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Single Nucleotide Polymorphisms in the Interleukin-10 Gene Promoter and Clinical Outcome of Chronic Hepatitis C Virus Infection

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ABSTRACT

To clarify the significance of polymorphisms of the interleukin (IL)-10 gene promoter region in determining clinical outcomes in chronic hepatitis C virus (HCV) infection, we analyzed single nucleotide polymorphisms by direct sequencing at positions -1082 (A/G), -819 (T/C), and -592 (A/C) in 143 patients with chronic HCV infection and 61 healthy volunteers. The frequencies of the TT genotype at position -819, the AA genotype at position -592, the T allele at position -819, and the A allele at position -592 were significantly lower in asymptomatic HCV carriers than in patients with chronic hepatitis or in healthy volunteers. However, serum levels of IL-10 did not differ with regard to pathogenesis or genotype. Our data suggest that the genotype of the IL-10 gene promoter at positions -1082, -819, and -592 and the haplotypes derived from these genotypes are important genetic factors determining the progression of liver disease resulting from chronic HCV infection.
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Key words : interleukin-10, promoter, polymorphism, hepatitis C virus, hepatitis

INTRODUCTION

Hepatitis C virus (HCV) is the most important causative pathogen of chronic hepatitis (CH), liver cirrhosis (LC), and hepatocellular carcinoma (HCC) in Japan[1,2]. The outcome of chronic HCV infection varies, with some patients becoming asymptomatic carriers (ASCs), and others having LC with or without HCC. More than 20% of Japanese infected with HCV will have LC within 25 years of infection[3], whereas 10% to 20% remain ASCs[4]. Serum aminotransferase levels in most of these patients remain within the normal range for long periods of time.

The reasons for such varied outcomes for chronic HCV infection are not fully understood. However, numerous factors can affect the outcome of HCV infection, such as gender, age at infection, alcohol consumption, and genetic factors[5].

The progression of chronic hepatitis C is dependent on the immune reaction against HCV-infected hepatocytes and the role of cytokines ; in particular, the balance of helper T (Th) 1 and Th2 cytokines is thought to be crucial[6,7]. The Th1 immune response promotes the generation of cytotoxic T cells that may eradicate HCV-infected hepatocytes and exacerbate inflammation[8], whereas the Th2 response inhibits the Th1 response[9]. Interleukin (IL)-10 is a potent Th2 cytokine which potently inhibits the effector mechanisms of Th1[10,11].

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The IL-10 gene is located at the junction of 1q31-q32[12]. The promoter region of the IL-10 gene contains three single nucleotide polymorphisms (SNPs) at positions -1082 (A/G), -819 (T/C), and -592 (A/C) which give rise to three haplotypes: GCC, ACC, and ATA. An *in vitro* lymphocyte stimulation study has shown that production of IL-10 depends on the combination of these haplotypes[13]. The combination ATA/ATA confers a low capacity for IL-10 production; ACC/ATA, ACC/ACC, and GCC/ATA confer an intermediate capacity; and GCC/GCC confers a high capacity.

In the present study, we examined the significance of SNPs in the IL-10 gene promoter region for determining the clinical outcomes of chronic HCV infection.

MATERIALS AND METHODS

1. *Patients*

A total of 143 patients with chronic HCV infection were enrolled. Patients were classified as ASCs when serum levels of alanine aminotransferase and aspartate aminotransferase had been within normal limits for 2 years or more and when no LC was found on routine clinical examination with abdominal sonography or computed tomography. All other patients were classified as having CH or LC depending on the results of abdominal ultrasonography or computed tomography. Habitual heavy drinkers, who were defined as consuming an equivalent of 65 g of pure ethanol per day for more than 5 years, were excluded. Thus, 46 patients were classified as ASCs, 61 as having CH, and 36 as having LC. Sixty-one unrelated healthy volunteers (29 men and 32 women) were enrolled as control subjects. All subjects were Japanese.

Written informed consent was obtained from all subjects. This study was reviewed and approved by the Ethics Committee of the Jikei University School of Medicine.

2. *DNA samples*

DNA was obtained from peripheral mononuclear cells, which were collected from 5 ml of heparinized peripheral blood by means of standard sediment centrifugation. DNA was extracted with a commercial kit (Talent Srl, Triste, Italy) according to the manufacturer's instructions.

3. *Detection of IL-10 promoter SNPs*

The IL-10 SNPs -1082 (A/G), -819 (T/C), and -592 (A/C) were detected with a direct sequencing technique.

