inhibitors of IRES-directed translation in HCV-infected cells, vitamin B12, a synthetic peptide derived from human La protein, and RNA molecules targeting IRES have been reported (42–44). However, little is known regarding the effect of natural product-derived polyphenolic compounds on HCV IRES-directed translation, and this possibility should be clarified in a future study. It should be noted that all proanthocyanidin-binding proteins identified in this study are intracytoplasmic and/or intranuclear proteins. However, it is not known whether proanthocyanidin can be efficiently translocated into the intracellular space despite its highly polymerized structure. Nonetheless, absorption of proanthocyanidin from the digestive tract has been reported (12, 45), suggesting the possibility of proanthocyanidin internalization into cells, and internalization of high molecular weight molecule via clathrin-mediated endocytosis, caveolae-mediated uptake or pinocytosis has been reported (46). Further studies are in progress, focusing on the intracellular uptake of proanthocyanidin.

The current therapies for hepatitis C patients are based on a combination of pegylated recombinant interferons and ribavirin. However, viral clearance is achieved by <60% of treated patients, and the therapies are limited by significant side effects and high costs (47, 48). Therefore, many novel anti-HCV drugs are currently under development, most of which target viral enzymes. For example, BILN-2061, VX-950, and SCH503034 are inhib-

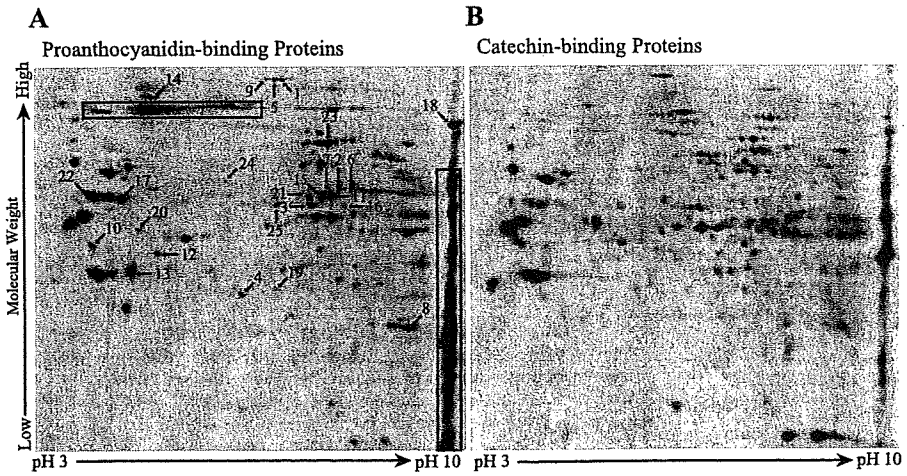

FIGURE 9. **Fluorescent two-dimensional-DIGE images of proteins with affinities to blueberry leaf proanthocyanidin (*A*) and catechin (*B*).** Protein extracts from replicon cells were treated with epoxy-activated Sepharose coupled to proanthocyanidin or catechin. The adsorbed proteins were eluted and then visualized as protein spots using fluorescent two-dimensional-DIGE. Fluorescent intensities were measured using Progenesis Discovery software. In the eluate from proanthocyanidin-coupled Sepharose (*A*), intensities of 32 spots were increased relative to those from catechin-coupled Sepharose (*B*). Twenty-seven spots were subjected to subsequent analysis and proteins derived from 25 spots (*spot nos. 1–25* in *A*) were identified with peptide mass fingerprinting. *Spot numbers* correspond to those in Table 4. Proteins in regions of high molecular weight and high pI (*rectangular regions*) were not sufficiently separated and were not analyzed.

TABLE 4
Proteins with higher affinity to blueberry proanthocyanidin than to catechin

Spot no.[a]	Intensity[b] (×10^5)		Ratio[c]	*p* value[d]	Protein name[e]	Accession number[f]	Coverage[g]	Molecular mass[h]	pI[i]
	Proanthocyanidin	Catechin					%	*kDa*	
1	5.69 ± 2.98	0.99 ± 0.24	5.73	0.0015	Eukaryotic translation initiation factor 3 subunit A (eIF3A)	Q14152	12.4	166.9	6.38
2	6.96 ± 1.33	1.39 ± 0.30	5.00	<0.0001	hnRNP Q	O60506	27.0	69.8	8.68
3	5.63 ± 0.71	1.15 ± 0.36	4.88	<0.0001	Splicing factor U2AF 65-kDa subunit	P26368	22.5	53.8	9.19
4	8.19 ± 2.57	1.68 ± 0.35	4.86	0.0001	eIF3H	O15372	41.2	40.1	6.09
5	5.22 ± 2.84	1.33 ± 0.36	3.91	0.0036	eIF3A	Q14152	15.1	166.9	6.38
6	8.02 ± 1.76	2.28 ± 0.90	3.52	<0.0001	hnRNP Q	O60506	24.4	69.8	8.68
7	2.45 ± 0.29	0.73 ± 0.24	3.35	<0.0001	hnRNP Q	O60506	16.4	69.8	8.68
8	17.11 ± 3.99	5.24 ± 4.26	3.26	<0.0001	hnRNP A2/B1	P22626	36.3	37.5	8.97
9	2.66 ± 1.70	1.00 ± 0.29	2.65	0.0202	eIF3A	Q14152	15.2	166.9	6.38
10	2.37 ± 0.82	0.96 ± 0.19	2.47	0.0010	eIF3F	O00303	28.0	37.7	5.24
11	5.40 ± 1.55	2.27 ± 0.56	2.38	0.0002	hnRNP L	P14866	20.2	64.7	8.46
12	6.77 ± 3.52	2.86 ± 0.68	2.37	0.0113	eIF3G	O75821	16.3	35.9	5.87
13	17.99 ± 9.05	7.89 ± 2.54	2.28	0.0104	eIF3M	Q7L2H7	32.6	42.9	5.41
14	9.28 ± 1.10	4.26 ± 0.71	2.18	<0.0001	Leucine-rich PPR motif-containing protein, mitochondrial	P42704	10.3	159.0	5.81
15	6.78 ± 1.96	3.20 ± 0.75	2.12	0.0005	hnRNP L	P14866	18.3	64.7	8.46
16	2.54 ± 0.55	1.24 ± 0.11	2.05	0.0001	Splicing factor U2AF 65-kDa subunit	P26368	20.0	53.8	9.19
17	17.65 ± 1.23	9.76 ± 1.87	1.81	<0.0001	hnRNP K	P61978	31.3	51.2	5.39
18	32.71 ± 6.34	19.20 ± 6.08	1.70	0.0003	Splicing factor, proline- and glutamine-rich	P23246	19.4	76.2	9.45
19	3.98 ± 0.35	2.34 ± 0.32	1.70	<0.0001	Heterogeneous nuclear ribonucleoprotein A/B (hnRNP A/B)	Q99729	17.5	36.3	8.22
20	3.57 ± 0.59	2.13 ± 0.52	1.68	<0.0001	Splicing factor 45	Q96I25	17.0	45.2	5.76
21	4.22 ± 1.18	2.52 ± 0.32	1.68	0.0023	hnRNP L	P14866	21.2	64.7	8.46
22	28.44 ± 2.99	17.40 ± 3.82	1.63	<0.0001	hnRNP K	P61978	29.8	51.2	5.39
23	18.62 ± 1.68	11.76 ± 3.01	1.58	<0.0001	ATP-dependent RNA helicase DDX1	Q92499	40.3	83.3	6.81
24	1.81 ± 0.46	1.18 ± 0.35	1.53	0.0052	Fragile X mental retardation syndrome-related protein 1	P51114	15.5	70.0	5.84
25	5.42 ± 0.57	3.60 ± 0.93	1.51	0.0001	Splicing factor U2AF 65-kDa subunit	P26368	26.3	53.8	9.19

[a] Spot numbers correspond to those in Fig. 9.
[b] Intensities of spots are shown as normalized volume ± S.D. (nine gels per group; proanthocyanidin and catechin).
[c] Ratio was calculated using Progenesis Discovery software and expressed as differences of spot intensity in proteins eluted from proanthocyanidin-coupled Sepharose compared with those from catechin-coupled Sepharose.
[d] Statistical difference were determined by Student's *t* test . Values of $p < 0.05$ were considered significant.
[e] Proteins were identified using Mascot with Swiss-Prot database.
[f] References for identified proteins.
[g] Percentage cover of the identified peptide in total tryptic digests.
[h] Theoretical molecular mass from Mascot search results.
[i] Theoretical isoelectric point (pI) from Mascot search results.

