ± 11.4	61.4 ± 10.2
Male:female	4:12	9:10	34:26*
Serum ALT levels (IU/L) medium (maximal–minimal)	24 (14–30)	44 (31–74)	173 (83–568)
Genotypes of HCV (1b:2a:2b:ND)[b]	10:2:1:3	12:3:1:3	35:8:6:11
HCV-RNA levels[c] (high:low:ND)[b]	15:0:1	15:3:1	51:5:4

[a] Maximal serum ALT levels were less than 30 IU/L in low-activity group, between 30 and 80 IU/L with no hepatoprotective treatment in medium-activity group, and higher than 80 IU/L irrespective of hepatoprotective treatment in the high-activity group.
[b] ND means not determined.
[c] HCV-RNA levels higher than 100 kIU/mL were defined as high, and the levels at 100 kIU/mL and lower than 100 kIU/mL as low.
[d] Mean ± SD.
* $p < 0.05$ vs low-activity group by Fisher's exact test.

Table 4
Prevalence of SNPs in the promoter region of osteopontin gene in 176 patients with chronic hepatitis C

SNPs	Number of patients (%)			
nt −155	G/G homozygotes 6 (3.5)	G/− heterozygotes[a] 70 (40.5)	−/− homozygotes[a] 97 (56.1)	ND[b] 3
nt −443	C/C homozygotes 29 (16.8)	C/T heterozygotes 88 (50.9)	T/T homozygotes 56 (32.4)	ND 3
nt −616	T/T homozygotes 6 (3.4)	T/G heterozygotes 70 (39.8)	G/G homozygotes 100 (56.8)	ND 0
nt −1748	G/G homozygotes 5 (2.8)	G/A heterozygotes 71 (40.3)	A/A homozygotes 100 (56.8)	ND 0

[a] − means deletion mutation.
[b] ND means not determined by Invader assay.

Table 5
Linkage disequilibrium coefficient (D' and r^2) among SNPs in the promoter region of osteopontin gene in patients with chronic hepatitis C

SNPs	D'			r^2		
	nt −155	nt −443	nt −616	nt −155	nt −443	nt −616
nt −443	−0.914	—	—	0.189	—	—
nt −616	0.983	−0.956	—	0.937	0.202	—
nt −1748	1.000	−0.955	0.984	0.953	0.198	0.953

Among 4 SNPs, SNPs at nt −155, −616, and 1748 showed linkage disequilibrium with D' and r^2 greater than 0.937 to each other (Table 5).

Table 6 shows the relation between hepatitis activity and 4 SNPs in the promoter region of *OPN*. SNP at nt −443 with T/T homozygotes was detected in 2 of 16 patients (12.5%) in the low-activity group. This prevalence was higher in the medium-activity group (8/19:42.1%) and in the high-activity group (25/57:43.9%) compared to the low-activity group ($p < 0.1$ and $p < 0.05$, respectively). In contrast, SNP at nt −443 with C/T heterozygotes was detected in 12 of 16 patients (75.0%) in the low-activity group, the prevalence was greater compared to the medium-activity group (8/19:42.1%) and the high-activity group (23/57:40.4%) ($p < 0.1$ and $p < 0.05$, respectively). However, there were no similar differences in SNPs at nt −155, −616, and −1748.

Multivariate and univariate logistic regression analyses were performed regarding the outcome variables between the low-activity group ($n = 16$) and the medium- and high-activity groups ($n = 69$). Age and sex of the patients, serum levels and genotypes of HCV-RNA, and alleles of SNPs at nt −155 and nt −443 were selected as predictor variables for univariate analysis. As shown in Table 7, sex of the patients and T/T homozygotes and C/T heterozygotes of SNP at nt −443 were significant variables on univariate analysis. G/− heterozygotes of SNP at nt −155 and C/T heterozygotes of SNP at −443 showing the smallest p values among alleles of each SNP on univariate analysis were selected as predictor variables, and multivariate analysis was done stepwise. Consequently, C/T heterozygotes of SNP at −443 were selected as an independent variable affecting hepatitis activity with odds ratio of 7.0876 ($p < 0.05$). Sex of patients also tended to affect hepatitis activity ($p < 0.1$).

Table 6
Maximal serum ALT levels and SNPs in the promoter region of osteopontin gene in patients with chronic hepatitis C

	Groups[a]		
	Low-activity	Medium-activity	High-activity
Number of patients:	16	19	60
SNP at nt −155 (G/G:G/−:−/−:ND)[b]	1:4:11:0	0:8:11:0	1:25:31:3
SNP at nt −443 (C/C:C/T:T/T:ND)	2:12:2:0	3:8:8:0*	9:23:25:3**
SNP at nt −616 (T/T:T/G:G/G:ND)	1:4:11:0	0:8:11:0	1:27:32:0
SNP at nt −1748 (G/G:G/A:A/A:ND)	1:4:11:0	0:8:11:0	1:27:32:0

[a] Maximal serum ALT levels were less than 30 IU/L in low-activity group, between 30 and 80 IU/L with no hepatoprotective treatment in medium-activity group, and higher than 80 IU/L irrespective of hepatoprotective treatment in the high-activity group.
[b] ND means not determined.
* $p < 0.1$ and ** $p < 0.05$ vs low-activity group by Fisher's exact test in the frequency of T/T homozygotes and C/T heterozygotes.

Table 7
Factors affecting serum ALT levels in patients with chronic hepatitis C

Variables	Parameter	SE	p value	Odds ratio (95% CI)	
Univariate logistic regression analysis					
Age (≥61:≤60)	−0.0634	0.2742	0.8172	0.8810	(0.2960–2.6200)
Sex (male:female)	0.6381	0.3100	0.0395	3.5832	(1.1377–13.6989)
HCV-RNA (low:high)[a]	−4.8606	47.8747	0.9191	0.00006	Cannot be calculated[b]
HCV genotype (2b:1b)	0.1852	0.4965	0.7092	1.4482	(0.2396–14.5213)
HCV genotype (2b:2a)	0.0280	0.6349	0.9648	1.0576	(0.0659–13.9989)
SNP −443 (C/C:others)	−0.1360	0.4094	0.7398	0.7619	(0.1107–3.2291)
SNP −443 (T/T:others)	−0.8406	0.3953	0.0335	0.1861	(0.0280–0.7276)
SNP −443 (C/T:others)	0.7356	0.3114	0.0182	4.3546	(1.3752–16.7276)
SNP −155 (−/−:others)	0.2886	0.2933	0.3252	1.7810	(0.5866–6.1075)
SNP −155 (G/−:others)	−0.4170	0.3110	0.1800	0.4343	(−1.0888–0.1595)
Multi variate logistic regression analysis (Stepwise method)					
Age (≥61:≤60)	−0.4210	0.3481	0.2265	0.4309	Cannot be calculated
Sex (male:female)	0.6603	0.3476	0.0575	3.4758	Cannot be calculated
HCV-RNA (low:high)[a]	−4.9114	42.6466	0.9083	0.0001	Cannot be calculated
SNP −443 (C/T:others)	0.9791	0.3889	0.0118	7.0876	Cannot be calculated
SNP −155 (G/−:others)	−0.3006	0.3460	0.3850	0.5481	Cannot be calculated

Outcome variable: the low-activity group ($n = 16$) vs the medium- and high-activity groups ($n = 79$).
[a] HCV-RNA levels higher than 100 kIU/mL were defined as high, and the levels at 100 kIU/mL and lower than 100 kIU/mL as low.
[b] 95% confidence interval (CI) cannot be calculated, because the parameter in serum HCV-RNA levels is unstable.

Discussion

In the present study, we analyzed the relation between polymorphisms of *OPN* and hepatitis activity in patients with chronic hepatitis C, because osteopontin is shown to be essential for initiation of Th1 immune response at the upstream of IL-18 and IL-12 in a cytokine network [14]. Genetic polymorphisms of *OPN* were determined in mice by restriction fragment length polymorphism (RFLP) survey using *Eco*RV and *Stu*I [15]. Considering that a restriction site for *Eco*RV is present within intron 5 of mouse *OPN* [21], polymorphisms in the intron may provoke diverse immunological response against rickettsial infection in mice through regulation of osteopontin expression in macrophages. In the present study, however, SNPs in the promoter region of *OPN* were analyzed, because nucleotide sequences in the introns are markedly different between human and mice [15], and the *cis*-acting enhancing elements are present in positions at nt −439 to −270, −124 to −80, and −55 to −39 in the promoter region in human [18].

To identify SNPs in the promoter region of human *OPN*, DNA fragments amplified by PCR were directly sequenced at the position between nt −1880 and −27 in 20 patients with chronic hepatitis C. As a result, 4 SNPs locating at nt −155, −443, −616, and −1748 were detected. Among them, SNPs at nt −155, −616, and −1748 had already been registered in a database of Japanese Single Nucleotide Polymorphisms (JSNP) and/or dbSNP (National Center for Biotechnology Information) as follows; SNPs at nt −155 as JST171776 and rs3841166, nt −616 as rs2853744, and nt −1748 as JST171775 and rs2728127 [22]. However, SNP at nt −443 locating at 13 bp-upstream of the *cis*-acting enhancing element was newly identified.

Four SNPs in 156 patients with chronic hepatitis C were evaluated by Invader assay, because DNA amplification by PCR is unnecessary, and thereby is convenient for SNP measurement of many samples [17]. As a result, SNPs at nt −155, −616, and −1747 showed linkage with disequilibrium with a coefficient greater than 0.9 to each other, but similar linkage was not found in the newly identified SNP at nt −443. As shown in Table 4, the frequencies of alleles in SNP at nt −443 were 16.8% for C/C homozygotes, 50.9% for C/T heterozygotes, and 32.4% for T/T homozygotes in Japanese patients with chronic hepatitis C.

In the evaluation of each SNP as a marker of hepatitis activity in chronic hepatitis C patients, hepatitis activity was based on serum ALT levels measured at intervals between 1 and 3 months at least for 2 years. The patients in whom the levels were constantly within the normal range (less than 30 IU/L) without hepatoprotective treatment were defined as the low-activity group. Although slight lymphocyte infiltration with fibrosis may exist in the liver even in HCV carriers with normal ALT levels [9,10], hepatitis activity would be less in the low-activity group than in the medium-activity or high-activity groups with abnormal ALT levels. The patients with maximal ALT levels less than 80 IU/L on hepatoprotective treatment were excluded from the evaluation, since hepatitis activity of such patients is not certain. Also, the patients with daily habit of drinking and with BMI greater than 25.0 kg/m^2 were excluded, because steatohepatitis can affect serum ALT levels. Considering that natural courses of HCV carriers differ

in age [23], the evaluation was done in patients older than 40 years.