First, the polymerase chain reaction (PCR) was performed in a thermal cycler (model 9,700, Perkin-Elmer Cetus, Norwalk, CT, USA) using AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) in total volumes of 50 μl using 50 to 100 ng of each genomic DNA template. The PCR conditions were initial denaturation (95°C, 10 minutes), followed by 40 cycles of denaturation (94°C, 30 seconds), annealing (60°C, 30 seconds), and extension (72°C, 30 seconds), with a final extension at 72°C for 5 minutes. The following primers were used: IL-10-1201F (forward) 5′-ACACTCCTCGTCGCAACCCA-3′ and IL-10-521R (reverse) 5′-GGTGGGCTAAATATCCTCAAAGTTCC-3′. The PCR products were purified on a Sephadex G-50 column (AM GIKEN Co., Tokyo) and sequenced with an ABI 3100 automated sequencer (Perkin-Elmer Cetus) and a ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturers' instructions. The following primers were used to sequence the promoter region in both directions: forward 5′-ACACTCCTCGTCGCAACCCA-3′ and reverse 5′-GATGGGGTGGAAGAAGTTGAAATAACAA-3′. Briefly, the cycle sequencing reaction was accomplished through the addition of 50 ng of PCR product, 4 pmol of primers, and 4 μl of BigDye terminator premix with the following protocol: 96°C for 30 seconds, 50°C for 15 seconds, and 60°C for 4 minutes, for a total of 30 cycles. After purification and denaturation of the product, the sequence was analyzed with an ABI 3100 automated sequencer.

4. *Estimation of the maximum likelihood of haplotype frequencies in the IL-10 gene promoter*

To estimate the maximum likelihood of the IL-10

haplotype frequencies for the three SNPs, the expectation-maximization algorithm according to Dempster et al.[14] was used. This calculation was performed with Arlequin computer software (http://acasun1.unige.ch/arlequin/).

5. Measurement of IL-10

Sera from 23 randomly selected ASCs and 53 patients with CH were frozen at −80°C until assay. Serum levels of IL-10 were measured with an enzyme-linked immunoassay test kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions. The minimum detectable level of IL-10 has been established as 0.1 pg/ml.

6. Statistical analysis

Allele and genotype frequencies were calculated by direct counting. The likely frequencies of genotypes at position -1082, -819 and -592 were predicted with the Hardy-Weinberg equation and compared with the observed frequencies in control subjects. Values are expressed as means ± standard deviations. Statistical differences were analyzed with Student's t-test, Fisher's exact probability test, and the χ^2 test. Odds ratios (OR) with 95% confidence intervals (95% CI) were also calculated.

Table 1. Clinical Characteristics of HCV carriers

	ASC	CH	LC
Gender (male : female)	13 : 33	35 : 26*	17 : 19
Age (y)	59.7±11.8	55.4±11.4	59.3±8.8
HCV serotype (group 1 : 2)	28 : 9	45 : 13	8 : 4
Amount of HCV RNA (kcopies/ml)	540.0±267.3	554.8±257.2	426.1±292.2
Platelet ($10^4/\mu$l)	19.9±4.8	16.2± 5.4**	8.1± 2.5**
Serum ALT (IU/L)	24.1±5.6	60.8±32.5**	76.7±47.1**

*$p=0.003$ vs ASC **$p<0.001$ vs ASC

Results

1. Clinical backgrounds of patients

Age, gender, HCV copy number in serum, and serotype of HCV in HCV carriers are listed in Table 1. Patient age was similar in the ASC, CH, and LC groups. However, the percentage of women was significantly higher among ASCs than among patients with CH ($p=0.003$). Neither the number of copies of HCV in serum nor the distribution of the HCV serotypes differed significantly among the ASC, CH, and LC groups.

2. Genetic polymorphisms in the IL-10 promoter

The observed genotype frequencies in control subjects at positions -1082, -819, and -592 were similar to those predicted with the Hardy-Weinberg equation, suggesting that alleles were being appropriately assigned.

The genotype distributions of the IL-10 gene promoter caused by single base substitutions (G/A at -1082, C/T at -819, and C/A at -592) are listed in

Table 2. Genotype frequencies of IL-10 gene promoter ( ) %

Position of Polymorphism		Control ($n=61$)	HCV carriers ($n=143$)	ASC ($n=46$)	CH ($n=61$)	LC ($n=36$)
-1082	A/A	54(89)	137(96)	44(96)	59(97)	34(94)
	A/G	7(11)	6(4)	2(4)	2(3)	2(6)
	G/G	0(0)	0(0)	0(0)	0(0)	0(0)
-819	T/T	*33(54)	68(48)	15(33)	†36(59)	17(47)
	T/C	25(41)	60(42)	24(52)	18(30)	18(50)
	C/C	3(5)	15(10)	7(15)	7(11)	1(3)
-592	A/A	**35(57)	69(48)	16(35)	††36(59)	17(47)
	A/C	23(38)	60(42)	24(52)	18(30)	18(50)
	C/C	3(5)	14(10)	6(13)	7(11)	1(3)