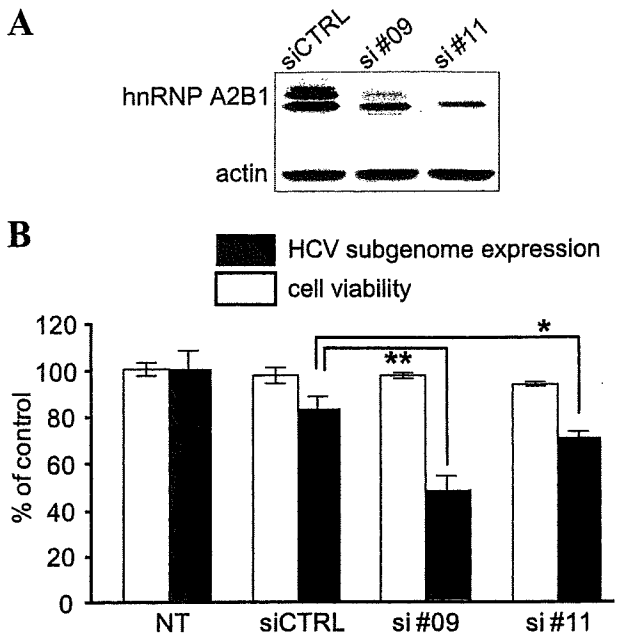

FIGURE 10. **Effects of hnRNP A2/B1 knockdown on HCV subgenome expression in replicon cells.** Results of two siRNA sequences (*si-#09* and *si-#11*) are shown. *A*, effects of siRNA on the expression of hnRNP A2/B1 protein. Same blot was also probed by anti-actin antibody. *B*, effects of siRNA on luciferase activity (HCV subgenome-expression activity) (*closed bars*) and cellular viability (*open bars*). The siRNA concentration is indicated as a logarithmic scale. Values are mean ± S.D. of triplicate experiments. *, $p < 0.05$; **, $p < 0.001$, Student *t* test.

itors of NS3/4A serine protease, and R1479 and HCV-796 are inhibitors of NS5B RNA-dependent RNA polymerase (41, 48–53). NA255 is also an HCV replication inhibitor targeting the host sphingolipid biosynthesis (10). These compounds are relatively low in molecular weight and can be manufactured by organic synthesis. On the other hand, the anti-HCV compound purified from blueberry leaves is a flavan-3-ol polymer with a molecular mass of ~2 kDa. The highly polymerized structure that is required for the efficient inhibition of HCV subgenome expression makes synthesizing the anti-HCV proanthocyanidin difficult. However, because proanthocyanidins are components of many plants and foods, daily intake of proanthocyanidin is possible and may be beneficial against HCV replication in hepatitis C patients. We estimate that fresh blueberry leaf is rich in proanthocyanidin, which accounts for 3–4% of the weight. Moreover, the polymerized status of blueberry leaf-derived proanthocyanidin appears to be suitable for the inhibition of HCV subgenome expression. Therefore, blueberry leaves might have potential as a source of anti-HCV proanthocyanidin.

In summary, we demonstrated that extracts of blueberry leaf possess strong suppressive effects against HCV subgenome expression in a replicon cell system. We identified the inhibitor as a proanthocyanidin oligomer with an mDP value of ~8. Further studies of the mechanism underlying proanthocyanidin-mediated HCV inhibition may open new ways to design novel anti-HCV drugs.

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Increased Rate of Death Related to Presence of Viremia Among Hepatitis C Virus Antibody–Positive Subjects in a Community-Based Cohort Study

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The overall mortality of patients infected with hepatitis C virus (HCV) has not been fully elucidated. This study analyzed mortality in subjects positive for antibody to HCV (anti-HCV) in a community-based, prospective cohort study conducted in an HCV hyperendemic area of Japan. During a 10-year period beginning in 1995, 1125 anti–HCV-seropositive residents of Town C were enrolled into the study and were followed for mortality through 2005. Cause of death was assessed by death certificates. Subjects with detectable HCV core antigen (HCVcAg) or HCV RNA were considered as having hepatitis C viremia and were classified as HCV carriers; subjects who were negative for both HCVcAg and HCV RNA (i.e., viremia-negative) were considered as having had a prior HCV infection and were classified as HCV noncarriers. Among the anti–HCV-positive subjects included in the analysis, 758 (67.4%) were HCV carriers, and 367 were noncarriers. A total of 231 deaths occurred in these subjects over a mean follow-up of 8.2 years: 176 deaths in the HCV carrier group and 55 in the noncarrier group. The overall mortality rate was higher in HCV carriers than in noncarriers, adjusted for age and sex (hazard ratio, 1.53; 95% confidence interval, 1.13-2.07). Although liver-related deaths occurred more frequently among the HCV carriers (hazard ratio, 5.94; 95% confidence interval, 2.58-13.7), the rates of other causes of death did not differ between HCV carriers and noncarriers. Among HCV carriers, a higher level of HCVcAg (≥100 pg/mL) and persistently elevated alanine aminotransferase levels were important predictors of liver-related mortality. *Conclusion:* The presence of viremia increases the rate of mortality, primarily due to liver-related death, among anti–HCV-seropositive persons in Japan. (HEPATOLOGY 2009;50:393-399.)

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Hepatitis C virus (HCV) was identified 20 years ago. It is now known that between 50% and 85% of acute HCV infections become chronic[1-3]; after developing chronic infection, spontaneous HCV clearance is very rare. Approximately 170 million people worldwide are infected with HCV, and chronic HCV infection is a major health problem. HCV is a common cause of fatal liver disease, including liver cirrhosis and hepatocellular carcinoma (HCC). However, the liver-related mortality rate associated with chronic HCV infection is highly variable across different populations. In

Abbreviations: ALT, alanine aminotransferase; anti-HCV, antibody to HCV; CI, confidence interval; GGT, gamma-glutamyltransferase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HCVcAg, hepatitis C virus core antigen; HR, hazard ratio; IFN, interferon.

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patients that have been infected for more than 20 years, the occurrence of liver cirrhosis, HCC, and liver disease–related mortality are reported to be 10%-50%, 1%-23%, and 4%-15%, respectively.[4-6]

The range in published HCV-related mortality rates is due in part to the variability in the natural history of HCV infection as well as in the subjects studied. Some HCV-positive individuals have persistently normal alanine aminotransferase (ALT) levels and exhibit no clinical symptoms. Persons with this phenotype were often not included in previous hospital-based studies that focused on liver-related mortality in patients with HCV-associated liver disease/cirrhosis.[4,5] A few studies have systematically examined the risk of causes of death after HCV infection in a community-based setting[7-9]; however, the status of HCV viremia was not clear in these studies. In addition, the age range of HCV-infected subjects followed for mortality can vary considerably, with some cohort studies conducted in subjects whose average age was younger than 45 years[1,10-13] and others among older individuals.[9,14,15] To overcome some of these limitations, we analyzed mortality in 1125 subjects positive for antibody to HCV (anti-HCV) with data on viremia status, who were enrolled in a population-based cohort study in an HCV hyperendemic area of Japan between 1995 and 2005. These subjects were followed prospectively until death or until the end of the study in December 2005.

Patients and Methods

Study Population. Since 1993, we have been following anti–HCV-seropositive residents in a hyperendemic area (Town C) of Japan. The overall prevalence of anti-HCV positivity is higher (20.6%) in this region than in the surrounding area.[16] Town C is a small town in mid-western Miyazaki Prefecture, Japan, and the Town C HCV Study is a cohort study examining the natural course of HCV infection.[17-20] A general health examination program, begun in 1993, has been conducted annually for residents over 20 years of age. An ultrasonography-based liver disease screening program was initiated in 1994 to detect HCC in Town C residents who were identified as positive for anti-HCV. A total of 1321 anti–HCV-positive residents were enrolled into the cohort from 1994 through the last liver disease screening in 2006. Informed consent was obtained from subjects at the time of enrollment. The study was approved by the human subjects committees of the Harvard School of Public Health, the University of Miyazaki Faculty of Medicine, the Boston University School of Public Health, and the Kagoshima University Graduate School of Medical and Dental Sciences.

Our analysis focuses on the 1125 subjects with hepatitis C viremia data between 1995 and February of 2005, who were followed for mortality from the beginning of 1996 through the end of 2005. Anti–HCV-seropositive subjects with detectable HCV core antigen (HCVcAg) or HCV RNA were considered to be persistently infected with HCV and were classified as HCV carriers. Anti–HCV-positive subjects who were negative for HCVcAg and HCV RNA were assumed to have had a prior HCV infection and were classified as noncarriers. Subjects who underwent oral or intravenous administration of medical herbs or other palliative therapies or who had received interferon therapy were not excluded from the analyses. A subgroup analysis was conducted on HCV carrier subjects with at least three independent ALT measurements obtained at an annual general health examination or liver disease screening; ALT levels ≥ 35 were considered abnormal.

Follow-Up. For this analysis, follow-up started at the date of first HCV viremia measurement (baseline) and ended at date of death or December 31, 2005. During the course of the study, 12 residents moved to other areas, and their follow-up time was censored at that point; no other subjects were lost to follow-up. Cause of death was based on the information from the death certificate and was classified into one of seven categories: HCC, liver disease excluding HCC, neoplasms excluding HCC (i.e., other neoplasms), stroke, heart disease, pulmonary disease excluding lung cancer, and other/unknown causes.

Laboratory Methods. Serum anti-HCV antibodies were detected using second-generation enzyme immunoassay testing (Immunocheck F-HCV antibody; International Reagents Co., Kobe, Japan) or third-generation chemiluminescence enzyme immunoassays (Lumipulse Ortho II; Ortho-Clinical Diagnostics, Tokyo, Japan). In the anti–HCV-positive residents, serum levels of HCVcAg were tested with a fluorescence enzyme immunoassay (Immunocheck F-HCV Ag Core; International Reagents Co., Kobe, Japan),[21] with a detection threshold of 8 pg/mL. The presence of HCV RNA was determined by reverse transcription polymerase chain reaction (Amplicor HCV Monitor, version 1.0 [Nippon Roche, Tokyo, Japan] or version 2.0 [Nippon Roche or Roche Diagnostics K.K., Tokyo, Japan]) in study subjects whose HCVcAg levels were below the detection threshold.

