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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 chronic¹⁻³; 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 setting⁷⁻⁹; 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 (\pm SD)	64.2 (\pm 11.1)	64.9 (\pm 10.6)	62.6 (\pm 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 \pm 42.8 (1062)	47 \pm 47.5 (719)	25.3 \pm 25 (343)	<0.001
GGT (IU/L)	35.8 \pm 46 (912)	39.1 \pm 50.7 (612)	29.2 \pm 33.6 (300)	<0.001
HCV core antigen level (pg/mL)				
Mean (\pm SD)		207.5 (\pm 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.

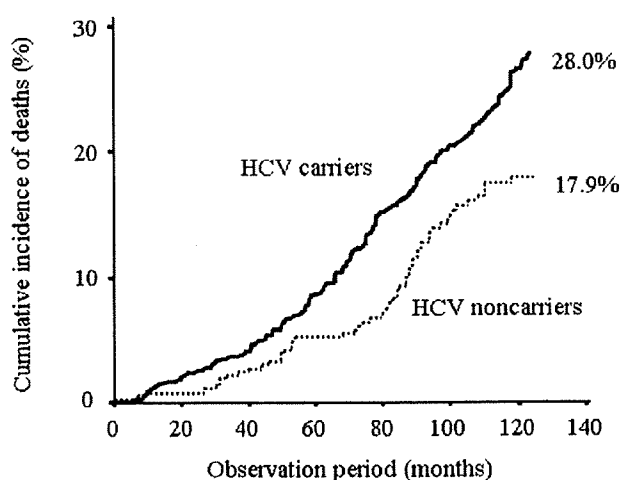


Fig. 1. Cumulative incidence of all-cause deaths in HCV carriers and noncarriers.

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 non-carrier 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 development¹⁴ 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 ($n = 539$; $18.4 \times 10^4/\mu\text{L} \pm 5.6 \times 10^4/\mu\text{L}$) 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 ($n = 94$; $14.5 \times 10^4/\mu\text{L} \pm 5.5 \times 10^4/\mu\text{L}$) 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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Proanthocyanidin from Blueberry Leaves Suppresses Expression of Subgenomic Hepatitis C Virus RNA^{*[S]}

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Hepatitis C virus (HCV) infection is a major cause of chronic liver disease such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma. While searching for new natural anti-HCV agents in agricultural products, we found a potent inhibitor of HCV RNA expression in extracts of blueberry leaves when examined in an HCV subgenomic replicon cell culture system. This activity was observed in a methanol extract fraction of blueberry leaves and was purified by repeated fractionations in reversed-phase high-performance liquid chromatography. The final purified fraction showed a 63-fold increase in specific activity compared with the initial methanol extracts and was composed only of carbon, hydrogen, and oxygen. Liquid chromatography/mass-ion trap-time of flight analysis and butanol-HCl hydrolysis analysis of the purified fraction revealed that the blueberry leaf-derived inhibitor was proanthocyanidin. Furthermore, structural analysis using acid thiolytic indicated that the mean degree of polymerization of the purified proanthocyanidin was 7.7, consisting predominantly of epicatechin. Proanthocyanidin with a polymerization degree of 8 to 9 showed the greatest potency at inhibiting the expression of subgenomic HCV RNA. Purified proanthocyanidin showed dose-dependent inhibition of expression of the neomycin-resistant gene and the NS-3 protein gene in the HCV subgenome in replicon cells. While characterizing the mechanism by which proanthocyanidin inhibited HCV subgenome expression, we found that heterogeneous nuclear ribonucleoprotein A2/B1 showed affinity to blueberry leaf-derived proanthocyanidin and was indispensable for HCV subgenome expression in replicon cells. These data suggest that proanthocyanidin isolated from blueberry leaves may have potential usefulness as an anti-HCV compound by inhibiting viral replication.

chronic hepatitis at high rates and finally results in liver cirrhosis and subsequent occurrence of hepatocellular carcinoma (1–3). The number of people worldwide who are infected by HCV is estimated to be over 200 million with 2 million infections in Japan (4). The South Kyushu area of Japan, including Miyazaki prefecture, has a high prevalence of this virus, and it is now recognized as a social problem. There is no vaccine effective for HCV at present. The elimination of HCV may be achieved by a combination of pegylated α -interferon and ribavirin, a broad spectrum antiviral drug (4–6). However, virological response to this combination therapy has been reported to be 80% for genotypes 2 and 3, but less than 50% for genotype 1 (7, 8). Moreover, α -interferon is associated with severe side-effects, including leucopenia, thrombocytopenia, depression, fatigue, and flu-like symptoms, and ribavirin is associated with side-effects such as hemolytic anemia (9). Therefore, establishment of a new modality of treatment without serious adverse effects is still required.

Considering the prolonged period (20–30 years) required for development of liver cirrhosis and hepatocellular carcinoma in individuals infected with HCV, we speculated that progression of the disease might be influenced by daily diet. Our research project focuses on the daily use of agricultural products that could cure or reduce the risk of disease progression by HCV. Thus, we screened local agricultural products (1700 samples from 283 species) for their suppressive activity against HCV subgenome expression using an HCV replicon cell system. We found a significant suppressive activity in extracts of blueberry leaves. Blueberries are classified in the genus *Vaccinium*, and the species are native only to North America. Blueberry leaves have high quinic acid and chlorogenic acid contents and also significant flavonol glycosides such as rutin. Thus, they are high in antioxidant activity. In our subsequent screening studies using various kinds of blueberry species, the most potent activity was observed in the leaf of rabbit-eye blueberry (*Vaccinium virgatum* Aiton), which is cultivated in southern areas of Japan.

Hepatitis C virus (HCV)² is often associated with the development of chronic liver diseases. Infection by HCV causes

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² The abbreviations used are: HCV, hepatitis C virus; hnRNP, heterogeneous nuclear ribonucleoprotein; HPLC, high-performance liquid chromatogra-

phy; PDA, photodiode array; EPMA, electron probe micro-analysis; LC/MS-IT-TOF, liquid chromatography/mass spectrometry-ion trap-time of flight; APCI, atmospheric pressure chemical ionization; mDP, mean degree of polymerization; IC₅₀, concentration required for 50% inhibition; CC₅₀, concentration required for 50% cytotoxicity; eIF3, eukaryotic translation initiation factor 3; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; IRES, internal ribosome entry site; DiGE, differential gel electrophoresis.

Blueberry Leaf Proanthocyanidin Suppresses HCV

In this study, extracts of rabbit-eye blueberry leaves were used in an effort to purify and identify the compound responsible for inhibition of the expression of subgenomic HCV RNA. We identified oligomeric proanthocyanidin with mean degree of polymerization (mDP) around eight as an inhibitor of HCV subgenome expression. We also analyzed cellular proteins that have affinity to the oligomeric proanthocyanidin in HCV replicon cells and identified heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 as one of candidate proteins involved in the proanthocyanidin-mediated inhibition of HCV subgenome expression.

EXPERIMENTAL PROCEDURES

Extraction of Blueberry Leaves—A lyophilized powder made from leaves of rabbit-eye blueberry (*V. virgatum* Aiton) was provided by Unkai Shuzo Co., Ltd. (Miyazaki, Japan). One gram of the lyophilized powder was extracted with 100 ml of methanol at room temperature with shaking for 15 min, and the supernatant was passed through filter paper (filter paper No.2, Toyo, Tokyo, Japan). The methanol extract was then extracted with 100 ml of chloroform, followed by centrifugation ($1750 \times g$ for 10 min), and the resultant precipitate and supernatant were collected. The precipitate was dissolved in methanol, concentrated *in vacuo*, and lyophilized (CMW-ppt). The supernatant was mixed with 150 ml of distilled water and methanol to perform a liquid-liquid extraction, and the water layer was collected and mixed with 150 ml of chloroform to repeat the chloroform extraction. The water layer was concentrated and lyophilized (CMW-W). The chloroform layer was also concentrated and lyophilized (CMW-C). Most HCV subgenome-expression inhibitory activity was recovered in the CMW-W fraction.