*$p=0.027$ *vs* ASC †$p=0.007$ *vs* ASC
**$p=0.020$ *vs* ASC ††$p=0.013$ *vs* ASC

Table 3. Allelic frequencies of IL-10 gene promoter

Position of Polymorphism		Control (n=61)	HCV carriers (n=143)	ASC (n=46)	CH (n=61)	LC (n=36)
-1082	A	0.943	0.979	0.978	0.984	0.972
	G	0.057	0.021	0.022	0.016	0.028
-819	T	*0.746	0.685	0.587	†0.738	0.722
	C	0.254	0.315	0.413	0.262	0.278
-592	A	**0.762	0.692	0.609	††0.738	0.722
	C	0.238	0.308	0.391	0.262	0.278

*p=0.014 *vs* ASC †p=0.019 *vs* ASC
**p=0.016 *vs* ASC ††p=0.045 *vs* ASC

Table 4. Genotype distribution of the IL-10 gene promoter in relation to gender (HCV carrier)

		Male (n=65)			Female (n=78)		
		ASC (n=13)	CH (n=35)	LC (n=17)	ASC (n=33)	CH (n=26)	LC (n=19)
-1082	A/A	13	35	16	31	24	18
	A/G	0	0	1	2	2	1
	G/G	0	0	0	0	0	0
-819	T/T	3	20	9	12	16	8
	T/C	8	10	8	16	8	10
	C/C	2	5	0	5	2	1
-592	A/A	3	20	9	13	16	8
	A/C	7	10	8	16	8	10
	C/C	3	5	0	4	2	1

Table 2. The distribution of genotypes in the IL-10 promoter did not differ significantly between patients infected with HCV and control subjects. However, the genotype frequency of TT at position -819 was significantly lower in the ASC group than in the CH group (OR=0.34, 95% CI=0.15-0.75, p=0.007) and the control group (OR=0.41, 95% CI=0.19-0.91, p=0.027). Similarly, the genotype frequency of AA at position -592 was significantly lower in the ASC group than in the CH group (OR=0.37, 95% CI=0.17-0.82, p=0.013) and the control group (OR=0.40, 95%CI=0.18-0.87, p=0.020). The distribution of genotypes at positions -819 and -592 in the LC group did not differ significantly from those in CH group or the ASC group. At position -1082, nearly all HCV carriers and control subjects had genotype AA.

The allelic frequencies are listed in Table 3. At position -1082 in the IL-10 gene promoter, most HCV carriers and control subjects had the A allele. A strong linkage disequilibrium with the allele in the IL-10 gene promoter was present, in which the T and C alleles at position -819 almost always corresponded to the A and C alleles at position -592. However, a few exceptional cases did not exhibit this type of linkage. The allelic frequency of T at position -819 was significantly lower in the ASC group than in the CH group (OR=0.51, 95%CI=0.28-0.90, p=0.019) and the control group (OR=0.48, 95%CI=0.27-0.87, p=0.014). Similarly, the allelic frequency of A at position -592 was significantly lower in the ASC group than in the CH group (OR=0.55, 95%CI=0.31-0.99, p=0.045) and the control group (OR=0.49, 95%CI=0.27-0.88, p=0.016). Allele frequencies at positions -819 and -592 in the LC group were similar to those in the CH and control groups.

Allele and genotype frequencies did not differ

Table 5. Maximum-likelihood haplotype frequencies of IL-10 Gene Promoter SNPs

Haplotype	Control (n=61)	HCV carriers (n=143)	ASC (n=46)	CH (n=61)	LC (n=36)
ATA	0.746	0.682	0.576	0.738	0.726
ACC	0.180	0.287	0.369	0.246	0.242
GCC	0.057	0.021	0.022	0.016	0.032
Others	0.016	0.010	0.033	0.000	0.000

significantly according to gender (Table 4).

3. *Maximum likelihood of haplotype frequencies in IL-10 promoter gene*

As the phase was unknown, the maximum likelihood of haplotype frequencies of the IL-10 promoter in HCV carriers and control subjects were defined according to the expectation-maximization algorithm (Table 5).

In control subjects, the most common haplotype was ATA, which was present in 75% of control subjects, followed by ACC in 18% ; GCC and other minor haplotypes together were present in less than 7% of subjects.