Serologically defined HCV genotype (HCV serotype) was determined with a serological genotyping assay kit (Immunocheck F-HCV Grouping; International Reagents Co., Tokyo, Japan). If the HCV serotype could not be determined, the HCV genotype was examined (HCV Core Genotype; SRL, Tokyo, Japan). HCV genotype 1b was included with serotype I, and genotypes 2a and 2b

Table 1. Baseline Characteristics of Anti-HCV Antibody-Positive Subjects in Town C HCV Study

Characteristics	All Patients (n = 1125)	HCV Carriers (n = 758)	HCV Noncarriers (n = 367)	*P* Value
Age (years)				
Mean (±SD)	64.2 (±11.1)	64.9 (±10.6)	62.6 (±11.9)	0.007
Range	28-97	32-97	28-90	
Sex				
Male	456 (40.5%)	313 (41.3%)	143 (39%)	0.46
Female	669 (59.5%)	445 (58.7%)	224 (61%)	
ALT (IU/L)	40 ± 42.8 (1062)	47 ± 47.5 (719)	25.3 ± 25 (343)	<0.001
GGT (IU/L)	35.8 ± 46 (912)	39.1 ± 50.7 (612)	29.2 ± 33.6 (300)	<0.001
HCV core antigen level (pg/mL)				
Mean (±SD)		207.5 (±208.4)	-	
Median		140	-	
Range		20-1445	-	
HCV serotype				
I		463 (64.5%)	-	
II		220 (30.6%)	-	
Indeterminate		35 (4.9%)	-	
HBs antigen				
Positive	6 (0.6%)	4 (0.6%)	2 (0.6%)	0.99
Negative	948 (99.4%)	638 (99.4%)	310 (99.4%)	
History of alcohol intake				
Daily	365 (34.3%)	236 (32.9%)	129 (37.2%)	
Occasionally	206 (19.4%)	140 (19.5%)	66 (19.0%)	0.37
None	493 (46.3%)	341 (47.6%)	152 (43.8%)	
History of blood transfusion				
Yes	165 (15.7%)	101 (14.3%)	64 (18.6%)	0.07
No	885 (84.3%)	605 (85.7%)	280 (81.4%)	

Abbreviations: ALT, alanine aminotransferase; GGT, gamma-glutamyltranspeptidase; HBs antigen, hepatitis B surface antigen; HCV, hepatitis C virus.

with serotype II. No other HCV genotype was detected in this study population.

Statistical Analysis. One-factor analysis of variance, χ^2 tests, Fisher's exact tests, and the Mann-Whitney U tests were used, when appropriate, for statistical comparisons of the baseline characteristics of the HCV carrier and noncarrier groups of subjects. Cox proportional hazards regression was used to obtain hazard ratios (HRs) and 95% confidence intervals (CIs) that were adjusted for age and sex; for the analyses of cause-specific mortality, subjects who died from a different cause were censored at the time of death. The cumulative incidence of death was analyzed by the Kaplan-Meier method, and differences in the survival curves were evaluated by the log-rank test. Statistical analyses were performed using Statistical Analysis System (SAS, version 9.1; SAS Institute, Cary, NC), STATVIEW (version 5.0; Abacus Concepts, Berkeley, CA), or SPSS (SPSS Inc., Chicago, IL) software programs. A *P* value less than 0.05 was considered to be statistically significant.

Results

Demographic Characteristics of Study Subjects. As shown in Table 1, 758 (67.4%) of the anti–HCV-positive subjects were HCV carriers (i.e., positive for HCVcAg or HCV RNA), with a mean age at enrollment of 64.9 years. The HCV noncarrier group, who were considered to have had a prior HCV infection, included 367 subjects whose mean age at enrollment was 62.6 years. On average, the HCV carriers were older and had higher levels of ALT and gamma-glutamyltransferase (GGT) than the noncarriers, at baseline. In contrast, there were no significant differences between the two groups with respect to sex, alcohol intake, or history of blood transfusions. The number of subjects positive for hepatitis B surface antigen was small and not significantly different between the two groups. Sixty-seven subjects reported that they had previously received interferon (IFN) therapy, all of whom were categorized as HCV carriers when they entered the study. Fifteen of these subjects were treated prior to entering the study, five were treated during the study, and one was treated both prior to and during the study; for the other 46 subjects, the timing of IFN treatment was unknown. Although the results of IFN therapy could not be fully determined for these 67 subjects, 41 of 44 with available data in 2005 were positive for HCV RNA at that time and only three (7%) were negative for HCV RNA.

Overall and Cause-Specific Mortality. Over an average of 8.2 years of follow-up, 231 deaths occurred among the 1125 subjects (Table 2). The overall mortality

Table 2. Cause of Death in Subjects Positive for Anti-HCV Antibody

Cause of Death	All Patients	HCV Carriers	HCV Noncarriers
All causes	231	176	55
1. All liver-related deaths	76	70	6
a. HCC	45	41	4
b. Non-HCC	31	29	2
2. Neoplasms excluding HCC	41	28	13
3. Stroke	30	20	10
4. Heart disease	22	13	9
5. Pulmonary disease excluding lung cancer	32	22	10
6. Other/unknown	30	23	7

Abbreviations: HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

rate was 25.0 per 1000 person-years in this study population. Most deaths were liver-related, with 45 due to HCC and 31 to other liver diseases including cirrhosis, hepatic failure, and ruptured esophageal varix. The next most frequent cause of death was other neoplasms (n = 41), followed by pulmonary disease excluding lung cancer (n = 32), stroke (n = 30), other/unknown causes (n = 30), and heart disease (n = 22).

Of the 231 deaths, 176 were in the HCV carrier group and 55 were in the noncarrier group (Table 2). After adjusting for age and sex, HCV carriers had a significantly higher overall mortality rate (HR, 1.53; 95% CI, 1.13-2.07), compared to HCV noncarriers (Table 3). The elevated mortality rate among the subjects with evidence of HCV viremia was due to a much higher occurrence of liver-related deaths (HR, 5.94; 95% CI, 2.58-13.7). In contrast, HCV viremia was not significantly associated with death from other malignancies, stroke, heart disease, or pulmonary disease. The cumulative risk of death, based on Kaplan-Meier estimates, was 28.0% for the HCV carrier group and 17.9% for the HCV noncarrier group over 10.3 years (Fig. 1), a statistically significant difference ($P < 0.001$).

Table 3. The Association of HCV Viremia with Causes of Mortality Among Anti-HCV Antibody-Positive Subjects in Town C HCV Study

Cause of Death	HR	95% CI
All causes	1.53	(1.13, 2.07)
1. All liver-related deaths	5.94	(2.58, 13.7)
a. HCC	4.85	(1.73, 13.5)
b. Non-HCC	8.11	(1.94, 33.8)
2. Neoplasms excluding HCC	1.04	(0.54, 2.02)
3. Stroke	0.89	(0.41, 1.90)
4. Heart disease	0.68	(0.29, 1.60)
5. Pulmonary disease excluding lung cancer	1.05	(0.50, 2.22)
6. Other/unknown	1.59	(0.68, 3.71)

Abbreviations: CI, confidence interval; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio.

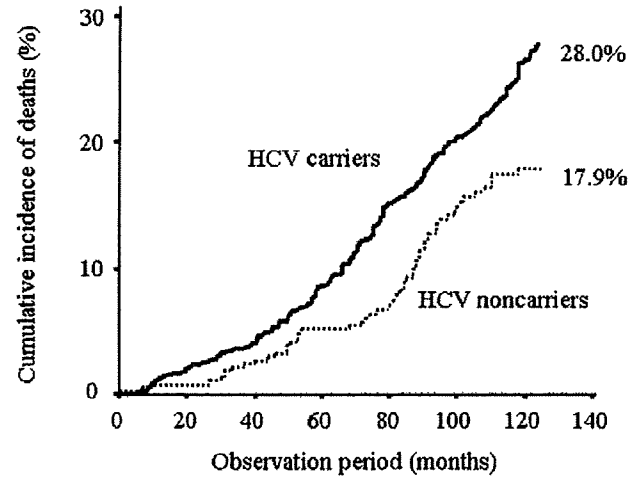

Fig. 1. Cumulative incidence of all-cause deaths in HCV carriers and noncarriers.

Predictors of Mortality Among HCV Carriers. The age-adjusted and sex-adjusted association between HCV serotype and HCVcAg level and mortality was examined among the subjects with HCV viremia. Compared to HCV serotype II, those with serotype I infection did not have a higher rate of overall (HR, 1.04) or liver-related mortality (HR, 1.12); however, having an indeterminate HCV serotype was related to both overall (HR, 3.59; 95% CI, 2.1-6.1) and liver-related death (HR, 2.12; 95% CI, 0.78-5.75). Of note, both serotype I infection (HR, 2.21; 95% CI, 0.91-5.33) and indeterminate HCV infection (HR, 3.89; 95% CI, 0.97-15.7) appeared to increase HCC mortality. In addition, a significantly increased rate of liver-related death was associated with a higher level (≥100 pg/mL) of HCVcAg (HR, 1.81; 95% CI, 1.08-3.06); the effect of higher HCVcAg level was stronger with respect to other liver-related death (HR, 2.58; 95% CI, 1.04-6.41) than to HCC death (HR, 1.48; 95% CI, 0.77-2.82). HCVcAg level had no effect on overall mortality among the HCV carriers (HR, 1.06).

In a subgroup analysis of 719 HCV carrier subjects who had data for at least three separate ALT measurements, 173 had persistently normal ALT levels while 141 had persistently abnormal levels. Subjects whose ALT levels fluctuated were not included in the analysis. Adjusting for age and sex, overall mortality (HR, 2.23; 95% CI, 1.37-3.61) and liver-related death (HR, 11.0; 95% CI, 4.35-27.9) were significantly higher for HCV carriers with persistently elevated ALT than for those with persistently normal ALT. The strongly elevated rate of liver-related mortality was evident for death due to both HCC (HR, 11.1) as well as other liver-related disease (HR, 14.5).