As shown in Table 3, there were no differences in demographic and clinical features of the patients among three groups except for male prevalence in the high-activity group compared to the low-activity group. Also, multivariate logistic regression analysis revealed that sex of the patients was a variable having a tendency to affect hepatitis activity in chronic hepatitis C patients. These observations are in line with the report that HCV carriers with normal ALT levels were frequently found in women than in men [24]. It is noteworthy that the prevalence of SNP at nt −443 differed in patients between the low-activity group and the medium- and high-activity groups (Table 6). The frequency of T/T homozygotes in the medium- and high-activity groups (42.1% and 43.9%, respectively) was about 3.5 times higher than that in the low-activity group (12.5%). In contrast, C/T heterozygotes prevailed in the low-activity group; the frequency (75%) was significantly higher than that in the high-activity group (40.4%). Such difference was not found in SNPs at nt −155, −616, and −1748. On multivariate logistic regression analysis, SNP at nt −443 was selected as a significant variable reflecting hepatitis activity (Table 7). Since SNP at nt −443 is locating just at the upstream of the *cis*-acting enhancing element of human *OPN* [18], such SNP can affect the expression of osteopontin in the liver and may diverse immunological response against HCV infection. This matter should be investigated in future by promoter assay with each allele at nt −443.

The present study is the first observation of SNP in the promoter region of *OPN* associated with human diseases, as reported associations with susceptibility to multiple sclerosis [25] and lupus erythematosus [26] are shown in SNPs in the exons of *OPN*. Yee et al. [27] reported that TNF2 allele was more frequently seen in cirrhotic patients with HCV infection than in patients with less severe liver diseases. The significance of SNPs in the promoter region of *OPN* must be studied in relation to SNPs of proinflammatory cytokine genes.

In conclusion, SNP at nt −443 in the promoter region of human *OPN* may be a useful marker reflecting hepatitis activity in chronic hepatitis C patients.

Acknowledgment

We thank Ms. Kayoko Naiki (Third Department of Internal Medicine, Saitama Medical School) for her technical help.

References

[1] Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium, J. Viral Hepat. 6 (1999) 35–47.

[2] J.M. Barrera, M. Bruguera, M.G. Ercilla, C. Gil, R. Celis, M.P. Gil, M. del Valle Onorato, J. Rodes, A. Ordinas, Persistent hepatitis C viremia after acute self-limiting posttransfusion hepatitis C, Hepatology 21 (1995) 639–644.

[3] P. Amoroso, M. Rapicetta, M.E. Tosti, A. Mele, E. Spada, S. Buonocore, G. Lettieri, P. Pierri, P. Chionne, A.R. Ciccaglione, L. Sagliocca, Correlation between virus genotype and chronicity rate in acute hepatitis C, J. Hepatol. 28 (1998) 939–944.

[4] E. Tanaka, K. Kiyosawa, Natural history of acute hepatitis C, J. Gastroenterol. Hepatol. 15 (2000) E97–E104.

[5] A. Cerny, F.V. Chisari, Pathogenesis of chronic hepatitis C: immunological features of hepatic injury and viral persistence, Hepatology 30 (1999) 595–601.

[6] M.J. Tong, N.S. el-Farra, A.R. Reikes, R.L. Co, Clinical outcomes after transfusion-associated hepatitis C, N. Engl. J. Med. 332 (1995) 1463–1466.

[7] H. Yoshida, Y. Shiratori, M. Moriyama, Y. Arakawa, T. Ide, M. Sata, O. Inoue, M. Yano, M. Tanaka, S. Fujiyama, S. Nishiguchi, T. Kuroki, F. Imazeki, O. Yokosuka, S. Kinoyama, G. Yamada, M. Omata, Interferon therapy reduces the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and non-cirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of hepatocarcinogenesis by interferon therapy, Ann. Intern. Med. 131 (1999) 174–181.

[8] M. Naito, N. Hayashi, H. Hagiwara, N. Hiramatsu, A. Kasahara, H. Fusamoto, T. Kamada, Serum hepatitis C virus RNA quantity and histological features of hepatitis C virus carriers with persistently normal ALT levels, Hepatology 19 (1994) 871–875.

[9] M. Persico, E. Persico, R. Suozzo, S. Conte, M. De Seta, L. Coppola, B. Palmentieri, F.C. Sasso, R. Torella, Natural history of hepatitis C virus carriers with persistently normal aminotransferase levels, Gastroenterology 118 (2000) 760–764.

[10] M.M. Jamal, A. Soni, P.G. Quinn, D.E. Wheeler, S. Arora, D.E. Johnston, Clinical features of hepatitis C-infected patients with persistently normal alanine transaminase levels in the Southwestern United States, Hepatology 30 (1999) 1307–1311.

[11] T. Uede, Y. Katagiri, J. Iizuka, M. Murakami, Osteopontin, a coordinator of host defense system: a cytokine or an extracellular adhesive protein? Microbiol. Immunol. 41 (1997) 641–648.

[12] R. Kawashima, S. Mochida, A. Matsui, Y. YouLuTuZ, K. Ishikawa, K. Toshima, F. Yamanobe, M. Inao, H. Ikeda, A. Ohno, S. Nagoshi, T. Uede, K. Fujiwara, Expression of osteopontin in Kupffer cells and hepatic macrophages and Stellate cells in rat liver after carbon tetrachloride intoxication: a possible factor for macrophage migration into hepatic necrotic areas, Biochem. Biophys. Res. Commun. 256 (1999) 527–531.

[13] Y. Wang, S. Mochida, R. Kawashima, M. Inao, A. Matsui, Y. YouLuTuZ, S. Nagoshi, T. Uede, K. Fujiwara, Increased expression of osteopontin in activated Kupffer cells and hepatic macrophages during macrophage migration in *Propionibacterium acnes*-treated rat liver, J. Gastroenterol. 35 (2000) 696–701.

[14] S. Ashkar, G.F. Weber, V. Panoutsakopoulou, M.E. Sanchirico, M. Jansson, S. Zawaideh, S.R. Rittling, D.T. Denhardt, M.J. Glimcher, H. Cantor, Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity, Science 287 (2000) 860–864.

[15] R. Patarca, R.A. Saavedra, H. Cantor, Molecular and cellular basis of genetic resistance to bacterial infection: the role of the early T-lymphocyte activation-1/osteopontin gene, Crit. Rev. Immunol. 13 (1993) 225–246.

[16] H. Okamoto, Y. Sugiyama, S. Okada, K. Kurai, Y. Akahane, Y. Sugai, T. Tanaka, K. Sato, F. Tsuda, Y. Miyakawa, et al., Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources, J. Gen. Virol. 73 (1992) 673–679.

[17] M. De Arruda, V.I. Lyamichev, P.S. Eis, W. Iszczyszyn, R.W. Kwiatkowski, S.M. Law, M.C. Olson, E.B. Rasmussen, Invader technology for DNA and RNA analysis: principles and applications, Expert Rev. Mol. Diagn. 2 (2002) 487–496.

[18] S. Yamamoto, N. Hijiya, M. Setoguchi, K. Matsuura, T. Ishida, Y. Higuchi, S. Akizuki, Structure of the osteopontin gene and its promoter, Ann. N. Y. Acad. Sci. 760 (1995) 44–58.

[19] M. Nagano, S. Yamashita, K. Hirano, M. Ito, T. Maruyama, M. Ishihara, Y. Sagehashi, T. Oka, T. Kujiraoka, H. Hattori, N. Nakajima, T. Egashira, M. Kondo, N. Sakai, Y. Matsuzawa, Two novel missense mutations in the CETP gene in Japanese hyperalphalipoproteinemic subjects: high-throughput assay by Invader assay, J. Lipid Res. 43 (2002) 1011–1018.

[20] B. Devlin, N. Risch, A comparison of linkage disequilibrium measures for fine-scale mapping, Genomics 29 (2) (1995) 311–322.

[21] Y. Miyazaki, M. Setoguchi, S. Yoshida, Y. Higuchi, S. Akizuki, S. Yamamoto, The mouse osteopontin gene: expression in monocytic lineages and complete nucleotide sequence, J. Biol. Chem. 265 (1990) 14432–14438.

[22] H. Haga, R. Yamada, Y. Ohnishi, Y. Nakamura, T. Tanaka, Gene-based SNP discovery as part of the Japanese Millennium Genome Project: identification of 190,562 genetic variations in the human genome. Single-nucleotide polymorphism, J. Hum. Genet. 47 (2002) 605–610.

[23] L.B. Seeff, Natural History of Chronic Hepatitis C, Hepatology 36 (2002) S35–S46.

[24] C. Puoti, A. Magrini, T. Stati, P. Rigato, F. Montagnese, P. Rossi, L. Aldegheri, S. Resta, Clinical, histological, and virological features of hepatitis C virus carriers with persistently normal or abnormal alanine transaminase levels, Hepatology 26 (1997) 1308–1393.

[25] M. Niino, S. Kikuchi, T. Fukazawa, I. Yabe, K. Tashiro, Genetic polymorphisms of osteopontin in association with multiple sclerosis in Japanese patients, J. Neuroimmunol. 136 (2003) 125–129.

[26] A.C. Forton, M.A. Petri, D. Goldman, K.E. Sullivan, An osteopontin (SPP1) polymorphism is associated with systemic lupus erythematosus, Hum. Mutat. 19 (2002) 459.

[27] L.J. Yee, J. Tang, J. Herrera, R.A. Kaslow, D.J. Van Leeuwen, Tumor necrosis factor gene polymorphisms in patients with cirrhosis from chronic hepatitis C virus infection, Genes Immun. 1 (2000) 386–390.

J Gastroenterol 2005; 40:381–388
DOI 10.1007/s00535-005-1558-3

Journal of Gastroenterology

SNPs in the promoter region of the osteopontin gene as a marker predicting the efficacy of interferon-based therapies in patients with chronic hepatitis C

Masashi Naito[1], Atsushi Matsui[1], Mie Inao[1], Sumiko Nagoshi[1], Makoto Nagano[2], Nobuko Ito[2], Tohru Egashira[2], Michie Hashimoto[3], Shunji Mishiro[1,3], Satoshi Mochida[1], and Kenji Fujiwara[1]

[1]Division of Gastroenterology and Hepatology, Internal Medicine, Saitama Medical School, 38 Morohongo, Moroyama-cho, Iruma-gun, Saitama 350-0495, Japan
[2]Department of Gene Science, BML, Inc., Saitama, Japan
[3]Department of Medical Sciences, Toshiba General Hospital, Tokyo, Japan

Background. The T-helper (Th)1 immune reaction is essential for the eradication of hepatitis C virus (HCV) during interferon (IFN) therapy in patients with chronic hepatitis C. Osteopontin is a cytokine crucial for the initiation of the Th1 response. Recently, we identified four single-nucleotide polymorphisms (SNPs) in the promoter region of the osteopontin gene (*OPN*), at nucleotide (nt) -155, -443, -616, and -1748, and suggested that the SNP at nt -443 was a marker reflecting hepatitis activity in patients with HCV. Therefore, we examined the possibility that SNPs in *OPN* were also markers predicting the therapeutic efficacy of IFN in patients with chronic hepatitis C. ***Methods.*** Blood was collected from 77 patients with chronic hepatitis C who had received either IFN monotherapy or IFN-ribavirin combination therapy (IFN-based therapies). SNPs in *OPN*, *MxA*, *MBL*, and *LMP7* were analyzed by Invader assay. ***Results.*** Promoter SNPs of *OPN* at nt -155, -616, and -1748 showed linkage disequilibrium at 100% to each other. Sustained virological response (SVR) was observed in 58% of all patients. The SVR rate was higher in patients with the G/G or G/A alleles in the *OPN* promoter SNP at nt -1748 than in those with A/A (85% vs 45%; $P < 0.05$). The SVR rate was also higher in patients with T/T at nt -443 than in those with C/C or C/T (86% vs 47%; $P < 0.05$). Such differences were particularly evident in patients with HCV genotype 1b who had a pretreatment viral load greater than 100 KIU/ml. All the patients who had G/G or G/A at nt -1748 and T/T at nt -443 obtained an SVR. On the other hand, there was no relationship between the efficacy of IFN-based therapies and SNPs in *MxA*, *MBL*, and *LMP7*, which had been shown to have association with the response to IFN monotherapies. ***Conclusions.*** SNPs in the promoter region of *OPN* may be useful as a marker to predict the efficacy of IFN-based therapies in patients with chronic hepatitis C, and further investigation regarding their real significance is warranted in a large series of patients.