In HCV carriers, the frequency of the ATA haplotype was similar to that in control subjects, but the ACC haplotype was present in as many as 30% of subjects.

In the ASC group the ATA haplotype was less common whereas ACC was more common and was present in 37% of ASC patients. In contrast, the frequencies of ATA haplotype (approximately 73%) and the ACC haplotype (approximately 24%) were

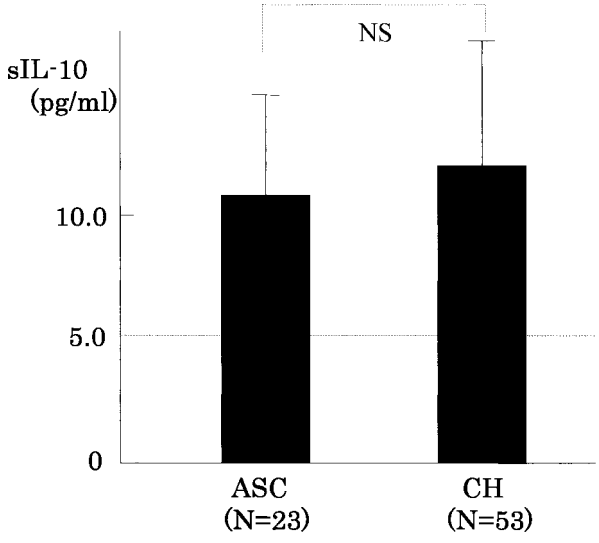

Fig. 1. Serum IL-10 levels in ASCs and patients with CH

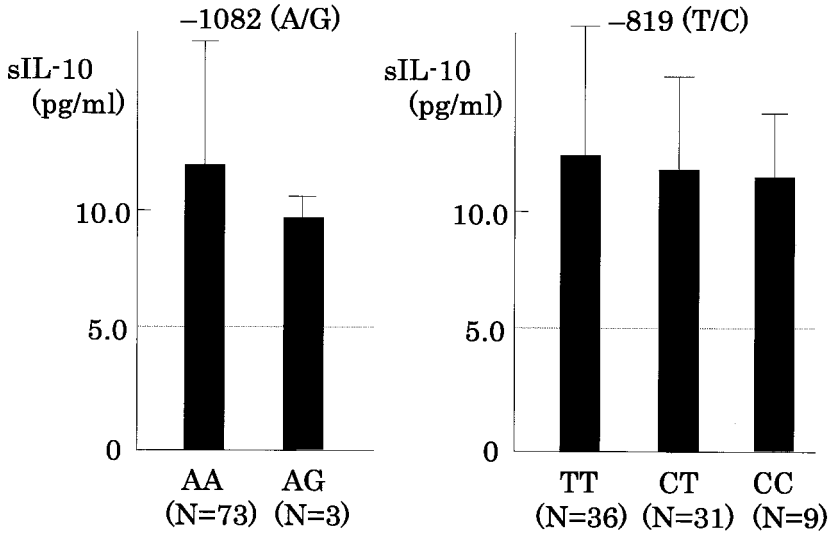

Fig. 2. Serum IL-10 levels in HCV carriers with various genotypes

similar in the CH and LC groups.

4. Relationship between serum IL-10 levels and SNPs of the IL-10 promoter gene

Serum levels of IL-10 did not differ significantly between the ASC group (10.5±4.7 pg/ml) and the CH group (12.0±5.1 pg/ml; Fig. 1). In addition, serum levels of IL-10 did not differ between subjects with the TT genotype and subjects with other genotypes (CT or CC) at position -819, or between those with the AA genotype and those with other genotypes (CA or CC) at position -592. Serum levels of IL-10 were measured in only 3 patients with genotype AG at position -1082 because genotype AG was detected in only 5% of the patients. Serum levels of IL-10 did not differ significantly between subjects with the AA genotype and those with the AG genotype (Fig. 2).

DISCUSSION

The frequencies of the TT genotype at position -819 and the AA genotype at position -592 of IL-10 gene promoter were significantly lower in ASCs than in patients with CH or in healthy control subjects. However, the frequencies of the TT genotype at position -819 and the AA genotype at position -592 in HCV-infected patients did not differ significantly from those in control subjects. These findings suggest that SNPs at -819 or -592 do not influence the susceptibility to HCV infection, although they may play a significant role in the progression of chronic liver disease induced by HCV.