Discussion

Our study indicated that liver-related mortality is strongly associated with the presence of HCV viremia among persons who are seropositive for anti-HCV antibodies and that HCVcAg and ALT levels were predictors of liver-related mortality in HCV carriers. In this study population, the age distribution of anti–HCV-positive subjects, the prevalence of viremia, and the frequency of HCV serotype I were similar to previously reported data in Japan.[22-25] Japan has the highest incidence rate of HCC attributed to HCV infection among developed countries. Tanaka et al. estimated that HCV infection was spread in Japan during the 1920s, whereas HCV was widely disseminated in the United States in the 1960s.[26] The authors suggested that the HCC burden in the United States will likely increase in the next two or three decades, possibly to a level equal to that currently experienced in Japan.

Several studies have examined mortality in patients with HCV. Seeff et al. provided mortality data for 222 transfusion-associated hepatitis C cases and 377 control patients after approximately 25 years of follow-up.[27] Kamitsukasa et al. also reported mortality data for 302 HCV-infected patients with tuberculosis sequelae who had received a blood transfusion.[15] Although both studies showed that liver-related mortality was significantly higher in the disease groups than in the control groups, liver-related mortality was not the main cause of death. Kamitsukasa et al. reported that the main cause of death for approximately 45% of the patients in their study was tuberculosis sequelae.[15] Similar results were obtained in patients with inherited bleeding disorders and hepatitis C, where the main cause of death was human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS).[28] Moreover, there was no significant difference between patients with and without hepatitis C in the overall mortality rates in the study by Seeff et al. In contrast, our study showed that all-cause mortality and liver-related mortality with or without HCC were significantly higher in the HCV carrier group than in the noncarrier group. The incidence of HCC in Caucasian patients with HCV-related cirrhosis has been reported to be 1.2% in the United States,[29] whereas the incidence in Japanese patients is reportedly between 6% and 7%.[30] Furthermore, HCV-related cases in some studies included subjects with previous HCV infections.[15,27] Ethnic-dependent and racial-dependent variation in the rates of HCC, the composition of the comparisons groups, and/or complications unrelated to liver disease, such as tuberculosis sequelae or HIV/AIDS, may have resulted in differences in the patient prognoses between our study and previous studies.

It has been reported that HCC was the main cause of liver-related death in patients with compensated cirrhosis due to HCV infection.[31,32] Kasahara et al. found that 74% of liver-related death in patients with chronic hepatitis C who had not received IFN therapy was due to HCC.[33] Although HCC was more frequently observed than other liver-related deaths in our study, the proportion of HCC among all liver disease deaths (59% in the HCV carrier group) was relatively low compared to that study.[33] This occurrence may have been because the causes of death were obtained from death certificates in our study and cases of severe hepatic failure due to HCC may have been classified as liver disease excluding HCC.

A large community-based linkage study that included 78,438 individuals with hepatitis C indicated that the risk of dying from drug-related causes was significantly greater than from liver-related causes; however, the incidence of liver-related deaths was greater than that of drug-related deaths in patients older than 45 years.[7] In addition, other studies have shown that age appears to be an important risk factor that affects HCC development[14] and that the risk of cirrhosis is related to the patient's age at the time of infection and to disease activity.[34,35] These reports, which focused on patients with transfusion-associated chronic hepatitis C, suggest that the younger the patients are at the time of infection, the lower the rate of progression. Although the exact dates of infection and HCC diagnosis were not clear in our study population, the median age at enrollment was older than 60 years. Thus, the incidence of liver-related deaths might be expected to be greater than deaths from other causes.

In our study, HCV serotype I, which included HCV genotypes 1a and 1b, was found in 64.5% of the HCV carrier subjects in whom serotype was measured, whereas serotype 2, which included genotypes 2a and 2b, was detected in 30.6% of patients. These results agree with the overall distributions of HCV genotypes and serotypes in the entire Japanese population, which show that genotype 1b is the most prevalent genotype at 70%.[36] Several studies have demonstrated that genotype 1b is associated with severe liver disease, including cirrhosis and HCC.[37,38] In this study, there was an apparent association between HCV serotype I infection and mortality due to HCC. Other studies, however, have not found an effect of HCV genotype on liver disease development.[39,40] In addition, although an association of indeterminate serotype with mortality was observed (HR = 3.6), the reason for this finding is not clear. A larger study is needed to elucidate the role of genotype in the prognosis of HCV infection.

HCV RNA levels have also been reported to be associated with the progression of chronic hepatitis C.[41,42] Although the level of HCV RNA was not quantified in this study, HCVcAg levels, which are known to correlate with HCV RNA levels,[21] were assessed by fluorescence enzyme immunoassay. We observed that high HCVcAg levels were predictive of liver-related mortality, including death due to HCC, in the HCV carriers. The precise mechanism underlying HCV infection–dependent hepatocarcinogenesis is not clear. However, a study of transgenic mice that express the HCV core protein demonstrated that this protein was important in HCC development.[43] Of interest, Moucari et al. reported that insulin resistance is a specific feature of chronic hepatitis C and associated with high serum HCV RNA levels.[44] A significant increase in the incidence of diabetes has also been seen in subjects with high titer of HCV core protein compared to subjects who were negative for anti-HCV.[45] Moreover, significant fibrosis is associated with insulin resistance,[44] and diabetes mellitus is known to increase the risk of primary liver cancer in the presence of other risk factors such as hepatitis C.[46] Thus, HCVcAg levels might be associated with liver-related mortality through the development of HCV-induced insulin resistance or diabetes mellitus.

We have previously shown that elevated ALT levels are an important predictor of HCC among HCV carriers in this study population.[19] In the current analysis, ALT, aspartate aminotransferase, and GGT levels at enrollment were significantly higher in subjects who died due to a liver-related disease compared with subjects who died from other causes (data not shown). In addition, after adjusting for age and sex, overall mortality (HR, 2.23) and liver-related death (HR, 11.0) were significantly higher for HCV carriers with persistently elevated ALT than for those with persistently normal ALT.

Our study had several limitations. First, data regarding liver histology were lacking. It is likely that HCV carriers had more cirrhosis than did HCV noncarriers, given that more HCV carriers died of HCC and non-HCC liver deaths (Table 2). However, we were unable to examine this possibility directly. Information on platelet counts, which are generally inversely correlated with hepatic fibrosis, was available for a subset of subjects. Based on data obtained in 1996, mean platelet counts were significantly lower in HCV carriers (n = 539; $18.4 \times 10^4/\mu L \pm 5.6 \times 10^4/\mu L$) than in HCV noncarriers (n = 277; 21.3 ± 6.0). In addition, data from the last examination attended after 2001 showed that the persistently elevated ALT group had lower mean platelet counts (n = 94; $14.5 \times 10^4/\mu L \pm 5.5 \times 10^4/\mu L$) than did the persistently normal ALT group (n = 123; 21.8 ± 7.3). These findings suggest that the presence of viremia may increase the rate of hepatic fibrosis, especially in HCV carriers with high ALT levels.

Second, although the effect of IFN therapy may have implications with respect to the overall death rates in the study population, information on treatment was limited. However, the proportion of treated subjects with an observed sustained viral response to IFN was small (7%). Data on socioeconomic factors, which are strongly related to mortality outcomes,[47] also were not available in this study. We would not expect much variation in socioeconomic status in the study population, because the cohort included only Japanese subjects who resided in a small rural community where farming is the principal occupation. In addition, all subjects in the study population had health insurance. Thus, we believe that socioeconomic factors and IFN therapy likely did not greatly affect the rate of mortality in our study population.

In conclusion, the results of this prospective 10-year follow-up study showed a strong effect of HCV carrier status on liver-related mortality among anti-HCV–seropositive individuals. Moreover, high HCVcAg and ALT levels were important predictors of liver-related death in this population. Monitoring HCV load and ALT level in HCV carriers may be important for identifying those individuals at increased risk for HCC or other liver disease, particularly among older carriers who are less likely to respond to HCV treatment.

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Association of genetic polymorphisms with interferon-induced haematologic adverse effects in chronic hepatitis C patients

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SUMMARY. Interferon (IFN)-based combination therapy with ribavirin has become the gold standard for the treatment of chronic hepatitis C virus infection. Haematologic toxicities, such as neutropenia, thrombocytopenia, and anaemia, however, frequently cause poor treatment tolerance, resulting in poor therapeutic efficacy. The aim of this study was to identify host genetic polymorphisms associated with the efficacy or haematologic toxicity of IFN-based combination therapy in chronic hepatitis C patients. We performed comprehensive single nucleotide polymorphism detection in all exonic regions of the 12 genes involved in the IFN signalling pathway in 32 healthy Japanese volunteers. Of 167 identified polymorphisms, 35 were genotyped and tested for an association with the efficacy or toxicity of IFN plus ribavirin therapy in 240 chronic hepatitis C patients. Multiple logistic regression analysis revealed that low viral load, viral genotypes 2 and 3, and a lower degree of liver fibrosis, but none of the genetic polymorphisms, were significantly associated with a sustained virologic response. In contrast to efficacy, multiple linear regression analyses demonstrated that two polymorphisms (*IFNAR1* 10848-A/G and *STAT2* 4757-G/T) were significantly associated with IFN-induced neutropenia ($P = 0.013$ and $P = 0.011$, respectively). Thrombocytopenia was associated with the *IRF7* 789-G/A ($P = 0.031$). In conclusion, genetic polymorphisms in IFN signalling pathway-related genes were associated with IFN-induced neutropenia and thrombocytopenia in chronic hepatitis C patients. In contrast to toxicity, the efficacy of IFN-based therapy was largely dependent on viral factors and degree of liver fibrosis.