Key words: osteopontin, promoter SNPs, chronic hepatitis C, interferon

Introduction

Persistent infection with the hepatitis C virus (HCV) frequently provokes hepatocyte necrosis by cytotoxic T lymphocytes, due to the T-helper (Th)1 immune reaction,[1] and extracellular matrix deposition in the space of Disse,[2] leading to hepatic fibrosis by the conversion of the normal architecture into structurally abnormal nodules, and finally, to liver cirrhosis. The incidence of hepatocellular carcinoma increased with the degree of hepatic fibrosis in patients with chronic hepatitis C and the annual incidence was 7.9% in patients with liver cirrhosis.[3] Therefore, antiviral therapies, with interferon (IFN) alone or in combination with ribavirin, are required to reduce the risk of carcinogenesis in patients with chronic hepatitis C.

It is well known that the efficacy of IFN monotherapy or IFN-ribavirin combination therapy (IFN-based therapies) depends on the HCV genotype and serum HCV-RNA level; genotype 1b, the predominant genotype of HCV in Japan,[4] had poorer responsiveness to IFN-based therapies than genotypes 2a or 2b, and having a viral load of 100 KIU/ml or more, by Amplicor monitor assay (Roche Diagnostica, Tokyo, Japan) was also an indicator of poor responsiveness to IFN.[5] On the other hand, serum HCV-RNA level was shown to decrease with a biphasic kinetic pattern within 2 weeks after the initiation of IFN-based therapies.[6,7] Neumann

Received: November 30, 2004 / Accepted: January 6, 2005
Reprint requests to: K. Fujiwara

Table 1. Therapies for chronic hepatitis C and genotype and serum level of HCV in patients

	Genotype 1b; <100 KIU/ml	Genotype 1b; ≥100 KIU/ml	Genotype 2a or 2b
(a) Pegylated-IFN-α2b + ribavirin	0[a]	6	0
(b) IFN-α2b + ribavirin	0	7	0
(c) IFN-α2b	1	5	4
(d) IFN-β	5	8	11
(e) IFN-β + IFN-alphacon	0	6	6
(f) IFN-β + IFN-α2b + ribavirin	1	10	7

The schedules for each therapy are outlined in the "Patients and methods" section
[a] Number of patients

et al.[6] suggested that the rapid decrease in the first phase, observed within 1 or 2 days, was due to the direct effect of IFN on HCV production or release from hepatocytes, while the slower decrease in the second phase resulted from immune-mediated elimination of hepatocytes infected with HCV.[6] Furthermore, it was shown that the slope of the decline in the second phase was high in all patients with a sustained virological response (SVR) and low in most non-responders to IFN therapies.[8] Thus, the efficacy of IFN-based therapies may be influenced not only by viral factors but also by host factors, such as the Th1 immune reaction.

Osteopontin is an extracellular matrix protein with an RGD motif, and it is physiologically expressed in the kidney and bone. Previously, we found that osteopontin was expressed in activated Kupffer cells and stellate cells, and that osteopontin contributed to the migration of macrophages into necrotic areas in injured rat liver.[9,10] On the other hand, it was shown that osteopontin also acted as a cytokine essential for the initiation of the Th1 immune response in mice,[11] and that genetic polymorphisms in the osteopontin gene (*OPN*) determined the magnitude of the immune reaction to bacterial infection in mice.[12] Recently, we found four single-nucleotide polymorphisms (SNPs) in the promoter region of human *OPN*, and suggested that SNP in *OPN* at nucleotide (nt) -443 affected hepatitis activity in patients with chronic hepatitis C.[13] From these observations, we assumed that SNPs in the promoter region of *OPN* might be markers to predict the efficacy of IFN-based therapies in HCV-infected patients.

In the present study, we analyzed SNPs in the promoter region of *OPN* in patients with chronic hepatitis C treated with IFN alone or with IFN plus ribavirin, and evaluated the significance of these SNPs as a marker predicting the efficacy of these therapies, as compared with SNPs in the myxovirus resistance protein A gene (*MxA*), the mannose-binding lectin gene (*MBL*), and the low-molecular-mass polypeptide 7 gene (*LMP7*), which had been reported to have associations with the response to IFN therapies.[14–17]

Patients and methods

Patients and interferon-based therapies

The patients were 77 Japanese with chronic hepatitis C who had medical examinations at the outpatient clinic of Saitama Medical School Hospital in August 2002, and who had finished IFN-based therapies by March 2004. All the patients were positive for HCV-RNA before the therapies and negative for hepatitis B virus surface antigen in the sera. The diagnosis of chronic hepatitis was made by histological findings in liver biopsy specimens and/or by serum biochemical tests and peripheral blood cell counts. Documented informed consent for gene analysis was obtained from all the patients.

Each patient received one of six schedules of therapy with IFN (Table 1), as follows; (a) subcutaneous injection of pegylated (Peg)-IFN-α2b, at 1.5 μg/kg body weight once a week, combined with daily oral administration of ribavirin at 600, 800, or 1000 mg/day for patients with a body weight of less than 60 kg, between 60 and 80 kg, and more than 80 kg, respectively, for 48 weeks; (b) intramuscular injection of IFN-α2b, at 6 MU daily for 2 weeks and three times a week for the following 46 weeks, combined with administration of ribavirin as in schedule (a) for 48 weeks; (c) injection of IFN-α2b at 10 MU daily for 4 weeks and three times a week for the following 20 weeks; (d) intravenous injection of IFN-β, at 3 MU, at 12-h intervals for 4 weeks and at 6 MU at 24-h intervals for the following 2 to 8 weeks; (e) injection of IFN-β at 3 MU at 12-h intervals for 4 weeks, and subcutaneous injection of IFN-alphacon at 18 MU three times a week for the following 24 weeks; or (f)

injection of IFN-β at 3MU at 12-h intervals for 4 weeks and injection of IFN-α2b at 10MU three times a week for the following 24 weeks, with administration of ribavirin at 600mg or 800mg for patients with body weights of less than 60kg or 60kg or more, respectively. The intervals between IFN-β injections, and the doses of IFN and ribavirin were changed when severe side effects occurred.

Analysis of serum HCV-RNA and determination of therapeutic efficacy of interferon alone or interferon plus ribavirin

The serum HCV-RNA level was measured using a polymerase chain reaction (PCR) Kit (Amplicor HCV Monitor; Roche Diagnostica, Tokyo, Japan). The HCV genotype was determined on the basis of the sequence of the core region, according to the method of Okamoto et al.[18]

Patients in whom serum HCV-RNA was not detected by PCR (Cobas Amplicor; Roche Diagnostica) for 6 months after the discontinuation of IFN-based therapies were classified as patients with an SVR, and the other patients were classified as those with non-response (NR).

Analysis of SNPs in the genes for osteopontin, myxovirus resistance protein A, mannose-binding lectin, and low-molecular-mass peptide 7

Genomic DNA was extracted from peripheral blood mononuclear cells. SNPs in the promoter region of *OPN*, at nt -155, -443, -616, and -1748; *MxA*, at nt -88 and -123; and *MBL*, at nt -221, and SNPs in *MBL* at G54D and *LMP7* at Q49K were determined by Invader assay.[19]

The Invader assay was done as described previously,[20] with minor modifications. Primer probes and the Invader oligonucleotides for each SNP (Tables 2 and 3) were designed with Invader Creator software (Third Wave Technologies, Madison, WI, USA) to have theoretical annealing temperatures of 63°C and 77°C, respectively. The reactions were performed using 384-well Invader assay fluorescence resonance energy transfer (FRET) detection plates (Third Wave Technologies), in which Cleavase XI enzyme, both F (FAM) dye and R (Redmond Red) dye (Epoch Biosciences, Redmond, WA, USA) FRET cassettes and reaction buffer were dried on each well. Three microliters of a mixture consisting of the appropriate primary probe, Invader oligonucleotide, and $MgCl_2$ was added to the wells, followed by the addition of 3μl of the heat-denatured genomic DNA (≥10ng/μl), and overlaid with 6μl of mineral oil (Sigma Chemical, St. Louis, MO, USA). The plates were incubated at 63°C for 4h in a DNA thermocycler (RTC-200; MJ Research, Watertown, MA, USA), and then kept at 4°C until fluorescence measurements were done. The fluorescence intensities were measured on a Cytofluor 4000 fluorescence plate reader (Applied Biosystems, Foster City, CA, USA) with excitation at 485/20nm (wavelength/bandwidth) and emission at 530/25nm for F dye detection, and excitation at 560/20nm and emission at 620/40nm for R dye detection.

Statistical analysis

The proportions of patients with an SVR were compared among patients with different alleles of the SNPs by Fisher's exact test. Also, these proportions were compared in patients with different backgrounds for viral markers. *P* values of less than 0.05 were regarded as statistically significant.