Preparative Fractionation by HPLC—To separate the components in the CMW-W fraction processing inhibitory activity against HCV RNA expression, we performed HPLC (Prominence System, Shimadzu, Kyoto, Japan). Preliminary fractionation of CMW-W to confirm the elution pattern of HCV expression suppressive components was carried out on a reversed-phase column (Atlantis dC18, 4.6 mm \times 150 mm, 3 μ m, Waters, Milford, MA) at 40 °C with UV detection at 254 nm. A gradient consisting of eluant A (0.05% trifluoroacetic acid) and eluant B (acetonitrile) was applied at a flow rate of 0.7 ml/min as follows: 15–25% B linear from 0 to 12.5 min, 25–100% B linear from 12.5 to 17.5 min followed by washing 100% B from 17.5 to 25 min. For purification, the first HPLC fractionation was performed on a reversed-phase column (Atlantis T3, 4.6 mm \times 150 mm, 3 μ m, Waters). A gradient consisting of eluant A and eluant B (acetonitrile) was applied at a flow rate of 0.7 ml/min as follows: 30% B from 0 to 7.5 min, 30–100% B linear gradient from 7.5 to 12.5 min, followed by washing with 100% B from 12.5 to 20 min. The CMW-W fraction dissolved in 30 ml of methanol was injected, and the eluted fractions (2.1 to 18.0 min, total 26 fractions) were collected. The gradient program for the second fractionation was 20% B from 0 to 7.5 min, 20–100% B linear from 7.5 to 12.5 min, followed by washing with 100% B from 12.5 to 20 min. Fractionation of the eluate was the same as the first HPLC program. In the third HPLC fractionation, the eluant B was replaced by methanol and

eluted with 40–65% B linear gradient from 0 to 12.5 min and 65–100% B linear gradient from 12.5 to 17.5 min. Fractions eluted from 2.2 to 17.5 min (total 26 fractions) were collected. In all experiments, suppressive activity of each fraction against HCV RNA expression was measured using replicon cells.

HCV Replicon Cells and Replicon Assay—The Huh-7/3-1 cell line carrying an HCV-replicon was used (10). The line was established from Huh-7 cells by stable transfection with subgenomic selectable RNA in which the encoding HCV structural proteins were replaced by the firefly luciferase gene, the internal ribosome entry site (IRES) of the *Encephalomyocarditis* virus and the neomycin phosphotransferase gene. With this HCV subgenome, the efficiency of subgenomic HCV expression could be estimated by measuring luciferase activity in the replicon cells. The HCV replicon cells were routinely grown in Dulbecco's modified Eagle's medium supplemented with Glutamax (Invitrogen), 10% fetal bovine serum, 1% penicillin/streptomycin (Invitrogen), and 500 μ g/ml G418 (Invitrogen). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂. For the HCV subgenome expression assay, the replicon cells in Dulbecco's modified Eagle's medium supplemented with Glutamax and 5% fetal bovine serum were seeded in 96-well plates (5000 cells/well) and incubated for 24 h. Then the cells were cultured with various concentrations of samples for 72 h. Quantification of the luciferase activity was performed using the Steady-Glo Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions, and the luminescence was measured by DTX 800 Multimode Detector (Beckman Coulter, Fullerton, CA). The inhibitory activity was expressed as the concentration required for 50% inhibition (IC₅₀). Specific activity was calculated as a reciprocal number of IC₅₀ (1/IC₅₀). Total activity was calculated by multiplying yielded weight by specific activity.

The cytotoxicity of the samples was measured by Cell Counting Kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, 10 μ l/well of Cell Counting Kit-8 reagent was added to the cells cultured in a 96-well plate, incubated at 37 °C for 60 min. The absorbance of each well was measured at 450 nm with a reference wavelength at 650 nm using an Emax Precision microplate reader (Molecular Devices Inc., Sunnyvale, CA). Cell viability was calculated as relative index of control cells, and effects of samples on cell viability were expressed as the concentration required for 50% cytotoxicity (CC₅₀).

Constitutive Analysis of Electron Probe Micro-analysis and Liquid Chromatography/Mass Spectrometry-ion Trap-time of Flight (LC/MS-IT-TOF)—For electron probe micro-analysis (EPMA-1600, Shimadzu), the excitation voltage and the beam current were kept at 15 kV and at 100 nA, respectively. The diameter of the electron beam was 50 μ m, and the sample was processed for carbon shadowing in advance.

Identification of the anti-HCV compound purified from blueberry leaves was done by HPLC-MSn fragmentation analyses. An HPLC System (Prominence System, Shimadzu) on a reversed-phase column (Atlantis T3, 2.1-mm inner diameter \times 100 mm, 3 μ m, Waters) was equipped with a photodiode array (PDA) detector scanning from 200 to 800 nm and mass spectrometry-ion trap-time of flight (MS-IT-TOF, Shimadzu)

detector. The mobile phase consisted of a gradient system 30 min of eluant A (0.05% trifluoroacetic acid) and eluant B (acetonitrile) at a flow rate of 0.25 ml/min. The elution program was 10–25% B linear from 0 to 7.5 min, 25–100% B linear from 7.5 to 12.5 min, followed by washing 100% B from 12.5 to 20 min. The column was maintained at 40 °C. Electrospray ionization conditions were recorded from $m/z = 200$ to 1500 in a negative ionization mode. Other MS conditions were as follows: interface voltage, –3.5 or –3.0 kV; nebulizer N_2 gas, 1.5 or 2.0 liters/min; drying N_2 pressure, 200 or 70 kPa, respectively. Heat block temperature and curved desolvation line temperature were both 200 °C. Analytical conditions were recorded from m/z 250 to 1500 in a negative ionization mode. Atmospheric pressure chemical ionization (APCI) probe temperature was set from 250 to 450 °C.

Analysis of Proanthocyanidin—Proanthocyanidins were characterized by a modified method of Porter *et al.* (11, 12), in which they were degraded to anthocyanidins by heating under acidic conditions. Briefly, 200 μ l of purified compound from blueberry leaves (0.1–2.5 mg/ml) was mixed with 750 μ l of *n*-butanol/HCl (95:5) and 50 μ l of 1% of $NH_4Fe(SO_4)_2 \cdot 12H_2O$ dissolved in 2 M HCl. The mixture was vortexed and heated in an oven at 105 °C for 40 min, and cooled in flowing water. Optical densities of the treated solution were recorded at 540 nm by spectrophotometer (UV-1700, Shimadzu). Procyanidin B2 (Sigma-Aldrich) was used as a standard. The hydrolysates generated by the modified Porter method were also analyzed using LC/MS-IT-TOF as described above. The elution program was 10–40% B linear from 0 to 15 min followed by washing 100% B from 15 to 22.5 min. Electrospray ionization conditions were recorded from m/z 200 to 1500 in a positive ionization mode. Interface voltage and nebulizer N_2 were 4.5 kV and 1.5 L/min, respectively. MS/MS conditions were set to auto system and recorded from m/z 50 to 1000. The parent MS was searched from m/z 200 to 1500, and ion accumulation was 30 ms. The data were analyzed by LCMS solution v3.41 software and Formula Predictor Software (Shimadzu).

Thiolysis Analysis—Thiolysis was performed by a previously described method (13, 14) with some modifications. Briefly, 50 μ l of purified samples (2 mg/ml in methanol) was mixed with 50 μ l of methanol acidified with HCl (3.3%) and 100 μ l of benzyl mercaptan (5% in methanol). The reaction was carried out at 50 °C for 30 min and then kept at ambient temperature for 3 h. Pure catechin or epicatechin solution (1.25 mg/ml in methanol) (Funakoshi, Tokyo, Japan) was also thiolized to obtain the epimerization rate to calculate the ratio of catechin and epicatechin in the terminal units. The reaction mixture was diluted 5-fold with methanol and analyzed by reverse-phase HPLC. An Atlantis T3 column (4.6 mm \times 150 mm, 3 μ m, Waters) was used at 40 °C as described above. UV detection was performed at 280 nm. The gradient program was 15–25% B linear from 0 to 10 min, 25–100% B linear from 10 to 30 min, followed by washing 100% B from 30 to 37.5 min and re-equilibration of the column 37.5 to 45 min under initial gradient conditions. To ascertain the elution pattern of thiolysis media and to estimate unknown peaks, LC/MS-IT-TOF was also employed in a negative ion mode. Flavan-3-ols and their benzylthio adducts obtained by thiolysis media of procyanidin B2 were used as a

standard. The mDP was calculated by the formula, $mDP = [\text{sum of (benzylthio adducts} \times n) + \text{sum of (free flavan-3-ol} \times n)] / [\text{total free flavan-3-ol}]$, in which “*n*” is DP of detected flavan-3-ol by thiolysis.