Keywords: haematologic adverse effect, hepatitis C, interferon, single nucleotide polymorphism, sustained virologic response.

INTRODUCTION

Hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1] and is a leading cause of chronic hepatitis, liver cirrhosis, and primary hepatocellular carcinoma [2]. Currently, combination therapy with ribavirin (RBV) and either conventional interferon (IFN)-α or pegylated-IFN-α (peg-IFN-α) is the gold standard of treatment for chronic HCV infection [3,4], but the overall rate of a sustained virologic response (SVR) with these therapies ranges from only 54% to 63% [5–7]. The limited therapeutic efficacy might be due to the poor virologic response in some patients or to adverse effects of the IFN-based therapy, leading to low treatment tolerance [5,6].

Predictive factors associated with a virologic response to IFN-based therapy include viral and host factors. Several studies have recently reported a possible association between the efficacy of IFN-based therapy and polymorphisms in genes encoding cytokines, chemokines, or their receptors [8–14]. The reported single nucleotide polymorphisms (SNPs) associated with a virologic response to IFN-based therapy include the *IFNAR1* [8], *IL-10* [9,10], *TNF-α* [11], *IFN-γ* [12], *CCR5* [13], *osteopontin* [14] and *TLR7* [15] genes. These data, however, are controversial and inconclusive, because most of the previous studies analysed a selected single target gene. Indeed, such limited evaluation of only one or two SNPs might not be sufficient in determining association of genetic polymorphisms with a virologic response to IFN-based therapy. Moreover, few studies have involved patients treated with combination therapy using peg-IFN-α and RBV [16,17].

Abbreviations: ALT, alanine aminotransferase; CI, confidence interval; HCV, hepatitis C virus; IFN, interferon; OR, odds ratio; PCR, polymerase chain reaction; RBV, ribavirin; SNP, single nucleotide polymorphism; SVR, sustained virologic response.

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Among the side effects of IFN plus RBV combination therapy, haematologic toxicities are frequently observed and sometimes treatment must be discontinued or the drug dose reduced, resulting in reduced efficacy of the combination therapy [5,6,18]. However, the mechanisms and predictive factors in the occurrence of these adverse effects, especially the critical decrease in blood cell count, are not clear at present.

Many studies have clarified the molecular pathway of action of IFN in detail [4,19,20]. Binding of IFN-α to its receptor induces IFNAR1 and IFNAR2 dimerization, followed by the activation of IFNAR-associated tyrosine kinases (JAK1 and TYK2). These tyrosine kinases phosphorylate STAT1 and STAT2 monomers, leading to the induction of multiple IFN-stimulated genes. Moreover, type I IFNs induce IRF7 and IRF3, which are responsible for type 1 IFN induction mediated by the virus or Toll like receptors [21]. On the other hand, the mechanisms of IFN induction in response to viral infection were recently determined [22,23]. In HCV-infected cells, the cytoplasmic RNA helicase, RIG-I, recognizes the viral dsRNA and interacts with IPS-1, leading to activation of the transcription factors, IRF3 and NF-kB, which in turn transcribe type I IFN genes. In contrast, IRF2 negatively regulates the IFN signalling pathway and recent studies suggest that IRF2 modulates the differentiation of haematopoietic cells [24–26]. Despite the unveiling of the molecular pathway of IFN signalling, it remains unclear why IFN-based therapy induces divergent efficacy or adverse haematologic toxicities in different patients.

In the present study, therefore, in order to determine the genetic factors associated with not only the efficacy but also haematologic toxicity of IFN-based therapy, we focused on the genes involved in the IFN signalling pathway, and performed a large-scale and comprehensive analysis of the genetic polymorphisms in 12 genes among chronic hepatitis C patients receiving IFN plus RBV therapy. To identify the predictors of efficacy or haematologic toxicity of IFN-based therapy, we carried out multivariate analyses using various clinicopathological factors and genetic polymorphisms.

MATERIALS AND METHODS

Patients

DNA for SNP screening was extracted from blood samples of 32 healthy Japanese volunteers under the auspices of the Pharma SNP Consortium (Tokyo, Japan). The participants comprised 240 Japanese adult chronic hepatitis C patients receiving conventional IFN-α 2b (n = 157) or peg-IFN-α 2b (n = 83) plus RBV combination therapy (Schering-Plough, Kenilworth, NJ, USA) at Kyoto University and affiliated hospitals from February 2002 to August 2007. In Japan, peg-IFN-α 2b plus RBV combination therapy was approved in October 2004. Thus, the patients who participated before and after October 2004 received conventional IFN-α 2b and peg-IFN-α 2b, respectively. Indications for IFN-based therapy included high serum values of alanine aminotransferase (ALT) and positivity for serum anti-HCV and HCV RNA. Histological examination of liver biopsy specimens was available for 165 (68.8%) of the 240 enrolled patients. Liver histology was assessed by an experienced hepatopathologist using the METAVIR score [27]; the fibrosis stage was defined as: F0 (no fibrosis), F1 (mild fibrosis), F2 (moderate fibrosis), F3 (severe fibrosis) and F4 (cirrhosis). The ethics committee at Kyoto University approved the studies, and informed consent for participation in the study was obtained from all patients.

IFN-α 2b or peg-IFN-α 2b plus RBV combination therapy

Patients receiving conventional IFN-α plus RBV therapy were treated with 6 million units of recombinant IFN-α 2b daily for 2 weeks and with 6 million units three times a week for the following assigned treatment period, in combination with daily oral RBV. The RBV dose was 600 mg/day in patients weighing less than 60 kg, and 800 mg/day in those weighing 60 kg or more. Patients receiving peg-IFN-α 2b plus RBV therapy were treated with peg-IFN-α 2b once per week, combined with daily oral RBV for the assigned period. The peg-IFN-α 2b dose was 1.5 μg/kg per week. Patients with genotype 1 received 48 weeks of combination therapy and patients with genotypes 2 and 3 received 24 weeks of combination therapy.

The dosage of IFN-α 2b or peg-IFN-α 2b was reduced by half if platelet counts dropped to <80 000/μL, if leucocyte counts dropped to <1500/μL, or if neutrophil counts dropped to <750/μL during therapy. IFN-α 2b or peg-IFN-α 2b was discontinued if platelet counts dropped to <50 000/μL, if leucocyte counts dropped to <1000/μL, or if neutrophil counts dropped to <500/μL during therapy. The RBV dosage was reduced to 400 mg/day or 600 mg/day if haemoglobin levels were less than 10 g/dL. RBV was discontinued if haemoglobin levels were less than 8.5 g/dL.

Sustained virologic response was defined as no detectable HCV RNA by qualitative assay for at least 24 weeks after cessation of therapy. Non-SVR was defined as no response or relapse after the cessation of therapy.

SNP screening of the IFN signalling pathway-related genes

We selected the following IFN signalling pathway-related genes, including seven genes involved in the intracellular IFN-mediated signalling pathway from the binding of IFN to its receptor to initiation of the transcription of various target genes [20]; four genes involved in the RIG-I signalling pathway, which triggers the IFN-induction pathway after viral infection [22,23], and one gene that negatively regulates the IFN signalling pathway [24] [IFNAR1 (NT_011512.10, NM_000629.2), IFNAR2 (NT_011512.10, NM_207585.1), JAK1 (NT_032977.7, NM_002227.1), TYK2 (NT_011295.10, NM_003331.3), STAT1 (NT_005403.15, NM_007315.2), STAT2 (NT_029419.10, NM_005419.2), IRF9 (NT_026437.11, NM_006084.3), RIG-I (NT_

008413.16, NM_014314.2), IPS-1 (NT_011387.8, NM_020746.1), IRF3 (NT_011109.15, NM_001571.2), IRF7 (NT_035113.6, NM_004031.1), and IRF2 (NT_0022792.17, NM_002199.3)]. Genomic DNA was extracted from blood samples of 32 healthy Japanese volunteers using a DNA extraction kit (Genomix Kit; TALENT, Trieste, Italy), and the 179 exons, including the 5′- and 3′-untranslated regions and adjacent intronic regions of the 12 candidate genes, were amplified. The resultant polymerase chain reaction (PCR) products were used as templates for direct sequencing on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). Segregating sites were identified and genotypes were confirmed directly from electrophorograms using Genalys (http://www.software.cng.fr/docs/genalys.html) [28].

SNP genotyping

Among the SNPs identified by the screening, we selected tag SNP markers that covered all of the common (>5% frequency) haplotypes using the minimal haplotype tagging method, one of the best methods to identify the smallest tagging set for an arbitrary region of the genome [29]. These tag SNPs allowed us to genotype the smallest possible number of SNPs for each gene while resolving all common haplotypes. We also included SNPs that existed in coding sequences or 5′ flanking regions with frequencies higher than 5%. These SNPs were genotyped using the ABI Taqman allelic discrimination method and an ABI 7900HT sequence detection system (Applied Biosystems). Primers and probes were designed by the manufacturer with SNP browser Software (Applied Biosystems), as shown in Tables S1 and S2. Amplification reactions were performed in a 3 μL volume, with 5 ng DNA, 1.5 μL universal PCR master-mix, and 0.0375 μL assay mix with the specific primers and probes. Seven SNPs that could not be detected using the Taqman assay were determined by direct sequencing of PCR products amplified with primers specific for each SNP (Table S3).