Table 2. Probes for Invader assay of SNPs in the promoter region of *OPN*

SNP	Type of Probe	Probe sequences
nt -155	Primary probe 1	acggacgcggagCCAAAAACGCACACAC
	Primary probe 2	cgcgccgaggCAAAAACGCACACACAC
	Invader probe	CCACACTTCCCCCTCTGGTTTTGTGGTTAAAACAAAAAAAAAT
nt -443	Primary probe 1	cgcgccgaggAAACTTGCCTCTGTCC
	Primary probe 2	acggacgcggagGAACTTGCCTCTGTCC
	Invader probe	GAAGGCTATTGTTCAAGCCTGCAAGGAGTTCAGAT
nt -616	Primary probe 1	acggacgcggagAAGGATGACTGCTTGAG
	Primary probe 2	cgcgccgaggCAGGATGACTGCTTGAG
	Inavder probe	GATGTTGCAGAAGTAAAGCAGTTTCTGACTGAGAGT
nt -1748	Primary probe 1	acggacgcggagGGACTTCCCTCCACTAA
	Primary probe 2	cgcgccgaggAGACTTCCCTCCACTAAA
	Invader probe	GGCACAGAGTAAACTACAGTAAATCCTGTGGAAATTTTGTTGTTTTT AGAATTTTCT

Lower-case letters indicate the flap sequences of primary probes
nt, nucleotide

Table 3. Probes for Invader assay of SNPs in the genes for Myxovirus resistance protein A (*MxA*), mannose-binding lectin (*MBL*), and low-molecular-mass peptide 7 (*LMP7*)

SNP	Type of probe	Probe sequences
MxA nt -88	Primary probe 1	acggacgcgagGCCCGGAGCCG
	Primary probe 2	cgcgccgaggTCCCGGAGCCGC
	Invader probe	GGGGCCAGGAGCTAGGTTTCGTTTCTGCC
MxA nt -123	Primary probe 1	acggacgcggagGCAGCACTTGCCTC
	Primary probe 2	cgcgccgaggTCAGCACTTGCCTCG
	Invader probe	CCTAGCTCCTGGCCCCGCACCTC
MBL nt -221	Primary probe 1	acggacgcggagGGAAAGCATGTTTATAGTCTTC
	Primary probe 2	cgcgccgaggCGAAAGCATGTTTATAGTCTTC
	Invader probe	GCACGGTCCCATTTGTTCTCACTGCCACT
MBL G54D	Primary probe 1	acggacgcggagGCACCAAGGGAGAAAAG
	Primary probe 2	cgcgccgaggACACCAAGGGAGAAAAG
	Invader probe	GCTTCCCAGGCAAAGATGGGCGTGATGT
LMP7 Q49K	Primary probe 1	acggacgcggagCAGGTCGGGGCAG
	Primary probe 2	cgcgccgaggAAGGTCGGGGCAG
	Invader probe	CCAGAGCTCGCTTTACCCCGGGGAATGT

Lower-case letters indicate the flap sequences of primary probes

Table 4. SNPs in the genes for osteopontin (*OPN*), Myxovirus resistance protein A (*MxA*), mannose-binding lectin (*MBL*), and low-molecular-mass polypeptide 7 (*LMP7*) in patients with chronic hepatitis C treated with interferon with or without ribavirin

SNP	Number of patients (%)		
OPN nt -155	G/G Homozygotes 1 (1.3)	G/- Heterozygotes 25 (32.5)	-/- Homozygotes[a] 51 (66.2)
OPN nt -443	C/C Homozygotes 15 (19.5)	C/T Heterozygotes 40 (51.9)	T/T Homozygotes 22 (28.6)
OPN nt -616	T/T Homozygotes 1 (1.3)	T/G Heterozygotes 25 (32.5)	G/G Homozygotes 51 (66.2)
OPN nt -1748	G/G Homozygotes 1 (1.3)	G/A Heterozygotes 25 (32.5)	A/A Homozygotes 51 (66.2)
MxA nt -88	G/G Homozygotes 36 (46.8)	G/T Heterozygotes 34 (44.2)	T/T Homozygotes 7 (9.1)
MxA nt -123	C/C Homozygotes 29 (37.7)	C/A Heterozygotes 44 (57.1)	A/A Homozygotes 4 (5.2)
MBL nt -221	G/G Homozygotes 67 (87.0)	G/C Heterozygotes 10 (13.0)	C/C Homozygotes 0
MBL G54D	G/G Homozygotes 57 (74.0)	G/A Heterozygotes 13 (16.9)	A/A Homozygotes 7 (9.1)
LMP7 Q49K	C/C Homozygotes 57 (74.0)	C/A Heterozygotes 19 (24.7)	A/A Homozygotes 1 (1.3)

[a] Deletion mutation

Results

Demographic and clinical features of the patients, and therapeutic efficacy of interferon alone or interferon plus ribavirin

The patients were 45 men and 32 women, aged 53.3 ± 10.0 years (mean ± SD), with a range of 25 to 70 years. The genotype of serum HCV-RNA was 1b in 49 patients (63.6%), 2a in 23 (29.9%), and 2b in 5 (6.5%). Of the 49 patients with HCV genotype 1b, 7 patients (14.3%) had a pretreatment viral load of less than 100 KIU/ml (patients with genotype 1b and low titer) and 42 patients (85.7%) had a pretreatment viral load of 100 KIU/ml or more (patients with genotype 1b and high titer).

SVR was achieved in 45 patients (58.4%). The SVR rate was significantly higher in patients with genotype

Table 5. SNPs in the promoter region of *OPN* and response to interferon-based therapies

SNP	Total			Genotype 1b; <100 KIU/ml			Genotype 1b; ≥100 KIU/ml			Genotype 2a or 2b		
nt -155	G/G	G/–	–/–[c]	G/G	G/–	–/–	G/G	G/–	–/–	G/G	G/–	–/–
SVR[a]	1	21	23	0	3	3	1	8	8	0	10	12
NR[b]	0	4	28	0	0	1	0	3	22	0	1	5
P values[d]												
G/G and G/– vs –/–		0.018			>0.999			0.006			0.355	
nt -443	C/C	C/T	T/T	C/C	C/T	T/T	C/C	C/T	T/T	C/C	C/T	T/T
SVR	7	19	19	1	4	1	4	5	8	2	10	10
NR	8	21	3	1	0	0	7	15	3	0	6	0
P values												
C/C and C/T vs T/T		0.002			>0.999			0.029			0.062	
nt -616	T/T	T/G	G/G	T/T	T/G	G/G	T/T	T/G	G/G	T/T	T/G	G/G
SVR	1	21	23	0	3	3	1	8	8	0	10	12
NR	0	4	28	0	0	1	0	3	22	0	1	5
P values												
T/T and T/G vs G/G		0.018			>0.999			0.006			0.355	
nt -1748	G/G	G/A	A/A	G/G	G/A	A/A	G/G	G/A	A/A	G/G	G/A	A/A
SVR	1	21	23	0	3	3	1	8	8	0	10	12
NR	0	4	28	0	0	1	0	3	22	0	1	5
P values												
G/G and G/A vs A/A		0.018			>0.999			0.006			0.355	

[a]SVR, Sustained virological response
[b]NR, Virological non-response
[c]Deletion mutation
[d]*P* values by Fisher's exact test

1b and a low titer than in those with genotype 1b and a high titer (85.7% vs 40.5%, respectively; $P = 0.041$). Twenty-two patients with genotype 2a or 2b obtained an SVR (78.6%).

SNPs in the genes for osteopontin, myxovirus resistance protein A, mannose-binding lectin and low-molecular-mass peptide 7

As shown in Table 4, of the four SNPs in the promoter region of *OPN*, SNPs at nt -155, -616, and -1748 showed linkage disequilibrium at 100% to each others. On the other hand, there was no relationship between the prevalence of the four SNPs in *OPN* and each SNP in the *MxA*, *MBL*, and *LMP7* genes.

Table 5 shows that the response to IFN-based therapies differed depending on the alleles the three SNPs with 100% linkage disequilibrium and the SNP at nt -443. In regard to the SNP at nt -1748, the SVR rate was 84.6% (22/26) in patients with the G/G or G/A alleles, and this was significantly higher than the rate in those with the A/A allele (45.1%; 23/51). The SVR rate in patients with the T/T allele in the SNP at nt -443 (86.4%; 19/22) was also significantly higher than the SVR rate in those with the C/C or C/T alleles (47.3%; 26/55). Similar results were found in patients with genotype 1b and a high titer. Furthermore, all 14 patients showing G/G or G/A in the SNP at nt -1748 and T/T in the SNP at nt -443 obtained an SVR after IFN-based therapies.

As shown in Table 6, there was no relationship between the SVR rate and SNPs in the *MxA*, *MBL*, and *LMP7* genes, except that the rate in patients with G/G or T/T in the promoter SNP of *MxA* at nt -88 tended to be higher than the rate in those with G/T in the group of patients with genotype 1b and a high titer.

Discussion

The present study was designed to evaluate the usefulness of SNPs in the promoter region of *OPN* at nt -155, -443, -616, and -1748 as a marker to predict the therapeutic efficacy of IFN alone or combined with ribavirin in patients with chronic hepatitis C. Alleles of SNPs were determined by the Invader assay, an assay that does not include DNA amplification by polymerase chain reaction (PCR), and which is applicable to the measurement of many samples.[19] Among the four SNPs

Table 6. SNPs in the genes for myxovirus resistance protein A (*MxA*), mannose-binding lectin (*MBL*), and low-molecular-mass polypeptide 7 (*LMP7*) and response to interferon-based therapies

SNP		Total			Genotype 1b; <100 KIU/ml			Genotype 1b; ≥100 KIU/ml			Genotype 2a or 2b	
MxA	G/G	G/T	T/T	G/G	G/T	T/T	G/G	G/T	T/T	G/G	G/T	T/T
At nt -88												
SVR[a]	22	19	4	3	3	0	10	4	3	9	12	1
NR[b]	14	15	3	1	0	0	8	14	3	5	1	0
P values[c]												
G/T vs C/G and T/T		0.816			>0.999			0.057			0.173	
MxA	C/C	C/A	A/A	C/C	C/A	A/A	C/C	C/A	A/A	C/C	C/A	A/A
At nt -123												
SVR	26	16	3	3	3	0	10	5	2	13	8	1
NR	18	13	1	1	0	0	12	12	1	5	1	0
P values												
C/C vs C/A and A/A		>0.999			>0.999			0.543			0.375	
MBL	G/G	G/C	C/C	G/G	G/C	C/C	G/G	G/C	C/C	G/G	G/C	C/C
At nt -221												
SVR	38	7	0	6	0	0	14	3	0	18	4	0
NR	29	3	0	1	0	0	22	3	0	6	0	0
P values												
G/G vs G/C		0.514			>0.999			0.672			0.549	
MBL G54D	G/G	G/A	A/A	G/G	G/A	A/A	G/G	G/A	A/A	G/G	G/A	A/A
SVR	33	8	4	5	1	0	11	4	2	17	3	2
NR	25	5	3	1	0	0	18	4	3	5	1	0
P values												
G/G vs G/C and A/A		>0.999			>0.999			0.738			>0.999	
LMP7 Q49K	C/C	C/A	A/A	C/C	C/A	A/A	C/C	C/A	A/A	C/C	C/A	A/A
SVR	33	11	1	4	2	0	13	3	1	16	6	0
NR	24	8	0	1	0	0	18	7	0	5	1	0
P values												
C/C vs C/A and A/A		>0.999			>0.999			>0.999			>0.999	

[a] SVR, Sustained virological response
[b] NR, Virological non-response
[c] *P* values by Fisher's exact test

described here, those at nt -155, -616, and -1748 had already been registered in a database of Japanese single-nucleotide polymorphisms (JSNP) and/or in the dbSNP (National Center for Biotechnology Information),[21] and we previously found that these SNPs showed linkage disequilibrium, with coefficients (D' and r^2) greater than 0.937 to each other in patients with chronic hepatitis C without any experience of IFN-based therapies.[13] The SNP at nt -443 was recently identified by our group,[13] and we found that the SNP at -443, but not the other three SNPs, had a close association with hepatitis activity in patients with chronic hepatitis C.[13] In the present study, the prevalence of the four SNPs of *OPN* (Table 4) was similar to that observed in patients without experience of IFN-based therapies,[13] and the SNPs at nt -155, -616, and -1748 showed 100% linkage disequilibrium to each other. These results suggested that there were no differences in genetic background, regarding *OPN*, between the patients with chronic hepatitis C who received IFN-based therapies and those without such experience at our hospital.