Preparation of Proanthocyanidin from Blueberry Leaves—To prepare proanthocyanidin from blueberry leaves, freeze-dried powder (100 g) was extracted with 1.2 liters of acetone for 10 min, and the supernatant was decanted. This procedure was repeated five times to remove the green pigment from the leaves, followed by washing in 1.2 liters of hexane for 30 min. The remaining residues were washed with ethyl acetate. The washed powder of leaves was extracted with 1.2 liters of methanol for 30 min, and the supernatant was filtered. This procedure was repeated four times, and the resulting crude methanol extracts were concentrated by rotary evaporator at 50 °C and lyophilized, finally resulting in ~30 g of solid powder. The crude methanol extract (15 g) was then dissolved in 1.0 liter of 60% methanol and placed on a Sephadex LH-20 column (50 mm \times 920 mm, Amersham Biosciences). Fractionation was performed using the following series of solvents: fraction I, 9.0 liters of 60% methanol (retrieved weight: 10.2 g); fraction II, 9.0 liters of 100% methanol (retrieved weight: 3.3 g); fraction III, 9.0 liters of 70% (v/v) acetone (retrieved weight: 1.3 g). The LC/MS-IT-TOF analyses of each fraction indicated that fraction I was primarily composed of quinic acid, chlorogenic acid, and flavonol glycosides such as rutin. Fraction II consisted of proanthocyanidin oligomers from tetramer to decamer as analyzed by thiolysis. Fraction III consisted of proanthocyanidin polymers that were decamers or greater. In each fraction, the eluate was divided into 28 subfractions/liter.

Northern Blot Analysis—Total RNAs from cultured replicon cells were prepared using RNeasy mini kits (Qiagen). RNAs were denatured at 65 °C for 15 min, cooled on ice, and then separated by 1% agarose-formaldehyde gel electrophoresis (2 μ g/lane) and transferred to a positively charged nylon membrane (Hybond- N^+ , Amersham Biosciences). The membrane was hybridized with a biotinized probe of the neomycin phosphotransferase gene. For detection of the bound probe, membranes were incubated with streptavidin-Alexa Fluor 680 conjugate (Invitrogen), and the bound fluorescence was detected by Odyssey Infrared Imaging System (LI-COR Biosciences). For internal control, β -actin mRNA-specific biotinized antisense RNA probe was used.

Western Blot Analysis—Cultured replicon cells were harvested, and total cellular proteins were extracted with Cel-Lytic-M (Sigma-Aldrich) containing 1% protease inhibitor mixture (Sigma-Aldrich). The samples were separated by SDS-PAGE using 10% gel under reducing conditions. The proteins were transferred electrophoretically to an Immobilon-P membrane (Millipore, Bedford, MA).

The membrane was treated with a blocking buffer for near infrared fluorescent Western blotting (Rockland, Gilbertsville, PA). Primary antibodies used were anti-human hnRNP A2/B1, hnRNP K, hnRNP L, and hnRNP Q and anti-human β -actin antibodies (EF-67, D-6, A-11, 18E4, and I-19, respectively, Santa Cruz Biotechnology, Santa Cruz, CA), anti-human eukaryotic translation initiation factor 3 (eIF3) F, eIF3G eIF3H polyclonal antibodies (Novus Biologicals, Littleton, CO), and

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anti-HCV NS-3 polyclonal antibody (10). The labeled proteins were visualized with Alexa Fluor 680 anti-rabbit or anti-mouse IgG (Invitrogen) or IRDyeTM 800CW anti-goat IgG (LI-COR Biosciences) and detected by using as Odyssey Infrared Imaging System.

Affinity Purification of Proanthocyanidin-binding Proteins—Purified blueberry leaf-derived proanthocyanidin or catechin was coupled with epoxy-activated Sepharose 6B (Amersham Biosciences) according to the manufacturer's instructions. Approximately 5×10^8 HCV replicon cells were extracted with lysis buffer (50 mM sodium phosphate (pH 7.5), 1% CHAPS, 5 mM EDTA, 150 mM NaCl, and protease inhibitor mixture (CompleteTM, Roche Diagnostics, Mannheim, Germany)). The total protein extract (90 mg) was added to the coupled Sepharose beads (3 ml) and incubated at 4 °C overnight with gentle rotation. The beads were centrifuged ($500 \times g$) for 1 min, and the pellet was washed six times with the lysis buffer. The absorbed proteins were eluted by incubation in 2% SDS with 50 mM dithiothreitol at 100 °C for 10 min. The eluate was concentrated with an Amicon Ultra-4 Ultracel-5k (Millipore), and the solvent was replaced by the lysis buffer. Protein concentration was determined by the *o*-phthalaldehyde method using bovine serum albumin as the standard.

Fluorescent Two-dimensional DIGE—Fluorescent two-dimensional-DIGE was performed using fluorescent dyes, IC3-OSu and IC5-OSu (Dojindo Molecular Technologies), with a modification of the methods reported elsewhere (15, 16). Briefly, 10 μ g of proteins per gel were precipitated using a two-dimensional clean-up kit (Bio-Rad) and then dissolved in 20 μ l of sample buffer (10 mM sodium phosphate (pH 8.0), 7 M urea, 2 M thiourea, 3% CHAPS, and 1% Triton X-100). After addition of 400 pmol of IC3-OSu or IC5-OSu, proteins were incubated at 40 °C for 30 min. The labeling reaction was quenched by incubation with 400 μ M lysine for 15 min, followed by addition of an equal volume of the sample buffer with 150 mM dithiothreitol, 0.4% Bio-Lyte 3–10 (Bio-Rad Laboratories), and 0.004% bromophenol blue. Two-dimensional gel electrophoresis was performed according to the manufacturer's instructions (Bio-Rad). The mixed samples were applied to ReadyStrip IPG strips (pH 3–10 NL, 7 cm, Bio-Rad) for separation in the first dimension. The second-dimensional separation was performed by SDS-PAGE using an 8% gel. Fluorescence imaging was performed on a ProxpressTM proteomic imaging system (PerkinElmer Life Sciences). IC3-OSu-labeled proteins were detected with 540/25 nm excitation and 590/35 nm emission filters. IC5-OSu-labeled proteins were detected with 625/35 nm excitation and 680/30 nm emission filters. In this study, while proteins from proanthocyanidin- or catechin-coupled Sepharose were labeled with IC5-OSu, a mixture of equal quantities of both samples was labeled with IC3-OSu and used as a reference for quantitation of IC5-OSu-labeled proteins as described (16). The fluorescent images were aligned using SameSpot TT900 S2S (Nonlinear Dynamics, Newcastle, UK) and then analyzed with Progenesis Discovery software (Nonlinear Dynamics). Each group (eluate from proanthocyanidin- or catechin-coupled Sepharose) was run on triplicate gels three times. Spot intensity in the IC5-OSu image was normalized to the intensity of the corresponding IC3-OSu image spot in the same gel. The average spot intensi-

ties \pm standard deviation (S.D.) from nine gels were calculated. Statistical differences were determined by Student's *t* test, and *p* values <0.05 were considered significant. The proteins having high affinity to proanthocyanidin but not to catechin were detected using a 1.5-fold change (*p* < 0.05) as the cut off.

Protein Identification—Protein identification by peptide mass fingerprinting was performed as described previously (17). Briefly, 100 μ g of proteins was separated by two-dimensional-DIGE gels and stained with Coomassie Brilliant Blue R-250. Protein spots of interest were excised from the gel and digested overnight with trypsin. Each peptide extract was deposited onto a thin layer of α -cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) and allowed to adsorb for 5 min, after which the layers were washed twice with 0.1% trifluoroacetic acid. Spectra were obtained using matrix-assisted laser desorption/ionization-TOF-TOF-MS, Autoflex II TOF/TOF (Bruker Daltonics) in positive-ion and reflectron mode. The data set was entered in an in-house Mascot search engine (Matrix Science, London, UK), to find the closest match with known proteins registered in the data base from the Swiss-Prot.