Statistical analysis

Genotype distributions were tested for Hardy–Weinberg equilibrium using exact tests. To identify predictors of SVR, we used univariate analysis of pre-treatment factors to compare all SVR and non-SVR patients who had completed the treatment. The following pre-treatment factors were considered: SNPs, sex (male *vs* female), age (in years), weight (in kilograms), serum ALT, IFN history (naive *vs* relapse *vs* nonresponse), HCV genotype (1 *vs* 2 and 3), HCV viral load (<100 *vs* 100 to <500 *vs* 500 to <850 *vs* ≥850 kIU/mL), and fibrosis stage (F0 *vs* F1 *vs* F3 *vs* F4). Allele and genotype frequencies were evaluated for their association with SVR using Fisher's exact tests. Sex, IFN history, and HCV genotype were evaluated using the chi-square test. Age, weight, and serum ALT were evaluated using the Mann–Whitney *U*-test. Fibrosis stage and viral load were evaluated using a trend chi-square test. We considered two-tailed *P*-values <0.05 to be statistically significant and calculated odds ratios (ORs) and 95% confidence intervals. Multiple logistic regression analysis was performed using STATISTICA (StatSoft, Tulsa, OK, USA) to evaluate the association between SVR and significant factors from the univariate analyses.

To identify predictors of cytopenia, we examined the association between decreased leucocyte, neutrophil, and platelet counts and haemoglobin levels, and the following patient characteristics and clinical features using linear regression analysis with STATISTICA: sex, age, weight, fibrosis stage and SNPs. Multiple linear regression analysis was performed to evaluate the association between the decreased peripheral blood cell numbers and significant factors from the univariate analyses.

RESULTS

Genetic variations and polymorphisms in IFN signalling pathway-related genes

By screening 32 healthy volunteers, we identified 167 genetic polymorphisms (153 SNPs and 14 insertions/deletions) in the 12 IFN signalling pathway-related genes (Table 1, Table S4). All identified polymorphisms were in Hardy–Weinberg equilibrium. Of these 167 polymorphisms, 60 (49 SNPs and 11 insertions/deletions) were novel and were not registered in Build 125 of the SNP database (http://www.ncbi.nlm.nih.gov) (Table 2). Among the 167 SNPs identified, 30 (16 nonsynonymous and 14 synonymous) were located in exons and we confirmed that 14 of the 30 SNPs identified in the exons were novel. Furthermore, we identified 10 novel nonsynonymous variants in the seven genes. Sixty-two SNPs were relatively uncommon (minor allele frequency <0.05) and were thus excluded from further analysis. Finally, 27 selected tag SNPs and eight additional SNPs that existed in coding sequences or 5′ flanking regions were subjected to further genotyping analyses in chronic hepatitis C patients (Table 2).

Variables associated with virologic response to IFN-based therapy

The relationship between baseline characteristics and virologic response to the IFN plus RBV combination therapy in chronic hepatitis C patients is summarized in Table 3. Combination therapy was discontinued in 37 patients during the assigned treatment period. These 37 patients were excluded from analysis of the virologic response. SVR was achieved in 98 of 203 (48.3%) patients, and 105 patients (51.7%) had a relapse of HCV infection after the end of therapy or showed no response to IFN-based therapy.

To determine the predictive factors for IFN-based therapy efficacy, we examined the correlation between virologic response, and clinical and viral factors. Of 56 patients with

Table 1 Classification of polymorphisms identified in the IFN-signalling related genes

Gene	Number of base pairs screened	SNP	Ins/del	Total polymorphisms	Distribution by gene structure					
					5′FL	5′UTR	CDS (sSNP, nsSNP)	Intron	3′UTR	3′FL
IFNAR1	7522	8	2	10	2	1	0 (0, 0)	5	2	0
IFNAR2	4849	6	0	6	0	0	1 (0, 1)	4	1	0
JAK1	11312	18	2	20	0	0	2 (2, 0)	18	0	0
TYK2	8270	19	0	19	0	0	8 (3, 5)	10	1	0
STAT1	10647	20	3	23	0	0	3 (3, 0)	18	1	1
STAT2	8646	13	1	14	0	0	1 (0, 1)	11	2	0
IRF9	3171	3	0	3	0	0	0 (0, 0)	2	1	0
RIG-I	8819	26	1	27	0	1	4 (1, 3)	19	3	0
IPS-1	5105	11	1	12	0	0	3 (1, 2)	2	7	0
IRF3	3968	8	2	10	0	3	2 (1, 1)	4	0	1
IRF7	2589	8	1	9	1	0	4 (2, 2)	4	0	0
IRF2	5668	13	1	14	0	0	2 (1, 1)	10	1	1
Total	74898	153	14	167	3	5	30 (14, 16)	107	19	3

SNP, single nucleotide polymorphism; ins, insertion; del, deletion; UTR, untranslated region; FL, flanking region; CDS, coding region; sSNP, synonymous SNP; nsSNP, nonsynonymous SNP.

Table 2 One hundred and sixty-seven polymorphisms in the IFN-signalling related genes

IFNAR1 (10 polymorphisms)	**−347**	**−6***	51	10595	**10848**	10927	24135	24469*
	33483*	**33741**						
IFNAR2 (6 polymorphisms)	14693	14983	22299*	**22687**	33267*	34057*		
JAK1 (20 polymorphisms)	91	365	12755	13212	13242*	21305	30599	30856*
	34934*	34999	35312	38993	39038	**40725**	40870	40871
	41498	42571*	46465	51217*				
TYK2 (19 polymorphisms)	2243*	12345*	12529	14003*	**14006**	14145*	15192*	15452
	15560	18074	18164*	18279	21293*	26247*	26378*	**26525**
	26561*	26854	29721*					
STAT1 (23 polymorphisms)	283	821*	**4270**	5384	6630*	6751	16036	16151
	16539	23416	24514	**27161***	27452	28838	30625	34532*
	35386*	35574*	37058	37178	39478	44152*	**45397**	
STAT2 (14 polymorphisms)	88	3706	3765*	**4757***	**4901***	9465	9488	9634
	9819	10543	11441*	16088*	18063	18306*		
IRF9 (3 polymorphisms)	621*	**1129***	4265*					
RIG-I (27 polymorphisms)	**90**	177	354	391*	408	**33794***	33971	**35083**
	35263	37764	38008*	38086	41043	46072	49075*	53235
	58363*	58590*	58615*	59861*	60046*	60133*	60139	**66873**
	69596	69667	70306					
IPS1 (12 polymorphisms)	10717	10748	**10952***	**15495**	**15538**	18908	**19354**	19653
	19836*	**20479***	20921*	20927				
IRF3 (10 polymorphisms)	**95**	175	188	244	418*	1389	2320	**2652**
	6206	6304*						
IRF7 (9 polymorphisms)	−198	390*	**457**	**789**	1335	1598*	2488	2686
	2829*							
IRF2 (14 polymorphisms)	45305	45371*	45420	55441*	56210	**66675**	75602*	**83546**
	83649*	83700*	83749*	**85509**	86327	87066		

Gene number is expressed as the nucleotide position from the first nucleotide of the transcriptional start codon. Polymorphisms in boldface are selected as tag SNP markers.
*Newly discovered polymorphisms.

Table 3 Demographic, virological and clinical features of patients with chronic hepatitis C treated by IFN plus ribavirin combination therapy

Variable	SVR (n = 98)*	Non-SVR (n = 105)*	P-value
Sex			
Male	64	50	**0.011**
Female	34	55	
Age (years)†	56.0 (24–72)	58.5 (27–74)	**0.023**
Weight (kg)†	61.8 (43–91)	61.9 (41–94)	0.821
Pre-treatment ALT (IU/L)†	69 (17–285)	57 (16–304)	0.170
Interferon history‡			
Naive	58 (51.8%)	54 (48.2%)	0.311
Relapse	30 (47.6%)	33 (52.4%)	
Nonresponse	10 (35.7%)	18 (64.3%)	
HCV genotype			
1	50 (34.0%)	97 (66.0%)	**0.00000002**
2, 3	48 (85.7%)	8 (14.3%)	
HCV RNA titre (kIU/mL)			
<100	15 (93.7%)	1 (6.3%)	**0.004**
100–500	35 (49.3%)	36 (50.7%)	
500–850	22 (44.9%)	27 (55.1%)	
850≤	26 (38.8%)	41 (61.2%)	
Fibrosis score			
0	5 (62.5%)	3 (37.5%)	**0.0005**
1	38 (59.4%)	26 (40.6%)	
2	19 (45.2%)	23 (54.8%)	
3	5 (20.0%)	20 (80.0%)	
4	2 (28.6%)	5 (71.6%)	

SVR, sustained virologic response. P-values in boldface are significant.

*SVR and non-SVR were evaluated in patients who had completed therapy for 24 or 48 weeks.

†Values are median (range).

‡One hundred six patients had received previous treatment with IFN-α monotherapy for 24 weeks, but failed to respond or relapsed.

HCV genotypes 2 and 3, 48 (85.7%) had SVR, whereas 50 of 147 (34.0%) patients with HCV genotype 1 had SVR, indicating that HCV genotype 1 was significantly associated with non-SVR (P = 0.00000002). In addition, a lower viral load before treatment (P = 0.004), male sex (P = 0.011), young age (P = 0.023), and lower degree of liver fibrosis (P = 0.0005) were significantly associated with SVR.

SNP genotyping analyses revealed that the frequencies of all 35 polymorphisms detected in the 240 hepatitis C patients were not significantly different from those in healthy volunteers. The success scores of the Taqman assay were 96.4–100% and those of direct sequencing were 95.8–100%. Univariate analyses of 35 polymorphisms revealed that a *TYK2* exon8 15560-G/T polymorphism (rs2304256) was significantly associated with virologic response to IFN-based therapy [P = 0.050, OR = 0.66 (0.44–0.99)] (Table S5).