As shown in Table 5, the SVR rate differed depending on the alleles of the four SNPs in the promoter region of *OPN*. Such differences were particularly evident in patients with genotype 1b and a high titer. Moreover, all the patients with G/G or G/A at nt -1748 and T/T at nt -443 obtained an SVR after IFN-based therapies. Therefore, it was suggested that the SNP in the promoter region of *OPN* at nt -443 and the three SNPs at nt -155, -616, and -1748 with linkage disequilibrium were useful as a marker to predict the therapeutic efficacy of IFN alone or IFN plus ribavirin, especially in patients with genotype 1b and a high titer.

In this study, the SVR rate in patients with T/T in SNP of *OPN* at nt -443 was 86%. Previously, we reported that the frequency of T/T at nt -443 was about 3.5 times higher in patients with chronic hepatitis C with serum alanine aminotransferase (ALT) levels higher than 80 IU/l than in those with an ALT level lower than

30 IU/l.[13] The SNP at nt -443 is located 13 base pairs (bp) upstream of the cis-acting enhancing element of human *OPN*.[22] Considering that the Th1 response is involved in the development of inflammation in chronic hepatitis C[1] and that hepatocytes infected with HCV are eradicated by Th1 response during IFN-based therapies,[6] the SNP in *OPN* at nt -443 may be crucial in provoking diverse Th1 immune reactions against HCV through the regulation of osteopontin expression in the liver. This matter should be investigated in future by carrying out promoter assays with each allele at nt -443.

Hijikata et al.[14,15] reported that G/G at nt -88 and C/C at nt -123 in the promoter region of *MxA* were observed more frequently in patients with NR than in those with an SVR after IFN therapy. *MxA* was shown to encode an IFN-inducible protein that inhibited the replication of single-stranded RNA viruses.[23,24] Matsushita et al.[16] showed that the frequencies of C/C at nt -221 in the promoter region of *MBL* and A/A at G54D in *MBL* were higher in patients with NR than in those with an SVR. Mannose-binding lectin is an acute-phase reactant protein inducing the phagocytosis of macrophages through binding to the surface of pathogens, and it is known to be essential for the innate immune system.[25,26] Sugimoto et al.[17] found that the frequency of C/A at Q49K in *LMP7* was higher in patients with an SVR than in those with NR, especially in patients with serum HCV-RNA levels less than 100 KIU/ml. Low-molecular-mass polypeptides were shown to play a crucial role in human leukocyte antigen (HLA) class I-restricted antigen-presenting systems.[27] However, we found no relationship between the SVR rate after IFN-based therapies and the alleles of these SNPs in patients with chronic hepatitis C. As outlined in Table 1 and the "Patients and methods" section, most patients received IFN-ribavirin combination therapy or IFN monotherapy with 3 MU of IFN-β injection given at 12-h intervals for 4 weeks as an induction therapy. This monotherapy was shown to be superior in HCV antiviral effects to 6 MU IFN-β injection given at 24-h intervals.[28] IFN-ribavirin combination therapy was also reported to show a higher SVR rate than IFN monotherapy.[29] The rate of SVR in patients with genotype 1b and a high titer in the present study was 40.5%. In previous studies regarding SNPs in *MxA*, *MBL*, and *LKP7*, all the patients received IFN-α for 24 weeks or less.[14–17] After IFN-α therapy for 24 weeks, the SVR rate was reported to be only 7%–8% in patients with genotype 1b and a high titer.[30] The differences in the antiviral effects of these therapies may produce discrepancies in the results regarding SNPs in *MxA*, *MBL*, and *LMP7*. This matter should be further investigated in a large series of patients in whom standardized therapy with Peg-IFN-α2b and ribavirin is done for 48 weeks.

In conclusion, four SNPs in the promoter region of *OPN* may be useful as a marker to predict the efficacy of IFN-based therapies in patients with chronic hepatitis C.

Acknowledgments. We thank Ms. Kayoko Naiki (Division of Gastroenterology and Hepatology, Internal Medicine, Saitama Medical School) for her technical help. This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS), Scientific Research (B)(2); 14370191.

References

1. Cerny A, Chisari FV. Pathogenesis of chronic hepatitis C: immunological features of hepatic injury and viral persistence. Hepatology 1999;30:595–601.
2. Friedman SL. Seminars in medicine of the Beth Israel Hospital, Boston. The cellular basis of hepatic fibrosis: mechanisms and treatment strategies. N Engl J Med 1993;328:1828–35.
3. Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, et al. Interferon therapy reduces the risk for hepatocellular carcinoma: National surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT Study Group. Inhibition of Hepatocarcinogenesis by Interferon Therapy. Ann Intern Med 1999;131:174–81.
4. Arase Y, Ikeda K, Chayama K, Murashima N, Tsubota A, Suzuki Y, et al. Fluctuation patterns of HCV-RNA serum level in patients with chronic hepatitis C. J Gastroenterol 2000;35:221–5.
5. Shiratori Y, Kato N, Yokosuka O, Imazeki F, Hashimoto E, Hayashi N, et al. Predictors of the efficacy of interferon therapy in chronic hepatitis C virus infection. Tokyo-Chiba Hepatitis Research Group. Gastroenterology 1997;113:558–66.
6. Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, et al. Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-α therapy. Science 1998;282:103–7.
7. Asahina Y, Izumi N, Uchihara M, Noguchi O, Tsuchiya K, Hamano K, et al. A potent antiviral effect on hepatitis C viral dynamics in serum and peripheral blood mononuclear cells during combination therapy with high-dose daily interferon α plus ribavirin and intravenous twice-daily treatment with interferon β. Hepatology 2001;34:377–84.
8. Zeuzem S, Herrmann E, Lee JH, Fricke J, Neumann AU, Modi M, et al. Viral kinetics in patients with chronic hepatitis C treated with standard or peginterferon α2a. Gastroenterology 2001;120:1438–47.
9. Kawashima R, Mochida S, Matsui A, YouLuTuZ Y, Ishikawa K, Toshima K, et al. Expression of osteopontin in Kupffer cells and hepatic macrophages and stellate cells in rat liver after carbon tetrachloride intoxication: a possible factor for macrophage migration into hepatic necrotic areas. Biochem Biophys Res Commun 1999;256:527–31.
10. Wang Y, Mochida S, Kawashima R, Inao M, Matsui A, YouLuTuZ Y, et al. Increased expression of osteopontin in activated Kupffer cells and hepatic macrophages during macrophage migration in *Propionibacterium acnes*-treated rat liver. J Gastroenterol 2000;35:696–701.
11. Ashkar S, Weber GF, Panoutsakopoulou V, Sanchirico ME, Jansson M, Zawaideh S, et al. Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. Science 2000;287:860–4.
12. Patarca R, Saavedra RA, Cantor H. Molecular and cellular basis of genetic resistance to bacterial infection: the role of the early T-lymphocyte activation-1/osteopontin gene. Crit Rev Immunol 1993;13:225–46.

13. Mochida S, Hashimoto M, Matsui A, Naito M, Inao M, Nagoshi S, et al. Genetic polymorphysms in promoter region of osteopontin gene as a marker for predicting hepatitis activity in chronic hepatitis C patients. Biochem Biophys Res Commun 2004;313:1079–85.
14. Hijikata M, Ohta Y, Mishiro S. Identification of a single nucleotide polymorphism in the MxA gene promoter (G/T at nt -88) correlated with the response of hepatitis C patients to interferon. Intervirology 2000;43:124–7.
15. Hijikata M, Mishiro S, Miyamoto C, Furuichi Y, Hashimoto M, Ohta Y. Genetic polymorphism of the MxA gene promoter and interferon responsiveness of hepatitis C patients: revisited by analyzing two SNP sites (-123 and -88) in vivo and in vitro. Intervirology 2001;44:379–82.
16. Matsushita M, Hijikata M, Matsushita M, Ohta Y, Mishiro S. Association of mannose-binding lectin gene haplotype LXPA and LYPB with interferon-resistant hepatitis C virus infection in Japanese patients. J Hepatol 1998;29:695–700.
17. Sugimoto Y, Kuzushita N, Takehara T, Kanto T, Tatsumi T, Miyagi T, et al. A single nucleotide polymorphism of the low molecular mass polypeptide 7 gene influences the interferon response in patients with chronic hepatitis C. J Viral Hepatitis 2002;9:377–84.
18. Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, et al. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol 1992;73:673–9.
19. De Arruda M, Lyamichev VI, Eis PS, Iszczyszyn W, Kwiatkowski RW, Law SM, et al. Invader technology for DNA and RNA analysis: principles and applications. Expert Rev Mol Diagn 2002;2:487–96.
20. Nagano M, Yamashita S, Hirano K, Ito M, Maruyama T, Ishihara M, et al. Two novel missense mutations in the *CETP* gene in Japanese hyperalphalipoproteinemic subjects: high-throughput assay by Invader ® assay. J Lipid Res 2002;43:1011–8.
21. Haga H, Yamada R, Ohnishi Y, Nakamura Y, Tanaka T. Gene-based SNP discovery as part of the Japanese Millennium Genome Project: identification of 190562 genetic variations in the human genome. Single-nucleotide polymorphism. J Hum Genet 2002;47: 605–10.
22. Yamamoto S, Hijiya N, Setoguchi M, Matsuura K, Ishida T, Higuchi Y, et al. Structure of the osteopontin gene and its promoter. Ann NY Acad Sci 1995;760:44–58.
23. Chang KC, Hansen E, Foroni L, Lida J, Goldspink G. Molecular and functional analysis of the virus- and interferon-inducible human MxA promoter. Arch Virol 1991;117:1–15.
24. Zhao H, De BP, Das T, Banerjee AK. Inhibition of human parainfluenza virus-3 replication by interferon and human MxA. Virology 1996;220:330–8.
25. Ezekowitz RA, Day LE, Herman GA. A human mannose-binding protein is an acute-phase reactant that shares sequence homology with other vertebrate lectins. J Exp Med 1998;167: 1034–46.
26. Hartshorn KL, Sastry K, White MR, Anders EM, Super M, Ezekowitz RA, et al. Human mannose-binding protein functions as an opsonin for influenza A viruses. J Clin Invest 1993;91:1414–20.
27. Arnold D, Driscoll J, Androlewicz M, Hughes E, Cresswell P, Spies T. Proteasome subunits encoded in the MHC are not generally required for the processing of peptides bound by MHC class I molecules. Nature 1992;360:171–4.
28. Fujiwara K, Mochida S, Matsuo S, Ogata I, Hayashi S, Sato Y. Randomized control trial of interferon-β injections at 12-h intervals as a therapy for chronic hepatitis C. Hepatol Res 1998;12: 240–51.
29. Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, et al. Randomized trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet 1998;352:1426–32.
30. Kasahara A. Treatment strategies for chronic hepatitis C virus infection. J Gastroenterol 2000;35:411–23.