Knockdown of Proanthocyanidin-binding Proteins Using siRNAs—ON-TARGETplus SMARTpools of duplex siRNAs targeting hnRNP L, hnRNP K, hnRNP A2/B1, hnRNP A/B, hnRNP Q, eIF3F, eIF3G, eIF3H, and non-targeting control siRNA were purchased from Dharmacon (Thermo Fisher Scientific, Tokyo, Japan). Individual sequence of hnRNP A2/B1 siRNAs was confirmed by two single siRNAs (Target #09: 5'-CGGUGGAAAUUUCGGACCA-3', Target #11: 5'-GGA-GAGUAGUUGAGCCAAA-3'). The replicon cells were transfected with each siRNA using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol. After 72 h incubation, the cells were assayed.

RESULTS

Purification of an Inhibitor of HCV Subgenome Expression from Blueberry Leaves—We screened 283 species of local agricultural products for their suppressive activity against the expression of subgenomic HCV RNA using an HCV replicon cell system, and found significant suppressive activity in the leaves of the blueberry (*Vaccinium virgatum* Aiton), peels of roots of Taro (*Colocasia esculenta* L.), and hulls of seeds of Japanese plum (*Prunus mume* Sieb. et Zucc). Among them, extracts of blueberry leaves contained the highest total activities. Therefore, we purified a compound from blueberry leaves that inhibited expression of subgenomic HCV RNA in replicon cells. An overall purification scheme is shown in Fig. 1, and a summary of the purification steps is shown in Table 1. From 1000 mg of lyophilized powder from the leaves, 440 mg of methanol extracts was obtained. The IC₅₀ value of the methanol extracts was 5.47 μ g/ml. The inhibitory activity was recovered in the CMW-W fraction (284.2 mg), in which the IC₅₀ value was 1.74 μ g/ml. The specific activity of CMW-W was 3-fold greater than that of the initial methanol extracts and the yield of the activity exceeded 200%, suggesting that an interfering substance had been removed.

The CMW-W fraction was subjected to a subsequent HPLC purification step in which a preliminary HPLC elution pattern

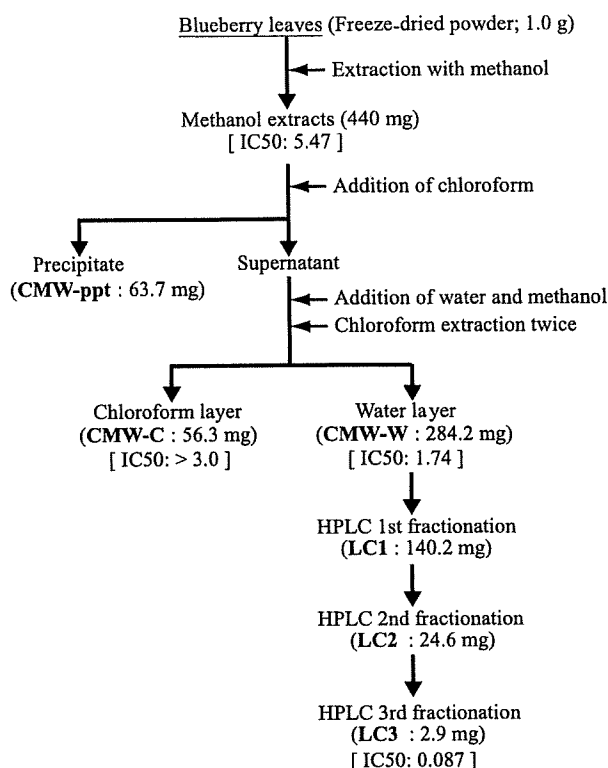


FIGURE 1. Fractionation of blueberry leaf extract for the inhibitor of HCV subgenome expression. The inhibitory activity was indicated under each fraction as the IC_{50} value (micrograms/ml).

TABLE 1
Purification of HCV subgenome expression inhibitory activity in blueberry leaf

	Total weight	Subgenome expression, IC_{50}	Specific activity	Purification factor	Total activity	Yield
	mg	$\mu\text{g/ml}$	$1/IC_{50}$		mg/IC_{50}	%
MeOH extract	440.0	5.47	0.18	1.00	80.44	100
Water layer	284.2	1.74	0.57	3.14	163.33	203.05
LC 1st	140.2	0.89	1.12	6.15	157.53	195.84
LC 2nd	24.6	0.54	1.85	10.13	45.56	56.63
LC 3rd	2.9	0.087	11.49	62.87	33.33	41.44

(a 15–100% gradient of acetonitrile) was used. The data indicated that a strong inhibitory activity eluted around 90% of acetonitrile (17 min) with some minor inhibitory activities broadly eluted earlier. Those results suggested the possible existence of multiple HCV subgenome expression inhibitors in the CMW-W fraction (Fig. 2A). To purify the most active component, we initially separated the CMW-W isocratic at 30% acetonitrile and collected the active fraction eluted at 3.3–5.2 min (Fig. 2B). After repeated collection, we obtained 140.2 mg of active fraction (LC1) from 440 mg of methanol extracts. The IC_{50} value of this fraction for HCV RNA expression was 0.89 $\mu\text{g/ml}$, yielding a specific activity 6-fold higher than that of the initial methanol extracts (Table 1). In the second round HPLC (Fig. 2C), we fractionated LC1 as follows: 20% acetonitrile from 0 to 7.5 min, followed by 20–100% linear gradient of acetonitrile from 7.5 to 12.5 min. A highly active fraction was eluted from 11.9 to 13.2 min and collected (LC2), yielding 24.6 mg with an IC_{50} value 0.54 $\mu\text{g/ml}$ (Table 1). In the third HPLC step (Fig. 2D), we applied LC2 and eluted with 40–65% methanol

instead of acetonitrile. The active fraction was eluted from 3.2 to 6.2 min and collected (LC3), finally yielding 2.9 mg of solid material with a dark flesh color. The IC_{50} value for HCV RNA expression of LC3 was 0.087 $\mu\text{g/ml}$, with a 63-fold increase in specific activity relative to the initial methanol extracts (Table 1). We also checked the cytotoxic effect on replicon cells. The CC_{50} value of the cytotoxicity of LC3 was 18.5 $\mu\text{g/ml}$, and the selective index, which was calculated by dividing CC_{50} by IC_{50} , was 212.6, showing a 16.5-fold higher selective index value compared with initial methanol extracts (Fig. 3).

The Inhibitor of HCV Subgenome Expression Is Proanthocyanidin—To analyze the constituent elements in the purified fraction LC3, EPMA was performed. This analysis indicated that the fraction is composed of carbon and oxygen, but not nitrogen (data not shown). In addition, trace amounts of calcium, sodium, potassium, and aluminum, which appeared to be contaminating elements, were also identified. Next, LC3 was analyzed by LC/MS-IT-TOF. Preliminary trials showed that analysis required the use of an APCI probe at 450 °C, and no signal was obtained at 250 °C. The mass spectrum data showed five peaks (Fig. 4), and $[M-H]^-$ at m/z 401.0494 and 689.1135 were considered to be trifluoroacetic acid adducts of m/z 287.0553 and 575.1196, respectively. From these spectra, the parent mass of this compound appeared to be $[M-H]^-$ at m/z 575.1196, which was estimated to be $C_{30}H_{24}O_{12}$ (error = 0.17 ppm), an A-type dimer of procyanidin. Given the fact that strict conditions (APCI probe temperature at 450 °C) were required to ionize the compound, it appeared that the isolate consisted of one or more polymers of procyanidin.

We next analyzed the purified LC3 fraction by butanol-HCl hydrolysis (Porter method) (11, 12). The reacted solution turned a red color, which is in accordance with the color of anthocyanidin generated by heating of procyanidin/proanthocyanidin under acidic condition. Using procyanidin B2 as a standard, the procyanidin content in the LC3 fraction was 86.33%. The hydrolysis solution was analyzed by LC/MS-IT-TOF. The main peak (retention time = 7.3 min) of the PDA chromatogram at 540 nm was observed at the same position as that of the cyanidin standard (Fig. 5A). Indeed, MS/MS spectra of this peak were identical to those of the cyanidin standard (Fig. 5B). These results revealed that the HCV RNA replication inhibitory compound present in the LC3 fraction from blueberry leaves was procyanidin. Because the hydrolysate of this compound also contained a trace amount of delphinidin (Fig. 5A, arrow), this compound was considered to be proanthocyanidin rather than procyanidin.