In contrast to the univariate analysis, however, multiple logistic regression analyses demonstrated that the rs2304256 was not significant (P = 0.675) (Table 4). As a host factor, only a lower degree of liver fibrosis before therapy (P = 0.007) was significantly associated with SVR in the multiple logistic regression model. On the other hand, HCV genotypes 2 and 3 (P = 0.00005) and a lower viral load before therapy (P = 0.027) were both significantly associated with SVR.

Genetic polymorphisms associated with the adverse effects of IFN-based therapy

A total of 132 of 240 (55.0%) patients required either a discontinuation or a dose reduction of IFN or RBV due to the following adverse events: anaemia (n = 50), neutropenia or leucocytopenia (n = 32), thrombocytopenia (n = 17), depression (n = 7), and other causes (malaise, alopecia, and abdominal discomfort). The relationship between baseline characteristics and occurrence of haematologic adverse effects of the IFN plus RBV combination therapy is summarized in Table S6.

To identify the host genetic polymorphisms associated with the haematologic adverse effects of IFN plus RBV therapy, we focused on decreases in blood cell counts during the therapy and analysed the association with the SNPs in IFN signalling pathway-related genes. Consistent with previous reports [30,31], leucocyte, neutrophil, and platelet counts and haemoglobin levels usually declined in the initial 2–4 weeks of treatment, then stabilized during treatment, and returned to baseline levels within 12 weeks from the end of treatment in patients receiving IFN plus RBV therapy (Fig. 1). Therefore, we evaluated the decreases in leucocyte, neutrophil, and platelet counts and haemoglobin level at 4 weeks of treatment. We first examined the predictive factors for neutropenia. In 240 patients, absolute neutrophil counts decreased by an average of 39.3% from baseline during the first 4 weeks of treatment. Univariate analyses of 32 polymorphisms and clinical features showed that two SNPs, an *IFNAR1* intron2 10848-A/G polymorphism (rs2243594), and a *STAT2* intron5 4757-G/T, were associated with neutropenia caused by IFN-based therapy [P = 0.038, P = 0.020] (Table 5, Table S7). Furthermore, multivariate linear regression analysis confirmed that both polymorphisms were significantly associated with the neutropenia (P = 0.013, P = 0.009). Next, we examined the predictive factors for leucocytopenia. Absolute leucocyte counts decreased by an average of 29.9% from baseline within the first 4 weeks of treatment. Univariate analyses indicated that an *IFNAR1* intron2 10848-A/G polymorphism (rs2243594), an *IRF2* intron6 66675-C/T polymorphism (rs2241500), and female sex were associated with leucocytopenia (P = 0.048, P = 0.026, P = 0.016,

Table 4 Univariate and multiple logistic regression analyses of SNPs and clinical factors associated with the efficacy of IFN plus ribavirin combination therapy

	Univariate analysis		Multiple logistic regression analysis	
Variable	*P*-value	OR (95% CI)	*P*-value	OR (95% CI)
SNPs				
TYK2 15660-G/T	**0.050**	0.66 (0.44–0.99)	0.675	0.48 (0.14–1.67)
Clinical variables				
Sex	**0.011**	2.07 (1.17–3.66)	0.082	2.09 (0.90–4.84)
Age	**0.023**	0.16 (0.04–0.65)	0.347	0.69 (0.11–4.22)
HCV genotype	**0.00000002**	11.6 (5.09–26.6)	**0.00005**	7.35 (2.54–21.2)
Viral load	**0.004**	0.25 (0.10–0.62)	**0.027**	0.22 (0.06–0.88)
Fibrosis stage	**0.0005**	12.0 (2.63–54.8)	**0.007**	10.3 (1.72–62.3)

P-values in boldface are significant. SNP, single nucleotide polymorphism.

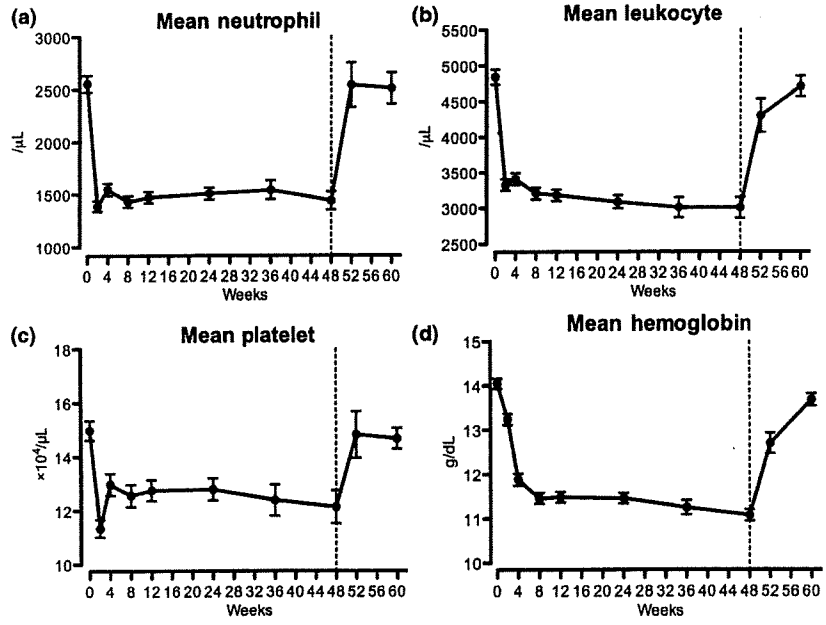

Fig. 1 Change in mean neutrophil (a), leucocyte (b), and platelet counts (c), and haemoglobin levels (d) during and after IFN plus RBV therapy. The results are shown as mean ± SEM.

respectively). Multivariate analysis, however, indicated that none of the factors, including *IFNAR1* rs2243594, *IRF2* rs2241500 and sex, were significant. Third, we examined the predictive factors for thrombocytopenia. Absolute platelet counts decreased by an average of 12.5% from baseline during the first 4 weeks of treatment. Univariate analyses showed that only an *IRF7* exon2 789-G/A (rs1061501) was associated with thrombocytopenia ($P = 0.031$). Finally, we examined the predictive factors for anaemia. Absolute haemoglobin concentration decreased by an average of 15.8% of baseline within the first 4 weeks of treatment. Univariate analyses revealed that anaemia was associated with older age ($P = 0.0004$), but not with any of the polymorphisms.

We examined the genotype results (variant allele carrier) of an *IFNAR1* intron2 10848-A/G polymorphism (rs2243594), a *STAT2* intron5 4757-G/T polymorphism, and an *IRF7* exon2 789-G/A polymorphism (rs1061501) for their association with various clinical and histologic features among 240 patients (Table S8). None of the factors, however, were associated with the SNPs identified.

DISCUSSION

In this study, we evaluated the influence of genetic polymorphisms on adverse effects and efficacy of IFN plus RBV combination therapy. Although several studies have evaluated the influence of host genetic polymorphisms on virologic response to IFN-based therapy, no studies have looked at possible association of adverse effects of the IFN-based therapy and host genetic polymorphisms. We report for the first time that certain SNPs in the IFN signalling pathway-related genes were associated with haematologic adverse effects in chronic hepatitis C patients undergoing IFN-based therapy.

Table 5 Univariate and multiple linear regression analyses of SNPs and clinical factors associated with leucocytopenia, neutropenia and thrombocytopenia

Variable	Unit of B coefficient	Univariate analysis			Multiple analysis		
		P-value	*B* coefficient	SE *B*	*P*-value	*B* coefficient	SE *B*
Neutropenia							
IFNAR1 10848-A/G	%	**0.038**	6.94	3.31	**0.013**	6.43	2.57
STAT2 4757-G/T	%	**0.020**	−14.3	6.09	**0.011**	−13.8	5.41
Leucocytopenia							
IFNAR1 10848-A/G	%	**0.048**	4.14	2.08	0.109	1.62	1.61
IRF2 66675-C/T	%	**0.026**	3.44	1.53	0.054	3.00	1.54
Sex	%	**0.016**	7.79	3.20	0.134	3.30	2.20
Thrombocytopenia							
IRF7 789-G/A	%	**0.031**	4.15	1.92	ND	ND	ND
Anaemia							
Age	%/year	**0.0004**	0.28	0.08	ND	ND	ND

P-values in boldface are significant. SE, standard error. ND, not done because only one factor was significant in the univariate analysis.