Original Paper

The Impact of Splenectomy or Splenic Artery Ligation on the Outcome of a Living Donor Adult Liver Transplantation using a Left Lobe Graft

Mitsuo Shimada MD, FACS, Hideki Ijichi MD, Yusuke Yonemura MD, Noboru Harada MD
Satoko Shiotani MD, Mizuki Ninomiya MD, Takahiro Terashi MD, Tomoharu Yoshizumi MD
Yuji Soejima MD, Taketoshi Suehiro MD, Yoshihiko Maehara MD
Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University
Fukuoka, Japan
Corresponding Author: Mitsuo Shimada, MD, PhD, Department of Surgery and Science
Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi
Higashi-ku, Fukuoka 812-8582, Japan
Tel: +81 92 642 5466, Fax: +81 92 642 5482, E-mail: mshimada@surg2.med.kyushu-u.ac.jp

ABSTRACT

Background Aims: The aim of this study was to clarify the impact of splenectomy or splenic artery ligation on the outcome in living donor adult liver transplantation (LDALT) using a left lobe graft.

Methodology: Forty-eight LDALT cases using a left lobe graft were enrolled in this study. The patients were classified into two groups; Group A (n=40), in which neither a splenectomy nor a splenic artery ligation was performed, and Group B (n=8), in which a splenectomy (n=6) or a splenic artery ligation (n=2) was performed. Indications for splenectomy were as follows; 1) demonstrating a hypersplenism and/or 2) having splenic aneurysms.

Results: None of the patients receiving a splenectomy or a splenic artery ligation experienced any septic complication in this series. The graft-recipient weight ratio in group B tended to be smaller than in group A. In group B, all patients were classified into Child's class C or B. The incidence of esophageal varices in group B was significantly higher than in group A. Moreover, the platelet count and the white blood cell count in group B were significantly lower than in group A. No statistical difference was found in postoperative functional cholestasis and intractable ascites. None of the participants in group B experienced both postoperative hyperbilirubinemia and intractable ascites, which were characterized as a small-for-size graft after LDALT. The patient survival rate in group B seems to be better than in group A. In a majority of the cases, the portal pressure as well as the portal vein flow after a splenectomy decreased in comparison to that before the splenectomy.

Conclusions: Splenectomy or splenic artery ligation is considered to be beneficial for improving the outcome in LDALT using a left lobe graft.

KEY WORDS:
Portal pressure; Portal flow; Small-for-size graft; Complication

ABBREVIATIONS:
Living Donor Adult Liver Transplantation (LDALT)

INTRODUCTION

Recently, living donor liver transplantation (LDLT) has been extended to include adult-to-adult liver transplantation (1). To avoid small-for-size graft, a right lobe graft was used in living donor adult liver transplantation (LDALT) (2-5). The ideal graft volume recommended is over 40% of the standard liver volume. In adult recipients, if a left lobe graft is selected, the graft volume is often less than 40% of the standard liver volume. In LDALT, several authors reported that graft size is one of the major factors in determining the outcome after LDALT (6-8). On the other hand, we have recently reported that a left lobe graft still remains an important option for LDALT (9).

One of the major causes of graft loss in small-for-size grafts is considered to be excessive portal venous flow to the graft, and Boillot *et al.* (10) have recently reported that a reduction of portal venous flow to the graft brought a good outcome in the small-for-size graft (10). To improve hypersplenism and to avoid graft congestion due to excessive portal venous flow, either a splenectomy or a splenic artery ligation should be taken into consideration. Up to now, splenectomy in liver transplantation has been mostly reported to have an adverse effect on the outcome of liver transplantation, due to the postsplenectomy septic complications (11-14). At our institution, laparoscopic splenectomy for cirrhotic patients with hypersplenism has also been a first choice of treatment (15,16). In cirrhotic patients with hepatocellular carcinoma and hypersplenism (17), simultaneous splenectomy has also been the best treatment strategy. Furthermore, none of the patients experienced an overwhelming septic complication in over one hundred of the cirrhotic patients who underwent either laparoscopic splenectomy or a simultaneous splenectomy

Hepato-Gastroenterology 2004; 51:625-629

TABLE 1 Comparison of Donor's, Graft's and Recipient's Variables between Groups A and B

Variables	Group A (n=40)	Group B (n=8)	*P* value
Donor variables			
Age (years)	33 (24, 48)*	35 (28, 47)	0.78
Gender (male:female)	29:11	6:2	0.99
Operative procedure (LL: LL+CL)	14:26	0:8	0.08
Operation time (min)	453 (375, 511)	436 (405, 462)	0.54
Allogenic blood transfusion	none	none	
Blood loss (mL)	853 (562, 1300)	638 (410, 900)	0.12
Postop. AST (IU/L)	244 (212, 358)	299 (223, 378)	0.30
Postop. ALT (IU/L)	271 (207, 362)	368 (291, 430)	0.13
Postop. bilirubin (mg/dL)	1.6 (1.3, 2.1)	1.8 (1.0, 2.2)	0.84
Postop. complication	1 (biliary stricture)	0	0.99
Postop. hospital stay (days)	11 (10, 14)	10 (7, 13)	0.23
Graft variables			
Graft weight (g)	440 (340, 495)	435 (395, 508)	0.41
GWRW ratio	0.84 (0.65, 0.93)	0.70 (0.62, 0.81)	0.20
Warm ischemic time (min)	38 (34, 42)	38 (34, 43)	0.97
Cold ischemic time (min)	59 (39, 76)	52 (41, 66)	0.61
HAF in recipient (mL/min)	100 (65, 125)	100 (70, 135)	0.71
PVF in recipient (mL/min)	980 (778, 1345)	1175 (970, 1650)	0.27
Recipient variables			
Age (years)	47 (38, 54)	53 (47, 60)	0.08
Gender (male:female)	15:25	5:3	0.25
Body weight (kg)	52.6 (48.0, 59.3)	66.6 (55.1, 79.8)	0.03
ABO blood type (identical:compatible)	31:9	7:1	0.67
Diagnosis			
FHF	19	0	
PBC	11	1	
LC (with/without HCC)	5	7	
PSC	2	0	
Others	3	0	
Child's Class** (A:B:C)	6:0:16	0:2:6	
Esophageal varices	12/40	7/8	<0.01
Platelet count (x10⁴/μL)	8.0 (4.9, 16.5)	3.9 (3.3, 4.8)	<0.01
WBC count (/μL)	5330 (3515, 8900)	2205 (1560, 3970)	<0.01
Operation time (min)	734 (623, 818)	884 (707, 949)	0.13
Blood loss (g)	4810 (2850, 6500)	6630 (5175, 11500)	0.06
Postop. hyperbilirubinemia (>10mg/dL at 14POD)	8/40	1/8	0.99
Intractable ascites (>1000mL at 30POD)	5/40	0/8	0.57
Postop. complications			
Biliary	7	1	
Bleeding	3	0	
Cytomegalovirus infection	1 (pneumonia)	1 (esophagitis)	
HAT	2	0	
PVT	1	0	
Severe delirium	0	1	
Graft *versus* host disease	1	0	
Acute cellular rejection	9 (23%)	2 (25%)	0.99

Group A was defined as a control in which neither splenectomy nor splenic artery ligation was performed. Group B was defined as a group in which either splenectomy or splenic artery ligation was performed. *: Data are expressed as medians (25th percentile, 75th percentile). **: fulminant hepatic failure was excluded in this entity. AST: aspartate aminotransferase; ALT: alanine aminotransferase; postop.: postoperative; LL: left hepatectomy; LL+CL: left hepatectomy plus caudate lobectomy; GW: graft weight; SLV: standard liver volume; GWRW: graft weight/recipient weight; HAF: hepatic artery flow; PVF: portal venous flow; FHF: fulminant hepatic failure; PBC: primary biliary cirrhosis; LC: liver cirrhosis; HCC: hepatocellular carcinoma; PSC: primary sclerosing cholangitis; WBC: white blood cell; POD; postoperative day.

with hepatectomy.

The aim of this study was to clarify the impact of splenectomy or splenic artery ligation on the outcome in LDALT using a left lobe graft.

METHODOLOGY

Sixty-seven LDALTs were performed at Kyushu University Hospital between October 1996 and May 2002. Among them, 48 LDALT cases, in which a left lobe graft was used, were enrolled in this study. Three retransplantations as well as one blood-type incompatible transplantation were excluded from this study. The indication for 48 LDALTs included fulminant hepatic failure in 19, primary biliary cirrhosis in 12, hepatitis C-cirrhosis in 12, familial amyloidotic polyneuropathy in 2, primary sclerosing cholangitis in 2, and another condition in 1. The donors were 3 husbands, 2 wives, 10 brothers, 5 sisters, 17 sons, 2 fathers, 2 mothers, 5 daughters, and others in two cases.

Regarding the selection of the graft, graft harvesting technique, recipient operation and perioperative patient management of recipients, including immunosuppression regimen, the conditions are described elsewhere (9,18). Immunosuppression commenced as either tacrolimus (Prograf, Fujisawa Pharmaceutical Co. Ltd., Japan)- or cyclosporine A (Neoral, Novartis Pharma. K.K., Japan)-based protocol.

All patients had a monthly follow-up, and the median follow-up period was 745 days with 190 days and 1060 days as a 25th percentile and 75th percentile, respectively. Patient survival was defined as the time period between LDALT and patient death.

The patients were classified into two groups: Group A (n=40), in which neither a splenectomy nor a splenic artery ligation was performed, and Group B (n=8), in which splenectomy (n=6) or splenic artery ligation (n=2) was performed. Among 6 splenectomized patients, one had an accessory spleen that was preserved and one received autotransplantation of a partial spleen (approximately 50g) in the retroperitoneal space of the left upper abdomen.

In a recent series of cirrhotic patients with portal hypertension, either a splenectomy or a splenic artery ligation has been principally used. Indications for a splenectomy were as follows: 1) demonstrating a hypersplenism and/or 2) having splenic aneurysms. If the platelet count is of over 50,000/μL, a splenic artery ligation was principally used.

Donor's, recipient's and graft's variables were compared between group A and group B.

Regarding the blood flowmetry, an electromagnetic blood flowmeter (MVF-3100, NIHON KOHDEN Corp., Tokyo, Japan) was used. All measurements were performed under the stable hemodynamic condition of the patients. In the recent cases, portal pressure was also monitored through a mesenteric vein during the operation.