Structural Analysis of the Inhibitory Proanthocyanidin by Thiolytic—To analyze the terminal and extension units and also define mDP of proanthocyanidin in the purified LC3 fraction of blueberry leaves, we combined thiolytic (13) with reversed-phase HPLC. When thiolytic products of purified proanthocyanidin in the LC3 fraction were analyzed in reversed-phase HPLC, several peaks (A–H) were identified (Fig. 6). The peaks A, C, and H were considered to be catechin, epicatechin, and benzylmercaptan, respectively, according to the retention time of each standard preparation. Other peaks were confirmed by analyzing mass spectra. The parent mass of peak E was $[M-H]^-$

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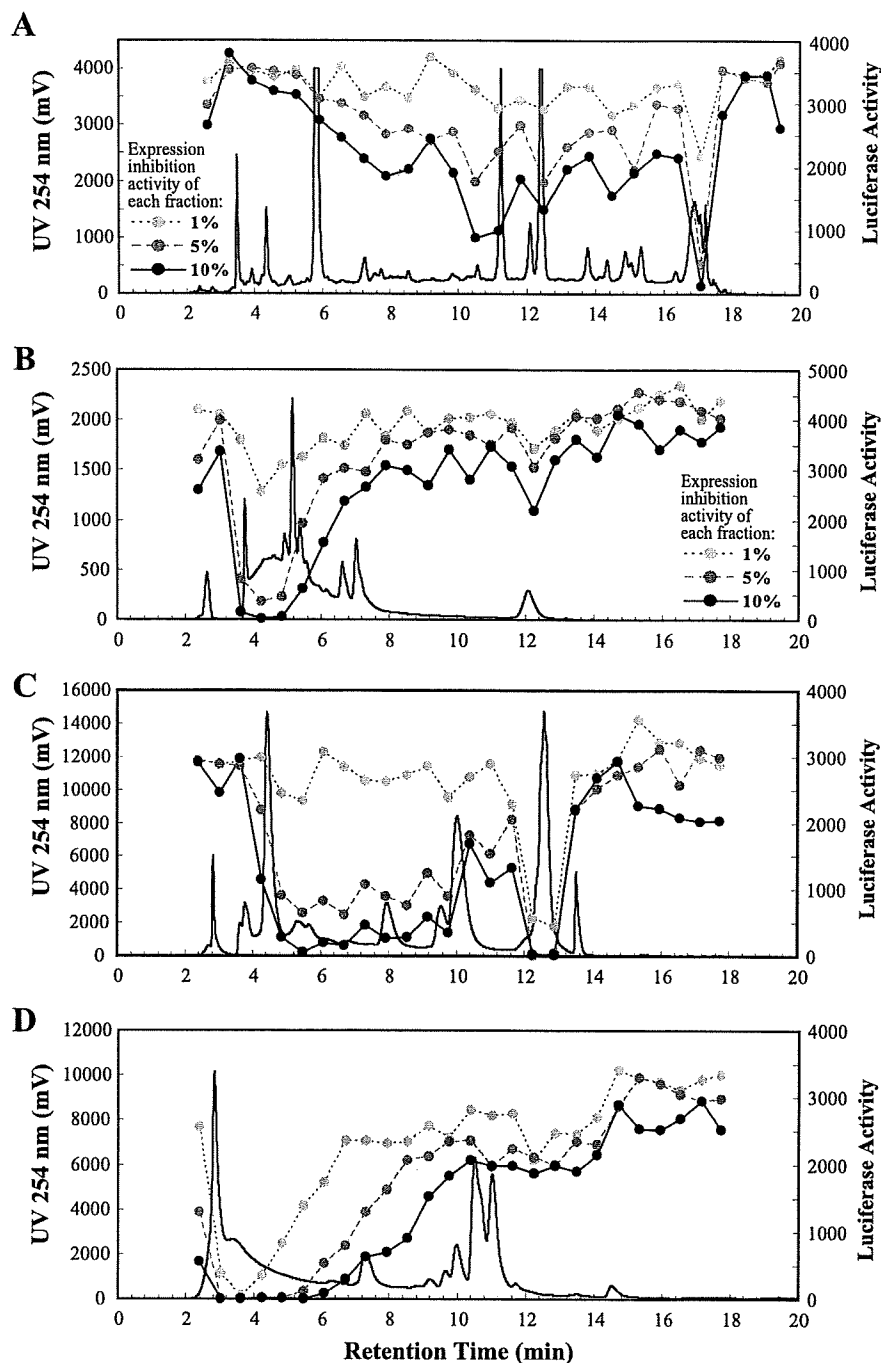


FIGURE 2. HPLC chromatogram and HCV subgenome expression-inhibitor activity. Replicon assays were performed using three different concentrations (1%, 5%, and 10%) of each eluted fraction and are indicated as luciferase activity. The elution conditions are indicated under "Experimental Procedures". *A*, preliminary HPLC chromatogram and suppressive activities against HCV subgenome expression in replicon cells. The applied sample was eluted with a 15–100% gradient of acetonitrile. *B*, first preparative fractionation (*LC 1st fractionation*). CMW-W fraction was applied, and the eluate was collected from 2.1 min to 18 min (445 μ l/fraction, total 26 fractions). Fractions with significant inhibitory activity, eluted from 3.3 to 5.2 min, were collected. *C*, second preparative fractionation (*LC 2nd fractionation*). The collected sample from the first LC fractionation was subsequently separated by HPLC, and fractions with significant inhibitory activity eluted from 11.9 to 13.2 min were collected. *D*, third preparative fractionation (*LC 3rd fractionation*). Sample collected in the second LC fractionation was further separated by HPLC, and fractions with significant inhibitory activity, eluted from 3.2 to 6.2 min, were collected.

MS/MS was 124.0382, which was in accordance with a benzylthio adduct. Thus, peak E appeared to be catechin or epicatechin benzylthioether. Because the retention time of epicatechin benzylthioether was the same as that of peak E, we considered peak E to be epicatechin benzylthioether. The parental mass of peak G was $[M-H]^-$ at m/z 697.1385 (predicted formula: $C_{37}H_{30}O_{12}S$), and its MS/MS was $[M-H]^-$ at m/z 573.0987. Again, the difference was 124.0398 and likely represented the benzylthio adduct. Thus, peak G was estimated to be a benzylthioether of A-type dimer consisting of catechin and/or epicatechin. Peak B was detected as parent MS $[M-H]^-$ at m/z 863.1822 with a predicted formula $C_{45}H_{36}O_{18}$ (error = -0.86 ppm). Because the formula of B-type procyanidin trimer is $C_{45}H_{38}O_{18}$ and that of A-type is $C_{45}H_{34}O_{18}$, this peak was likely a trimer in which A-type and B-type interflavan bonds coexisted. Peak D was suggested to be an A-B type trimer similar to peak B but with a benzylthio adduct. The parental mass of peak F was $[M-H]^-$ at m/z 605.1449, and its MS/MS was $[M-H]^-$ at m/z 481.1109, so that a benzylthio adduct was also present in peak F. However, we could not obtain the predicted formula of the parental mass of peak F. The structural analysis of the HCV inhibitor proanthocyanidin from blueberry leaves (fraction LC3) is summarized in Table 2. The mDP of proanthocyanidin in this fraction was estimated to be 7.7. Because the predicted formula of peak F was undefined, peak F is indicated as "unknown" in Table 2.