The representative side effect of IFN-based combination therapy with RBV that causes poor therapeutic tolerance is haematologic toxicity, such as anaemia, neutropenia, and thrombocytopenia [4,32]. In fact, several studies reported that less than half the patients with hepatitis C were able to complete IFN plus RBV combination therapy at the assigned dose of both drugs, causing reduced therapeutic efficacy [5,6]. One thing to be noted is that the decrease in neutrophil and platelet counts induced by IFN-based therapy varies among patients, and thus it is difficult to predict the risk of haematologic toxicities in chronic hepatitis C patients receiving IFN-based therapy. The molecular mechanism of IFN-induced haematologic toxicities, however, is unknown. Several studies suggested the possibility that IFN treatment causes bone marrow suppression [33,34]. In agreement with this hypothesis, it was shown that a significant drop in platelet count after the initiation of IFN therapy is accompanied by a moderate increase in thrombopoietin levels in the failing liver, which may be insufficient to counteract the myelosuppressive action of IFN [35]. Another study suggested that IFN-mediated cytopenia may be due to rapid sequestration of platelets and leucocytes in the capillary beds of the liver and spleen [36]. Our current findings suggest that some of the IFN signalling pathway-related genes are involved in the decrease in neutrophil and platelet counts in response to IFN treatment. Interestingly, a recent study demonstrated that an intrinsic program for apoptosis controls platelet survival and dictates life span [37]. They revealed that platelets are genetically programmed to die by apoptosis and the antagonistic balance between antiapoptotic and proapoptotic molecules determines platelet life span. It is well known that IFN signalling induces the expression of multiple IFN-stimulated genes including molecules with proapoptotic or antiapoptotic function, such as tumour necrosis factor-related apoptosis-inducing ligand Fas, and X-linked inhibitor of apoptosis-associated factor 1 [38]. Thus, it is possible that IFNAR1, STAT2, and IRF7 contribute to the occurrence of neutropenia and thrombocytopenia by regulating the magnitude of IFN signalling involved in the apoptotic pathway in the haematopoietic cells in patients receiving IFN-based treatment.

In this study, three SNPs were associated with cytopenia in chronic hepatitis C patients receiving IFN plus RBV combination therapy. Among them, rs1061501 in the *IRF7* gene was located in the exon region but is a synonymous SNP. Recently, Kimchi-Sarfaty *et al.* demonstrated [39] that a synonymous SNP that did not affect amino acid sequence was capable of changing the function of the resultant protein. Indeed, the presence of a rare codon marked by a synonymous SNP in the *Multidrug Resistance 1* gene affects the timing of cotranslational folding and thereby alters the structure of substrate. Thus, it is possible that the synonymous rs1061501 contributes to a functional change in the IRF7 protein. On the other hand, rs2243594 in the *IFNAR1* gene and the SNP in the *STAT2* gene associated with neutropenia were located in an intronic region. In general, intronic SNPs provide little evidence for changes in protein structure or function, but an intronic mutation in the *p53* gene could have functional consequences by regulating gene expression, suggesting that the effect is mediated by a nonsynonymous and disruptive coding change in linkage disequilibrium with the associated intronic SNP or by a change in RNA splicing, editing, or expression [40]. Thus, it is possible that two intronic SNPs associated with neutropenia contribute to functional changes in the IFNAR1 and STAT2 proteins.

In contrast to the adverse effects of IFN plus RBV combination therapy, none of the host genetic polymorphisms in the IFN signalling pathway-related genes analysed were

associated with therapeutic efficacy. The results indicated that viral factors, including viral genotype and pre-treatment viral load, and histological fibrosis grade were likely to have critical roles in treatment response. Consistent with many previous reports [41–43], we found that HCV genotypes 2 and 3, low viral load, and early fibrosis stage predict a favourable virologic response to IFN plus RBV combination therapy. On the other hand, it was reported that several SNPs in certain genes are associated with efficacy in IFN-based therapy [8–14,16,17]. Many of these previous studies, however, evaluated the association between the SNP and the treatment response using only univariate and not multivariate analyses that included viral factors. In fact, in our univariate analysis, one *TYK2* SNP (rs2304256) showed a possible association with therapeutic efficacy. Multivariate analysis, however, revealed that this SNP was not significant. Taken together, these findings suggest that the viral factors and host histological grade of liver fibrosis are important predictors of the treatment response in chronic hepatitis C infection. Although no significant association was observed between the efficacy and the IFN signalling pathway-related genes examined, it is possible that polymorphisms of other genes might play a role in the treatment response to IFN-based therapy.

In conclusion, we demonstrated that the SNPs in the *IFNAR1* and *STAT2* genes were associated with neutropenia and the SNP in the *IRF7* gene was associated with thrombocytopenia in chronic hepatitis C patients receiving IFN plus RBV combination therapy. In contrast, the virologic factors and histological grade of liver fibrosis are important predictors for virologic response to the IFN-based therapy, whereas no host genetic polymorphisms in IFN signalling pathway-related genes analysed affected the therapeutic efficacy. Further analyses are required to clarify the mechanisms of how those polymorphisms affect the biologic function of the IFN signalling and contribute to the occurrence of haematological adverse effects in IFN-treated patients.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Oligonucleotide sequences for primers and probes used for Taqman SNP genotyping assay.

Table S2 Assay ID of primers and probes used for Taqman SNP genotyping assay.

Table S3 Oligonucleotide sequences for primers used for PCR amplification and sequencing.

Table S4 List of discovered polymorphisms in 12 IFN-signalling related genes.

Table S5 Genotype frequency in the genotyped 35 polymorphisms of the IFN-signalling related genes.

Table S6 Demographic, virological and clinical features of patients with chronic hepatitis C treated by IFN plus ribavirin combination therapy.

Table S7 Linear regression analyses of 35 SNPs and clinical factors associated with haematologic adverse effects.

Table S8 Demographic, and clinical features according to three polymorphisms significantly associated with IFN-induced neutropenia and thrombocytopenia.

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The *HBS1L-MYB* intergenic interval associated with elevated HbF levels shows characteristics of a distal regulatory region in erythroid cells

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***HBS1L-MYB* intergenic polymorphism *(HMIP)* on chromosome 6q23 is associated with elevated fetal hemoglobin levels and has pleiotropic effects on several hematologic parameters. To investigate potential regulatory activity in the region, we have measured sensitivity of the sequences to DNase I cleavage that identified 3 tissue-specific DNase I hypersensitive sites in the core intergenic interval. Chromatin immunoprecipitation with microarray (ChIP-chip) analysis showed strong histone acetylation in a defined interval of 65 kb corresponding to the core *HBS1L-MYB* intergenic region in primary human erythroid cells but not in non–*MYB*-expressing HeLa cells. ChIP-chip analysis also identified several potential *cis*-regulatory elements as strong GATA-1 signals that coincided with the DNase I hypersensitive sites present in *MYB*-expressing erythroid cells. We suggest that *HMIP* contains regulatory sequences that could be important in hematopoiesis by controlling *MYB* expression. This study provides the functional link between genetic association of *HMIP* with control of fetal hemoglobin and other hematologic parameters. We also present a large-scale analysis of histone acetylation as well as RNA polymerase II and GATA-1 interactions on chromosome 6q, and α and β globin gene loci. The data suggest that GATA-1 regulates numerous genes of various functions on chromosome 6q. (Blood. 2009;114:1254-1262)**

Introduction

Variable levels of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) persist into adulthood, and although they have no clinical consequences in otherwise healthy individuals, high HbF levels have a major impact on the principal β hemoglobin disorders—β thalassemia and sickle cell disease. Increased HbF production mitigates the severity of both diseases.[1-3] The level of HbF in adults is inherited as a quantitative trait, and is largely genetically controlled with a heritability of 0.89.[4]

Three loci—*HBS1L-MYB* intergenic region on chromosome 6q23, *BCL11A* on chromosome 2p16, and the β globin cluster on chromosome 11—account for up to 50% of the variation in HbF levels in patients with sickle cell anemia or β thalassemia and in healthy European whites.[5-7] The *HBS1L-MYB* intergenic region alone contributes approximately 20% of the overall trait variance in healthy European whites,[5,8] and 3% to 7% of the trait variance in African-American and Brazilian patients with sickle cell anemia.[6]

The panel of single nucleotide polymorphisms in the *HBS1L-MYB* region that account for the effects of the 6q locus[6,8-10] reside in a nearly contiguous segment of 79 kb distributed in 3 linkage disequilibrium blocks, referred to as *HBS1L-MYB* intergenic polymorphism *(HMIP)* blocks 1, 2, and 3.[8] Genetic variants that show the strongest effects are concentrated in 24 kb of *HMIP* block 2, located 33 kb upstream of *HBS1L* and 65 kb upstream of *MYB*.[8] The mechanisms through which these variants operate to increase HbF are still not clear, but studies suggest that the biologic effects are likely to involve regulation of the flanking genes—*HBS1L* and *MYB*. *MYB* and *HBS1L* expression was significantly reduced in erythroid cultures of individuals with high HbF levels, whereas overexpression of *MYB* in K562 cells inhibited γ-globin expression supporting *MYB*'s role in HbF regulation.[11] Further, *HBS1L* and *MYB* expression was positively correlated in erythroid progenitor cells, and *HBS1L* expression correlated with the genetic variants associated with HbF.[8] Variability in *HMIP* block 2 was subsequently shown to have a pleiotropic effect on erythrocyte count and volume, and platelet and monocyte counts in healthy Europeans.[12] These observations suggest that the *HBS1L-MYB* intergenic region is functionally active, containing distal regulatory sequences for the flanking genes—*HBS1L* and *MYB*. The function of *HBS1L* is unknown but it encodes a protein with apparent GTP-binding activity, involved in the regulation of a variety of critical cellular processes,[13] and *MYB* encodes a transcription factor involved in oncogenesis and with an essential role in erythropoiesis.[14-16]

Initially, we investigated the regulatory potential of *HMIP* block 2 by measuring sensitivity of the sequences in this region to DNase I cleavage that identified multiple DNase I hypersensitive sites in the region in K562 cells. We then proceeded to a comprehensive analysis of the regulatory potential of a large region of chromosome 6q using chromatin immunoprecipitation (ChIP) and microarray (ChIP-chip) analysis on primary human erythroid progenitor cells. We identified strong signals of histones H3 and H4 acetylation in the *HBS1L-MYB* intergenic region (indicative of active chromatin) especially concentrated in block 2, in basophilic erythroblasts when the globin genes and *MYB* are fully active. Tissue specificity of the regulatory activity in the intergenic region

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