Using the patients with data available for both portal pressure and portal vein flow before and after splenectomy, the changes in portal pressure and portal vein flow were investigated.

Statistics

The data were expressed as medians (25th percentile and 75th percentile). The Mann-Whitney U-test with continuous variables and the chi-squared test of independence with categorical variables were used to compare the two groups. The survival was calculated by the product limit method of Kaplan and Meier, and the differences in the survival between the groups were then compared using the log-rank test. The software of StatView (Version 4.11; Abacus Concepts, Inc., Berkeley, CA94704-1014, U.S.A.) was used for all analyses on a Macintosh computer. A $p<0.05$ was considered to be significant.

RESULTS

No patient, who received a splenectomy or a splenic artery ligation, experienced any septic complication in this series. **Table 1** shows a comparison of donor's, recipient's and graft's variables between groups A and B. Regarding the donor's variables, the rate of operative procedure of left hepatectomy plus caudate lobectomy in Group B tended to be higher than in group A. No other significant differences were observed between the two groups. No allogenic blood transfusion was conducted, furthermore, at our institution there was only one significant postoperative complication consisting of a biliary stricture requiring reoperation in the initial period of LDALT. Regarding the graft's variables, no difference in graft volume was found between the two groups. The recipient's body weights in group B were heavier than in group A, therefore, a graft-recipient weight ratio in group B tended to be smaller than in group A. No other significant differences were found in warm ischemic time, cold ischemic time, hepatic blood flow, and portal flow, between the two groups. Regarding the recipient's variables, the age of the members in group B tended to be higher than the members of group A. In their diagnoses, half of the patients in group A had fulminant hepatic failure and had no portal hypertension. On the other hand, in group B, all patients had liver cirrhosis. In group B, all patients were classified into Child's class C or B. The incidence of esophageal varices in group B was higher than in group A. Moreover, the platelet count and white blood cell count in group B were lower than in group A. In group B, the operation time tended to be longer than in group A, and the blood loss tended to be larger than in group A. No statistical difference was found in the postoperative functional cholestasis and intractable ascites. None of the members of group B experienced either postoperative hyperbilirubinemia or intractable ascites, which were characterized as a small-for-size graft syndrome after LDALT.

The five-year patient survival rate in overall patients was 82.2%, and three-year patient survival rate in group A and group B were 79.6% and 100%, respectively (**Figure 1**). The patient survival rate in group B seems to be better than in group A.

Figure 2 depicts a comparison of portal pressure and portal vein flow before and after a splenectomy. The portal pressure after a splenectomy mostly decreased in comparison to that before a splenectomy. The portal vein flow after a splenectomy also decreased in comparison with that before a splenectomy.

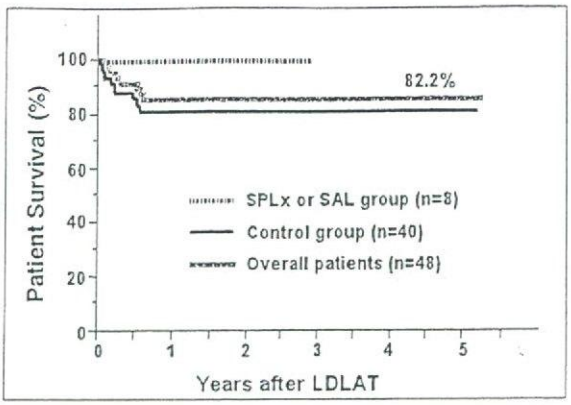

FIGURE 1 Patient's survival rates. Five-year patient's survival rate in overall patients was 82.2%. The patient's survival, in those who underwent a splenectomy or a splenic artery ligation, seemed better than that in the control group.

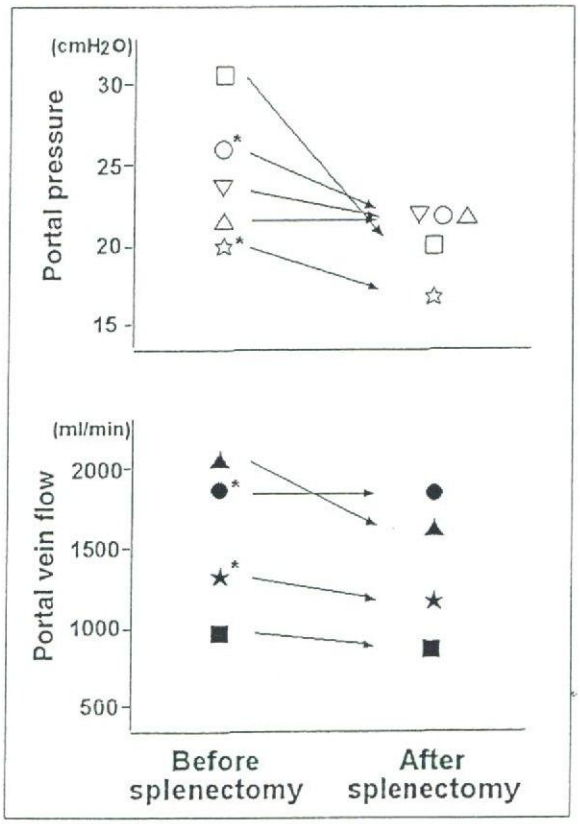

FIGURE 2 Changes in portal pressure and portal vein flow between before and after a splenectomy or a splenic artery ligation.
The portal pressure mostly decreased after a splenectomy or a splenic artery ligation. The portal vein flow also decreased after a splenectomy or a splenic artery ligation. Asterisk (*) indicates patients who underwent a splenic artery ligation.

DISCUSSION

It is of great interest that small-for-size left lobe graft in LDALT (group B; a graft-recipient weight

ratio with 0.70 as a median value) had an excellent outcome thanks to a splenectomy or a splenic artery ligation. One of the most important reasons why such an excellent outcome was obtained is considered to be the reduction of the excessive portal vein flow or the portal pressure. Judging from the changes in the portal vein flow and the portal pressure before and after a splenectomy, both the portal pressure and the portal vein flow in most cases was proven to decrease after a splenectomy. Actually, from France, a new technique of adult liver transplantation using a small-for-size graft was reported in order to avoid graft congestion and failure by overperfusion, in which the superior mesenteric venous flow was diverted by a mesocaval shunt with downstream ligation of the superior mesenteric vein (10). The facts suggest that an excessive portal flow gives an adverse effect on a partial liver graft, especially in the small-for-size graft. Therefore, from the standpoint of saving the small-for-size graft, a splenectomy or a splenic artery ligation should be recommended. Regarding the relationship between the graft size and the outcome after LDLT, Makuuchi *et al.* (19) recommended that a larger graft volume is required for critically-ill patients with primary biliary cirrhosis. Miller *et al.* (7) reported that graft functioning and survival were influenced not only by graft size, but also by pretransplantation disease severity. Transplant recipients with Child's class B or C require a graft-recipient weight ratio greater than 0.85% to avoid small-for-size syndrome and related complications. Furthermore, Tanaka *et al.* (8) reported that the use of small-for-size grafts (less than 1% of graft-recipient weight ratio) leads to lower graft survival, probably through enhanced parenchymal cell injury and reduced metabolic and synthetic capacity. Those reports are all considered important in selecting donors or grafts in LDALT. However, this study suggests that a splenectomy or a splenic artery ligation may alter the abovementioned criteria of the minimum requirement for the graft volume in LDALT.

Regarding the past reported results on splenectomy in liver transplantation, some authors (11-14) reported that a splenectomy in the liver transplantation was closely related to septic complications and a poorer prognosis. Neumann *et al.* (11) reported that a splenectomy was a major risk factor for the development of opportunistic pneumonia caused by *Legionella pneumophila, Pnuemocystis carini, Aspergillus* species, and cytomegarovirus after the liver transplant. Neuhaus *et al.* (12) therefore, recommended splenectomy only for very select number of patients and to investigate the banding of the splenic artery as an alternative. In contrast to increased septic complications reports, splenectomy was reported to bring the following merits: reducing graft congestion leading to improvement of the hepatic renal functions; improving thrombocytopenia persistence after liver transplantation, and; avoiding bleeding episodes related to left-sided portal hypertension (13). Samimi *et al.* (14) reported concomitant splenectomy raised one-month sepsis-related mortality, however, in our series, none of the patients experienced septic complications to date. Moreover, in our experience of more than 100 cirrhotic patients with hypersplenism who underwent either a laparoscopic splenectomy or a simultaneous open splenectomy at hepatectomy, no septic complications have been observed (15-17). The reason why Japanese patients hardly ever suffer from septic complications after a splenectomy still remains unclear, and the difference in races between Caucasian and Japanese may be related to low incidence of septic complications after splenectomy.

On the other hand, in case of splenic arterial aneurysm, the splenectomy including the aneurysm was usually performed to avoid the risk of rupture in the postoperative period. Recently, a new treatment option known as percutaneous embolization of the aneurysm followed by laparoscopic splenectomy in liver transplant patients has been reported (20). Another great merit of the splenectomy and the splenic artery ligation is considered to be prevention of splenohepatic arterial steal syndrome (21), leading to graft loss. This syndrome was characterized by an arterial malperfusion of the hepatic graft caused by a marked diversion of blood flow to a significantly enlarged spleen, which leads to major ischemic damage of the hepatic graft. In other situations of severe persistent thrombocytopenic states in cirrhotic patients early after liver transplantation, splenectomy can be used (22). Makuuchi *et al.* (23) also recently reported that splenectomy in LDLT is an acceptable treatment option in patients with thrombocytopenia or when hepatopedal portal flow is necessary to be obtained by closure of spleno-renal shunt. Furthermore, in Japan, LDALT has been widely indicated for liver cancer (hepatocellular carcinoma) with liver cirrhosis, and most of the cirrhotic patients have a significant hypersplenism. Different from cadaveric whole liver transplantation, a partial graft (small-for-size graft) could not correct portal hypertension with hypersplenism early after LDALT, therefore, some patients could not receive any adjuvant chemotherapy due to persistent thrombocytopenia after LDALT. Therefore, splenectomy has to be done to avoid such an unfortunate situation.

To avoid lethal septic complications, several authors have advocated autotransplantation of the spleen after splenectomy (24-28). In rat experiments, spleen autotransplantation can be expected to enable an improved humoral response against pathogens (28). Leemans *et al.* (23) reported that approximately 30g of spleen autotransplantation revealed a significant increase in the anti-pneumococcal antibody and is expected to protect the splenectomized patients against overwhelming postsplenectomy infection or sepsis. Zhang *et al.* (29) recently reported that approximately 40g of spleen fragment (7x5x1cm) autotransplantation in the left subphrenic space near the splenic pedicle can preserve immune function of the spleen in patients with portal hypertension and liver cirrhosis. We have never experienced an overwhelming septic complication up to this point, however, we have started a clinical trial of an autotransplantation of splenic tissues in case of splenectomy.

In conclusion, a splenectomy or a splenic artery ligation is considered to be beneficial for improving the outcome in LDALT using a left lobe graft. Further investigation is required regarding the significance of autotransplantation of the splenic fragments after splenectomy.