Role of Polymerized Structure of Proanthocyanidin in the Inhibition of HCV Subgenome Expression—Because the purified HCV expression-inhibitory proanthocyanidin of blueberry leaf was oligomer with mDP 7.7, we asked whether the polymerization was required for inhibitory activity. First, the inhibitory activities of monomers such as catechin, epicatechin, and epigallocatechin-gallate, all of which were constituents of proanthocyanidin, and also of the dimer (procyanidin B2) were

at m/z 411.0892, with an estimated formula of $C_{22}H_{20}O_6S$ (error = -3.8 ppm), and its MS/MS spectrum was $[M-H]^-$ at m/z 287.0510. The difference between the parental mass and

tory activities of monomers such as catechin, epicatechin, and epigallocatechin-gallate, all of which were constituents of proanthocyanidin, and also of the dimer (procyanidin B2) were

tested by HCV replicon assay. These monomers and the dimer of procyanidin lacked inhibitory activity (Table 3).

We then determined how the degree of polymerization of proanthocyanidin affected the inhibition. The crude fraction of proanthocyanidins was obtained by the extraction of three low polarity solvents (acetone-hexane-ethyl acetate) as described under "Experimental Procedures." The IC_{50} of HCV RNA expression of this proanthocyanidin-enriched fraction was 3.20 $\mu\text{g}/\text{ml}$, showing greater activity than the crude methanol extract. After fractionation on a Sephadex LH-20 column, each eluant was analyzed by LC/MS-IT-TOF and thiolysis to determine the components and mDP of proanthocyanidin (supple-

mental Fig. S1). Then, the blueberry leaf-derived proanthocyanidins with different mDP were assessed for HCV inhibitory activity. The inhibitory activity of blueberry leaf proanthocyanidin was clearly dependent on the polymerization level, and the peak activity was observed at a polymerization level of ~ 8 to 9 (IC_{50} : 0.05 $\mu\text{g}/\text{ml}$) (Fig. 7).

Effect of Purified Blueberry Proanthocyanidin on the Expression of NS3 HCV Protein in Replicon Cells—In our system, HCV RNA expression in replicon cells was expressed as luciferase activity. Thus, the observed inhibitory activity may have resulted from nonspecific inhibition of luciferase by proanthocyanidin. Therefore, we examined the effect of the purified proanthocyanidin (fraction LC3) on the expression levels of the neomycin-resistant gene and the NS3 protein gene, both of which were encoded in the HCV subgenome of replicon cells. The purified blueberry proanthocyanidin suppressed the expression of the neomycin-resistant gene and also the levels of NS3 protein in a concentration-dependent manner, indicating that the proanthocyanidin purified from blueberry leaves in fact suppressed the expression of HCV subgenome in the replicon cells (Fig. 8).

hnRNP A2/B1, Which Has Affinity to Proanthocyanidin, Is Indispensable for Expression of Subgenomic HCV RNA—To investigate the molecular mechanism underlying the suppression of HCV RNA expression by proanthocyanidin, we comprehensively identified proteins having affinity to the purified proanthocyanidin from blueberry leaves. The protein extract from replicon cells was treated with proanthocyanidin-coupled Sepharose, and then the adsorbed proteins were eluted. The extract was also treated with Sepharose beads coupled to catechin, a structural unit of proanthocyanidin, but HCV subgenome-expression inhibitory activity was not observed (Table 3). The proteins having higher affinity to proanthocyanidin than catechin were detected with fluorescent two-dimensional-DIGE (Fig. 9). In the eluate from proanthocyanidin-coupled Sepharose, intensities of 32 spots were increased compared with those from catechin-coupled Sepharose. Twenty-seven spots were cut from Coomassie-stained gels and subjected to peptide mass fingerprinting using MS, and we successfully identified proteins derived from 25 spots (Nos. 1 to 25 in Fig. 9A and Table 4). Although other possible candidate spots were also suggested in a rectangular portion (Fig. 9A), they were not subjected to protein identification due to insufficient separation.

From the list of identified proteins (Table 4), most could be categorized into two groups. The first group consisted of subunits of eukaryotic translation initiation factor 3 (eIF3). They included eIF3A (spot Nos. 1, 5, and 9), eIF3F (No. 10), eIF3G (No. 12), eIF3H (No. 4), and eIF3M (No. 13). Although eIF3A was identified from multiple protein spots (Nos. 1, 5, and 9), this may be due to post-translational modification and protein processing. The second group of proteins consisted of hnRNPs such as hnRNP A/B (No. 19), hnRNP A2/B1

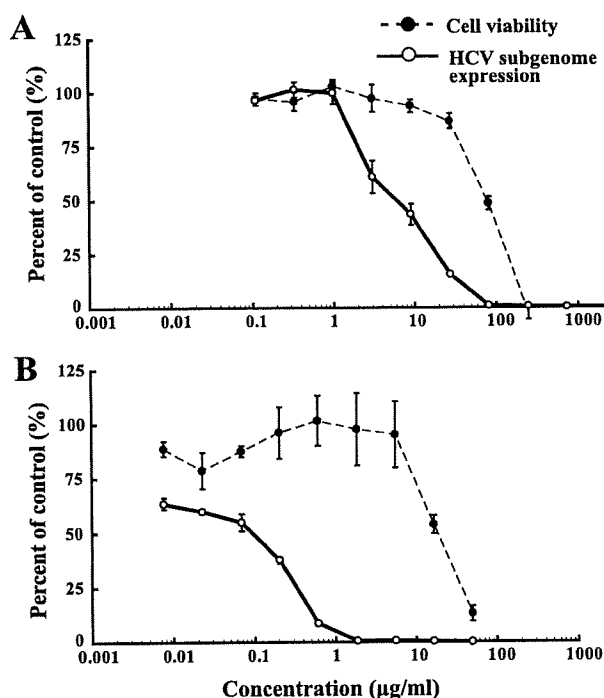


FIGURE 3. Dose-dependent effects of blueberry-derived samples on subgenomic HCV RNA-expression inhibition and viability of replicon cells. A, dose-dependent effects of methanol extracts of blueberry leaves. Concentrations of the sample from 0.112–2200 $\mu\text{g}/\text{ml}$ were tested. IC_{50} for HCV expression and CC_{50} for cytotoxicity were 5.47 $\mu\text{g}/\text{ml}$ and 70.61 $\mu\text{g}/\text{ml}$, respectively, and the selective index was 12.9. B, dose-dependent effects of purified sample (LC3rd fractionation). Concentrations of the sample from 0.01 to 50 $\mu\text{g}/\text{ml}$ were tested. The IC_{50} values for HCV subgenome expression and cytotoxicity were 0.087 $\mu\text{g}/\text{ml}$ and 18.50 $\mu\text{g}/\text{ml}$, respectively, and the selective index was 212.6.

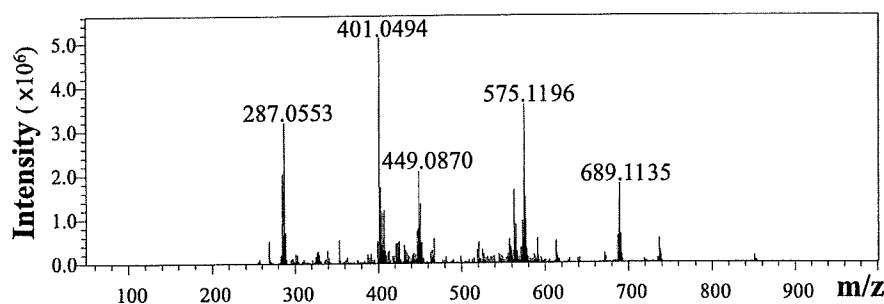


FIGURE 4. APCI MS spectra of the LC3 fraction. The total-ion chromatogram of the LC3 fraction was further analyzed by APCI MS. Peaks of m/z 401.0494 and m/z 689.1135 were considered to be trifluoroacetic acid adducts of m/z 287.0553 and m/z 575.1196, respectively. Parental MS of this compound was estimated at m/z 575.1196, and the formula was assumed to be $C_{30}H_{24}O_{12}$.