In contrast, the frequencies of genotypes at -1082 were similar for the ASC, CH, and LC groups and healthy control subjects. Most HCV carriers and control subjects had the A allele, a finding that suggests the frequency of genotypes at -1082 does not play an important role in the disease progression in HCV-infected patients. However, Lio et al. report[15] that genotype -1082 GG was significantly more common in patients who spontaneously recover from chronic HCV infection. Moreover, in Pakistan, the AG genotype is more prevalent in patients with HCV-RNA and normal serum levels of alanine aminotransferase[16]. Recent reports suggest genotype frequencies at -1082 vary greatly in different populations, particularly those of different ethnicity[17-19]. For example, -1082 G is a common allele present in 50% of whites but is a minor allele present in only 5% to 10% of Japanese[20]. Therefore, the genotype at position -1082 may have little significance in the Japanese population.

Of the haplotypes derived from -1082 A/G, -819 T/C, and -592 A/C, ATA was less common in the ASC group than in the CH group or healthy control subjects. A previous study identified only 3 haplotypes[13]. The ATA haplotype is associated with low capacity for IL-10 production after concanavalin A stimulation of peripheral blood mononuclear cells in vitro, whereas ACC is associated with intermediate capacity and GCC is associated with high capacity[13]. Therefore, determining haplotypes in this region is important for evaluating the genetic capacity for IL-10 production and susceptibility to progression of chronic inflammatory diseases, such as HCV-related liver disease.

The relation between response to antiviral therapy and SNPs of the IL-10 gene promoter has been examined. A sustained response to antiviral therapy is indicative of the ATA haplotype, which is associated with diminished IL-10 expression[21]. Similarly, the ATA haplotype is associated with a favorable response to interferon-α therapy[19]. In addition, levels of serum IL-10 are negatively correlated with the degree of response to interferon treatment in patients with chronic HCV infection[22]. Because the persistence of HCV replication is associated with an intensive release of IL-10[23], which inhibits induction of cytotoxic T cells in the antiviral response, the amount of IL-10 is an important factor in the persistence of HCV and the prognosis in chronic HCV infection. We hope to evaluate SNPs of the IL-10 gene promoter in relation to the response to antiviral therapy.

We found that the frequencies of -819TT, -592AA, and haptotype ATA were significantly lower in ASCs than in patients with CH. However, Miyazoe et al.[20] report that the frequencies of -819TT, -592AA, and haplotype ATA in ASCs of the hepatitis

B virus (HBV) are significantly higher than those in patients with CH. This difference may indicate that patients who are genetically predisposed to lower IL-10 production have a more favorable outcome in chronic HBV infection.

Theoretically, immunologic mechanisms do not differ between HBV infection and HCV infection. However, our findings suggest that outcome may differ between patients with chronic HCV infection and those chronic HBV infection who have the same genetic capacity for IL-10 production. Therefore, co-infection with HCV and HBV might not result in rapid disease progression[24].

We did not find any differences in serum IL-10 levels between ASCs and patients with CH. Additionally, genotypes at -1082 and -819 were not correlated with serum levels of IL-10 in HCV-infected patients. In contrast, IL-10 plasma levels are higher in patients with the ATA haplotype who are infected with Epstein-Barr virus[25]. This finding suggests that IL-10 production *in vivo* is not solely determined by genetic factors. In HCV infection, Kupffer cells and stellate cells secrete large amounts of IL-10 and contribute directly to the immune-mediated viral eradication and inflammation in the liver[26,27]. Thus, IL-10 concentrations should be measured in the liver.

In chronic inflammation, the serum IL-10 levels increase[28] with Th1 cytokines, such as IL-12 and interferon-γ. However, in CH, serum levels of IL-12 increase as liver disease progresses whereas those of IL-10 do not[29]. The serum levels of IL-10 in our study were consistent with this finding and suggest that serum levels of IL-10 should be compared with levels of Th1 cytokines[29]. In another study, administration of IL-10 induced marked changes in serologic markers, suggesting reductions in the immune response and fibrogenesis[30] in CH. This finding suggests that a disproportionate increase in IL-10 production may cause inflammation and fibrosis to subside.

We found no decrease in serum levels of IL-10 with progression of liver disease. However, taking into consideration upregulated Th1 cytokines in the progression of liver disease, the level of IL-10 might be disproportionately lower in CH than in ASC. Therefore, our findings for serum levels of IL-10 did not conflict with those showing that the ATA haplotype, which is associated with a reduced capacity for IL-10 production, was more common in patients with CH or LC than in ASCs.

In conclusion, the genotype of the IL-10 promoter at positions -1082, -819, and -592 and the haplotypes derived from them are important genetic factors in determining the progression of liver disease resulting from chronic HCV infection. Moreover, IL-10 gene promoter SNPs may have prognostic significance in patients with chronic HCV infection.

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