ACKNOWLEDGEMENTS

The authors thank Miss. Rie Nakao for her assistance in preparing this manuscript.

This study was supported in part by a Grant-in-aid for scientific research (No. 13357011) from the Ministry of Education, Science, Sports and Culture.

REFERENCES

1 **Lo CM, Fan ST, Chan JK, Wei W, Lo RJ, Lai CL:** Minimum graft volume for successful adult-to-adult living donor liver transplantation for fulminant hepatic failure. Transplantation 1996; 62:696-698.
2 **Lo CM, Fan ST, Liu CL, Wei WI, Lo RJ, Lai CL, Chan JK, Ng IO, Fung A, Wong J:** Adult-to-adult living donor liver transplantation using extended right lobe grafts. Ann Surg 1997; 226:261-269.
3 **Wachs ME, Bak TE, Karrer FM, Everson GT, Shrestha R, Trouillot TE, Mandell MS, Steinberg TG, Kam I:** Adult living donor liver transplantation using a right hepatic lobe. Transplantation 1998; 66:1313-1316.
4 **Fan ST, Lo CM, Liu CL:** Technical refinement in adult-to-adult living donor liver transplantation using right lobe graft. Ann Surg 2000; 231:126-131.
5 **Inomata Y, Uemoto S, Asonuma K, Egawa H:** Right lobe graft in living donor liver transplantation. Transplantation 2000; 69:258-264.
6 **Sugawara Y, Makuuchi M, Takayama T, Imamura H, Dowaki S, Mizuta K, Kawarasaki H, Hashizume K:** Small-for-size grafts in living-related liver transplantation. J Am Coll Surg 2001; 192:510-513.
7 **Ben-Haim M, Emre S, Fishbein TM, Sheiner PA, Bodian CA, Kim-Schluger L, Schwarte ME, Miller CM:** Critical graft size in adult-to-adult living donor liver transplantation: impact of the recipient's disease. Liver Transpl 2001; 7:948-953.
8 **Kiuchi T, Kasahara M, Uryuhara K, Inomata Y, Uemoto S, Asonuma K, Egawa H, Fujita S, Hayashi M, Tanaka K:** Impact of graft size mismatching on graft prognosis in liver transplantation from living donors. Transplantation 1999; 67(2):321-327.
9 **Shimada M, Shiotani S, Ninomiya M, Terashi T, Hiroshige S, Minagawa R, Soejima Y, Suehiro T, Sugimachi K:** Characteristics of liver grafts in living donor liver transplantation: comparison between right and left lobe grafts. Arch Surg. (In press)
10 **Boillot O, Delafosse B, Mechet I, Boucaud C, Pouyet M:** Small-for-size partial liver graft in an adult recipient; a new transplant technique. Lancet 2002; 359(9304):406-407.
11 **Neumann UP, Langrehr JM, Kaisers U, Lang M, Schmitz V, Neuhaus P:** Simultaneous splenectomy increases risk for opportunistic pneumonia in patients after liver transplantation. Transpl Int 2002; 15:226-232.
12 **Lusebrink R, Blumhardt G, Lohmann R, Bachmann S, Knoop M, Lemmens HP, Neuhaus P:** Does concommitant splenectomy raise the mortality of liver transplant recipients? Transpl Int 1994; 7(Suppl 1):S634-636.
13 **Troisi R, Hesse UJ, Decruyenaere J, Morelli MC, Palazzo U, Pattyn P, Colardyn F, Maene L, de Hemptinne B:** Functional, life-threatening disorders and splenectomy following liver transplantation. Clin Transplant 1999; 13(5):380-388.
14 **Samimi F, Irish WD, Eghtesad B, Demetris AJ, Starzl TE, Fung JJ:** Role of splenectomy in human liver transplantation under modern-day immunosuppression. Dig Dis Sci 1998; 43(9):1931-1937.
15 **Hashizume M, Tomikawa M, Akahoshi T, Tanoue K, Gotoh N, Konishi K, Okita K, Tsutsumi N, Shimabukuro R, Yamaguchi S, Sugimachi K:** Laparoscopic splenectomy for portal hypertension. Hepatogastroenterology 2002; 49:847-852.
16 **Shimada M, Hashizume M, Shirabe K, Takenaka K, Sugimachi K:** A new surgical strategy for cirrhotic patients with hepatocellular carcinoma and hypersplenism: performaing a hepatectomy after laparoscopic splenectomy. Surg Endosc 2000; 14:127-130.
17 **Shimada M, Yamashita Y, Hamatsu T, Gion T, Hasegawa H, Utsunomiya T, Shirabe K, Sugimachi K:** The effect of splenectomy on the long-term prognosis and liver function after hepatectomy in patients with hepatocellular carcinoma. Hepatogastroenterology. (In press)
18 **Nishizaki T, Ikegami T, Hiroshige S, Hashimoto K, Uchiyama H, Yoshizumi T, Kishikawa K, Shimada M, Sugimachi K:** Small graft for living donor liver transplantation. Ann Surg 2001; 233:575-580.
19 **Sugawara Y, Makuuchi M, Takayama T, Imamura H:** Living-related liver transplantation for primary biliary cirrhosis. Transplantation 2001; 72(6):1087-1091.
20 **DeRoover A, Sudan D:** Treatment of multiple aneurysms of the splenic artery after liver transplantation by percutaneous embolization and laparoscopic splenectomy. Transplantation 2001; 72(5):956-958.
21 **Geissler I, Lamesch P, Witzigmann H, Jost U, Hauss J, Fangmann J:** Splenohepatic arterial steal syndrome in liver transplantation: clinical features and management. Transpl Int 2002; 15(2):139-141.
22 **Altaca G, Scigliano E, Guy SR, Sheiner PA, Reich DJ, Schwartz ME, Miller CM, Emre S:** Persistent hypersplenism early after liver transplant: the role of splenectomy. Transplantation 1997; 64(10):1481-1483.
23 **Cescon M, Sugawara Y, Takayama T, Seyama Y, Sano K, Imamura H, Makuuchi M:** Role of splenectomy in living-donor liver transplantation for adults. Hepatogastroenterology 2002; 49(45):721-723.
24 **Leemans R, Manson W, Snijder JA, Smit JW, Klasen HJ, The TH, Timens W:** Immune response capacity after human splenic autotransplantation: restoration of response to individual pneumococcal vaccine subtypes. Ann Surg 1999; 229(2):279-285.
25 **Weber T, Hanisch E, Baum RP, Seufert RM:** Late results of heterotopic autotransplantation of splenic tissue into the greater omentum. World J Surg 1998; 22(8):883-889.
26 **Szendroi T, Miko I, Hajdu Z, Acs G, Kathy S, Furka I, Szabo L:** Splenic autotransplantation after abdominal trauma in childhood. Clinical and experimental data. Acta Chir Hung 1997; 36(1-4):349-351.
27 **Yamataka A, Fujiwara T, Tsuchioka T, Kurosu Y, Sunagawa M:** Heterotopic splenic autotransplantation in a neonate with splenic rupture, leading to normal splenic function. J Pediatr Surg 1996; 31(2):239-240.
28 **Zhang H, Chen J, Kaiser GM, Mapudengo O, Zhang J, Exton MS, Song E:** The value of partial splenic autotransplantation in patients with portal hypertension: a prospective randomized study. Arch Surg 2002; 137(1):89-93.
29 **Leemans R, Harms G, Rijkers GT, Timens W:** Spleen autotransplantation provides restoration of functional splenic lymphoid compartments and improves the humoral immune response to pneumococcal polysaccharide vaccine. Clin Exp Immunol 1999; 117(3):596-604.

Transpl Int (2004) 17: 310–316
DOI 10.1007/s00147-004-0720-9

ORIGINAL ARTICLE

M. Shimada
H. Ijichi
Y. Yonemura
N. Harada
S. Shiotani
M. Ninomiya
T. Yoshizumi
Y. Soejima
T. Suehiro
Y. Maehara

Is graft size a major risk factor in living-donor adult liver transplantation?

Received: 9 May 2003
Revised: 6 August 2003
Accepted: 9 March 2004
Published online: 22 June 2004

M. Shimada (✉)
Department of Digestive and Pediatric Surgery, Institute of Health Bioscience, Tokushima University,
770-8503 Tokushima, Japan
E-mail:
mshimada@clin.med.tokushima-u.ac.jp
Tel.: +88-633-7137
Fax: +88-631-9698

H. Ijichi · Y. Yonemura · N. Harada
S. Shiotani · M. Ninomiya
T. Yoshizumi · Y. Soejima
T. Suehiro · Y. Machara
Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University,
812-8582 Fukuoka, Japan

Abstract Graft size is known to be a major risk factor in living donor adult liver transplantation (LDALT). The aim of this study is to reassess whether graft size is a critical factor in LDALT or not. A series of 75 LDALTs excluding auxiliary transplantation and ABO blood-type incompatible transplantation were analyzed. The patients were divided into two groups, according to graft volume (GV) and standard liver volume (SLV): group 1 (small-size group) (GV/SLV: <40%), and group 2 (non-small-size group) (≥40%). Perioperative clinical data were compared between the two groups, including graft survival and postoperative complications. These parameters were also compared under the conditions of cirrhotic recipients. No difference in graft survival was found between the two groups. No difference was found in incidence of postoperative complications, such as intractable ascites and persistent hyperbilirubinemia. Even in cirrhotic patients with Child–Pugh's class C, there was no difference in graft survival between the two groups. Risk factors related to graft loss were a preoperative urgent status due to chronic liver disease, pre-operative hyperbilirubinemia of over 10 mg/dl, and ABO blood type of not identical but compatible combination between donor and recipient. Graft size is not always considered to be a major risk factor in LDALT, although the number of patients was small in this study. Therefore, a left-lobe graft, even a "small-for-size" graft for adult recipients, remains a feasible option in LDALT.

Keywords Small-for-size graft · Left-lobe graft · Graft survival · Cholestasis · Intractable ascites

Introduction

Graft size is known to be a major risk factor in living donor adult liver transplantation (LDALT) [1, 2, 3, 4]. Kawasaki et al. [5] reported that a GV and SLV ratio of more than 30% is a safe limit. Lo et al. [6] reported that a graft with GV/SLV of less than 40% should be regarded as a marginal graft that would have a lower success rate. Together with other reports [1,,2, 3, 4], the graft volume is considered to be ideally over 40% of the standard liver volume. To avoid a "small-for-size" graft, right-lobe grafts have been increasingly used in LDALT [7, 8, 9, 10], however, a critical comment has been raised that mortality of the right-lobe donor was significantly high [11]. From an ethical point of view, the risks for the living donor have to be minimized. The removal of a left lobe of the liver for donation is a more conservative surgical procedure than right lobe removal. The potential risks in right-lobe donors have been reported to be higher than those in left-lobe or lateral-segment donors [12, 13, 14]. We previously reported that a "small-for-size" graft in which GV/SLV is less than 30% can be