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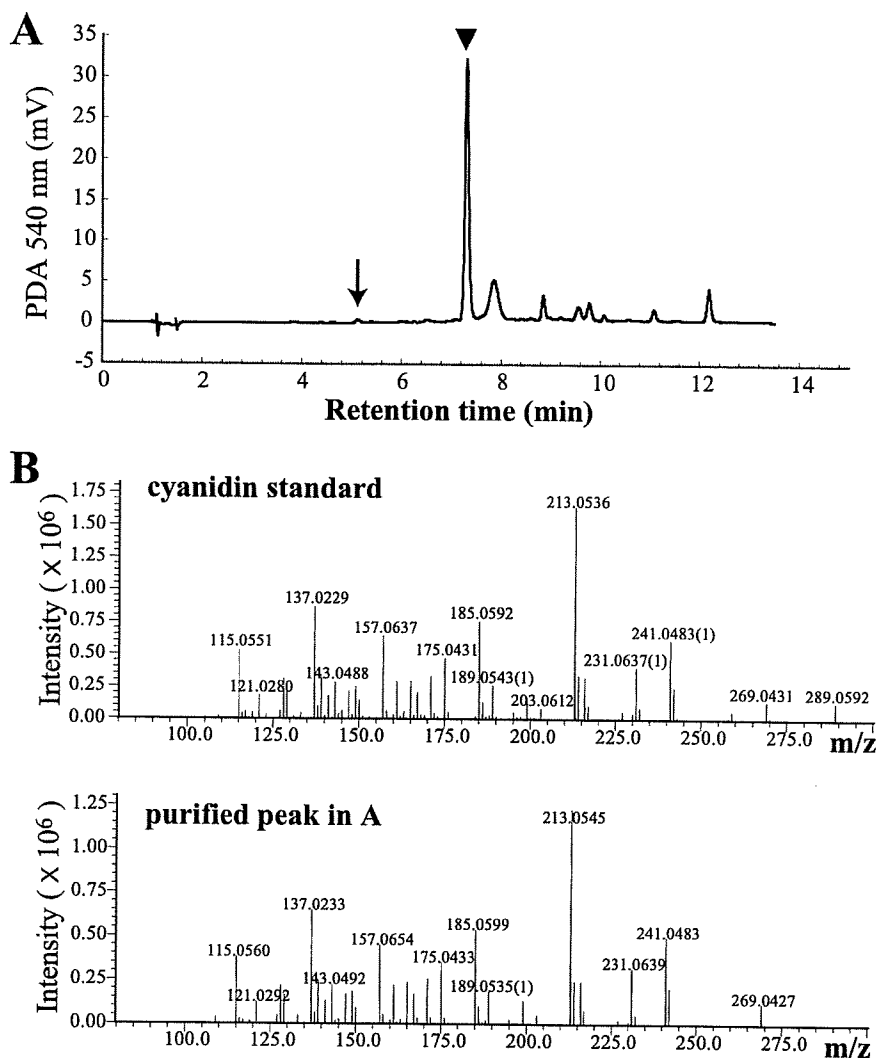


FIGURE 5. PDA chromatogram and MS/MS spectra of the hydrolysate of the purified fraction by the Porter method. **A**, PDA chromatogram at 540 nm of hydrolysate of purified fraction from blueberry leaves. The main peak (arrowhead; retention time = 7.3 min) is located at the same position as the cyanidin standard. Other peaks were estimated to be methoxylated cyanidins from MS and MS/MS spectra. The arrow indicates the position of delphinidin. **B**, MS/MS spectra (positive ion mode) of hydrolysate of cyaniding standard (upper panel; parent MS at 287.0550) and the purified peak in A (lower panel; parent MS at 287.0555).

(No. 8), hnRNP K (Nos. 17 and 22), hnRNP L (Nos. 11, 15, and 21), and hnRNP Q (Nos. 2, 6, and 7) also known as NS1-associated protein 1. Importantly, eIF3 has been reported to bind directly to the HCV internal ribosome entry site (IRES), leading to translation initiation of viral proteins (18). Moreover, all hnRNPs identified have been reported to be associated with HCV genomic RNA such as IRES and non-translated regions (19–25). These results imply that proanthocyanidin may target cellular proteins such as eIF3 and hnRNPs. To further clarify the relationship between these proteins and HCV subgenome expression, we examined the effects of siRNA-based knockdown of these proteins (supplemental Fig. S2). First, we selected three eIF3 subunits (eIF3F, eIF3G, and eIF3H), which are thought to be involved in IRES binding of eIF3 (26). However, knock-down of these subunits did not affect the luciferase activity in replicon cells. Then, we targeted all hnRNPs identified. Among them, siRNA pool targeting hnRNP A2/B1 significantly suppressed the luciferase activity of HCV subgenomic replicon cells (supplemental Fig. S2), and this result was confirmed using two kinds of single siRNA (Fig. 10). Weak suppressive activities were also suggested by siRNAs targeting other hnRNPs such as hnRNP A/B, K, and L (supplemental Fig. S2).

DISCUSSION

The HCV infection is a major cause of chronic liver disease, which eventually results in end-stage liver diseases such as cirrhosis and hepatocellular carcinoma. A crude extract from rabbit-eye blueberry (*V. virgatum* Aiton) leaves exhibited significant inhibitory activity against HCV RNA expression when analyzed in HCV subgenomic replicon cells. In this study, we attempted to purify a compound that suppresses HCV subgenome expression from the blueberry leaves. The final purified product was identified as proanthocyanidin, and it was effective at concentrations that are two orders of magnitude below the toxic threshold in replicon cells. The mDP of the proanthocyanidin in purified anti-HCV expression fraction was 7.7 with a high proportion of epicatechin as the monomeric components. Subsequent analysis indicated that the blueberry leaf-derived proanthocyanidin with a degree of polymerization of ~8–9 shows the highest inhibitory activity. Finally, the purified pro-

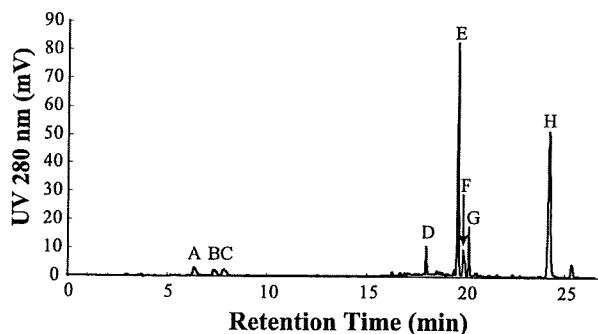


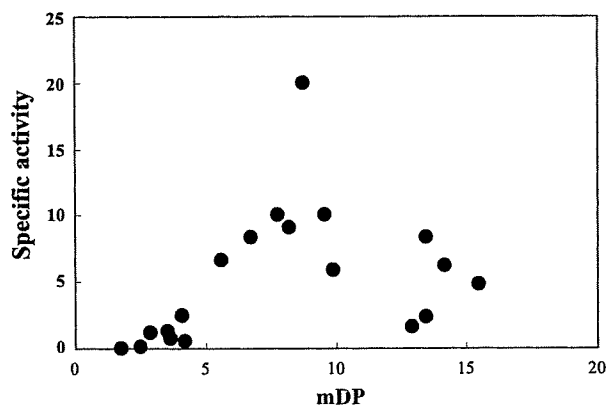
FIGURE 6. HPLC chromatogram at 280 nm of thiolysis products of LC3 fraction from blueberry leaves. Peaks A, C, and H were identified as catechin, epicatechin, and benzylmercaptan, respectively. Peak E was identified as epicatechin benzylthioether. Peaks B and D were estimated to be procyanidin trimer with coexistence of A-type and B-type linkages and its benzylthioether, respectively. Peak G was procyanidin A-type dimer. Peak F was not identified.

TABLE 2
Thiolysis results of purified fraction (LC3) from blueberry leaves

mDP	Terminal				Extension						
	C ^a	EC ^b	AB-3 ^c	Total	C ^a	EC ^b	A-2 ^d	Unknown	AB-3 ^c	Total	
	%										
LC3	7.7	20.4	65.1	14.5	100	0.8	58.1	11.9	23.2	6.0	100

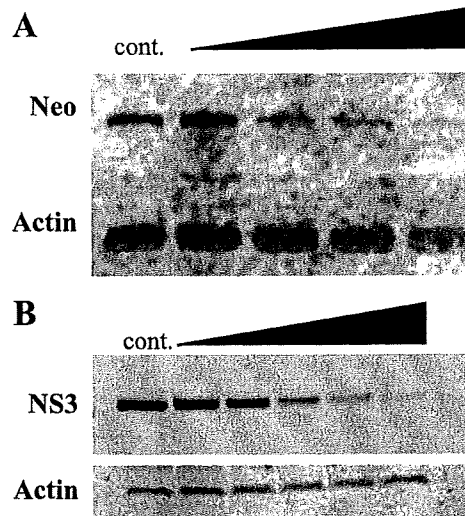
^a Catechin.^b Epicatechin.^c Trimer consisting of both A-type and B-type interflavan bonds.^d A-type dimer.**TABLE 3**
Effects of constitutional units of proanthocyanidin on expression of HCV subgenome in replicon cells

Compounds	DP or mDP	Subgenome expression, IC ₅₀	Cytotoxicity, CC ₅₀	Ratio, CC ₅₀ /IC ₅₀
		μg/ml	μg/ml	
Catechin	1	16.18	100.4	6.2
Epicatechin	1	27.32	113.8	4.2
Epigallocatechin-gallate	1	14.61	41.68	2.9
Procyanidin B2 ^a	2	>25.0	>25.0	–
Purified proanthocyanidin from blueberry leaf (LC3 fraction)	7.7	0.087	18.5	212.0

^a Epicatechin dimer.**FIGURE 7. Scatter plot of mDP and specific activity of subgenomic HCV RNA-expression inhibition.** The mDP was estimated by thiolysis of each fraction. The specific activity was calculated from IC₅₀ value of each fraction.

anthocyanidin from blueberry leaf extracts suppressed the expression of the neomycin phosphotransferase gene and the NS-3 protein gene in HCV subgenome replicon cells in a dose-dependent manner. These data suggest the potential value of blueberry leaf proanthocyanidin for the treatment of HCV infection.

Proanthocyanidin is a polyphenol that shows polymerization of more than two units of flavan-3-ol such as catechin and epicatechin (supplemental Fig. S3). There are two interflavan bonds in proanthocyanidin, in which the B-type has one linkage of interflavan bond (C4 → C8 or C4 → C6) and the A-type has two linkages of bonds (C4 → C8 and O7 → C2) (27). Proanthocyanidins were previously known as condensed tannin and are present in various plants and foods. They contribute to organoleptic properties such as stability, astringency, and bitterness (28, 29). There are a number of foods and nutritional supplements that contain proanthocyanidins with health-promoting benefits, and their value has been described in the literature and patent documents. For example, proanthocyanidin contained in blueberries increases the lifespan of the nematode (*Caenorhabditis elegans*) (30). Sangre de Grado extracted from *Croton*

**FIGURE 8. Suppressive effects of purified blueberry leaf proanthocyanidin (LC3 fraction) on the expression of the neomycin resistant gene and NS-3 protein in replicon cells.** A, Northern blot analysis of the neomycin-resistant gene expression (*Neo*) in the presence of 0 μg/ml (control) to 3.3 μg/ml proanthocyanidin in a 3-fold dilution series. The expression of β-actin mRNA is also indicated as a normalization control. B, Western blot analysis of the expression of NS-3 protein (*NS3*) in the presence of 0 μg/ml (control) to 10 μg/ml proanthocyanidin in a 3-fold dilution series. The β-actin protein levels are also shown as a normalization control.

lechleri resin is a traditional natural medicine in the upper Amazon and contains hydrolyzing flavonoids, proanthocyanidins, and other polyphenols (31, 32), which have been shown to possess anti-viral activities against influenza, parainfluenza, herpes simplex viruses, and respiratory syncytial virus (33–38). However, to the best of our knowledge, this report is the first study to demonstrate that proanthocyanidin inhibits the expression of subgenomic HCV RNA.

Regarding the mechanism underlying the anti-viral activities, proanthocyanidins from *Croton lechleri* resin and prodelfinidin B-2 3'-O-gallate from green tea leaf inhibit herpes simplex viruses infection by preventing the attachment and penetration of the virus into the target cells (37, 39). Recently, the grapefruit flavonoid naringenin was reported to inhibit apolipoprotein B-dependent HCV secretion (40). However, in this study, we evaluated the inhibitory effect on HCV subgenome expression by measuring luciferase activity in replicon cells without using actual viral particles. Therefore, the mode of anti-HCV action of proanthocyanidin is different from that in herpes simplex viruses infection mentioned above and is also different from the inhibitory mechanism of naringenin. Instead, our study suggests that blueberry leaf-derived proanthocyanidin may interact with hnRNP A2/B1, a factor required for HCV subgenome expression in our replicon assay. In accordance with this observation, recent study has shown that hnRNP A1, a protein highly homologous to hnRNP A2/B1, facilitates HCV replication, and the double knockdown of hnRNP A1 and hnRNP A2 significantly suppresses replication (23). Alternatively, proanthocyanidin may bind to the translational initiation complex associated with HCV IRES and thereby suppresses the HCV subgenome expression, because a number of translational regulatory proteins are included in our list of proanthocyanidin-binding proteins. To date, for the

Blueberry Leaf Proanthocyanidin Suppresses HCV

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-

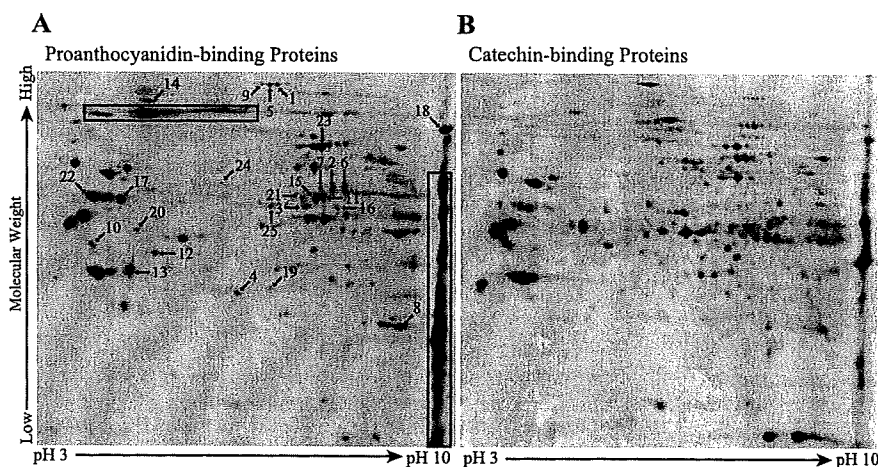


FIGURE 9. Fluorescent two-dimensional-DIGE images of proteins with affinities to blueberry leaf proanthocyanidin (A) and catechin (B). Protein extracts from replicon cells were treated with epoxy-activated Sepharose coupled to proanthocyanidin or catechin. The adsorbed proteins were eluted and then visualized 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							
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	Q96125	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.

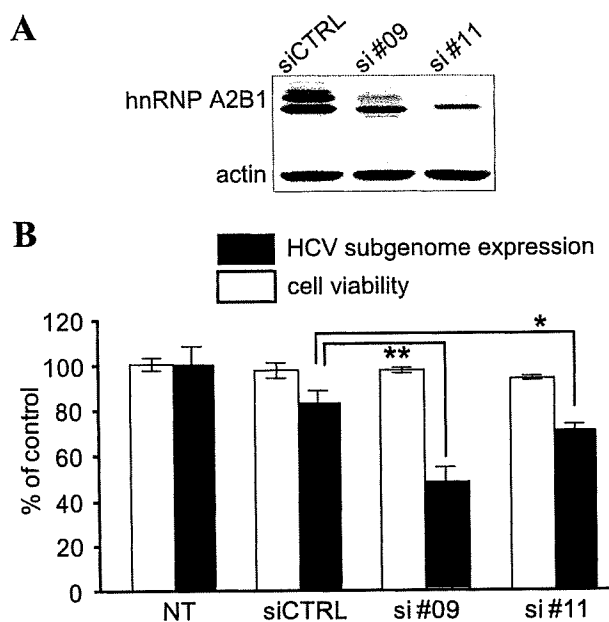


FIGURE 10. Effects of hnRNP A2/B1 knockdown on HCV subgenome expression in replicon cells. Results of two siRNA sequences (si#09 and si#11) are shown. *A*, effects of siRNA on the expression of hnRNP A2/B1 protein. Same blot was also probed by anti-actin antibody. *B*, effects of siRNA on luciferase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