related liver diseases have become the leading cause of death in Japanese hemophiliacs (*10*).

The only curative treatment for end-stage liver disease is liver transplantation. In the pre-HAART era, HIV infection was considered an absolute or relative contraindication for transplantation. Several cases were reported during that period (*11*, *12*), but the outcomes were not always satisfactory. In the HAART era, more than 50 cases of HIV-positive liver transplantation have been reported (*13–21*), and survival after liver transplantation seems to be more promising.

The absolute number of deceased donor livers in Japan is small, and living donor liver transplantation (LDLT) is the mainstay of liver transplantation. We reported the first LDLT in an HIV-positive hemophiliac in 2002 (*22*). Here, we present a series of six cases of LDLT in HIV/HCV-coinfected hemophiliacs performed at the University of Tokyo Hospital between 2001 and 2004.

RESULTS

Survival

The 1-, 3-, and 5-year survival rates were 66%, 66%, and 50%, respectively. Two patients (cases 2 and 5) died on postoperative day (POD) 99 and 156, respectively. The causes of early death were graft failure and bleeding from cytomegalovirus (CMV) enteritis (case 2) and graft failure suspected to be cholestatic hepatitis (case 5). One patient died 50 months after LDLT due to recurrent HCV-related cirrhosis.

Results of Antiviral Therapy for Recurrent Hepatitis C in the Graft

After LDLT, all but one (case 2) patients received combination therapy with IFN (standard or pegylated form) and ribavirin. Case 3 was treated for biopsy-proven recurrent hepatitis C, whereas the other four were treated preemptively (started on POD, 10–70 days). Duration of anti-HCV therapy was 12 months in case early viral response was achieved. Cases 1 and 3 achieved sustained viral response (SVR). Case 3 suffered from HCV-related cholestatic hepatitis on POD 38, which responded well to combination therapy with IFN and ribavirin and he eventually achieved SVR. The other patients did not achieve SVR. Cases 4 and 6 showed a biochemical response and were on maintenance antiviral therapy. In case 6, tacrolimus was switched to cyclosporine A 15 months after LDLT to suppress HCV replication. This lead to a transient 10-fold decrease in HCV-RNA, but it returned to the previous value within several months.

Results of Antiretroviral Therapy After LDLT

Antiretroviral therapy was transiently terminated during the perioperative period. The timing of reintroduction was individualized according to the CD4 count, HIV viral load, general status such as surgical complication and the result of liver function tests. One patient (case 1) has continued to maintain a high CD4 count without antiretroviral therapy. One patient (case 2) died before antiretroviral reintroduction.

The remaining four patients started antiretroviral therapy at a median of 56.5 days after LDLT (range, 43–485 days). The choice of the antiretroviral drug was individualized according to each patient's antiretroviral history and accumulated resistance mutations. A protease inhibitor-based combination was selected in all cases. All but one patient (case 5) tolerated antiretroviral therapy and had an excellent response. The blood concentration of the immunosuppressant increased drastically from the first day of protease inhibitor administration, which was controlled by close monitoring and dosage modification.

Elevation of serum alkaline-phosphatase and gamma-glutanyl-transpeptidase values was observed in all patients after antiretroviral reintroduction. Other significant adverse effects include severe allergic reaction to lamivudine (case 3) and liver failure, which was clinically diagnosed to be cholestatic hepatitis as an immune reconstitution inflammatory syndrome against HCV (case 5).

One patient (case 3) developed Burkitt leukemia 38 months after LDLT. His CD4 count at that time was 480/μL and HIV-RNA was undetectable. Combination chemotherapy using cyclophosphamide, vincristine, doxorubicin, and dexamethasone (*23*) was effective, and he eventually achieved complete remission. Other opportunistic infections included multiple abscess formation at the surgical site in two patients (case 2 by methicillin-resistant *Staphylococcus aureus* and case 5 by multi-drug resistant *Pseudomonas aeruginosa*). Positive CMV antigenemia was observed in all cases. However, only one patient (case 2) presented with clinically overt organ damage.

Restoration of Coagulation After LDLT

Except for case 5, replacement became unnecessary within 1 week after operation. In case 5, in addition to insufficient endogenous coagulation factor production, re-operation was necessary several times, and the coagulation factor replacement could not be withdrawn. Cases 2 and 6 again required coagulation factor replacement after graft failure became apparent.

Outcome of the Donors

All donors were alive without major complications at the point of analysis. Two donors were considered obligate carriers of hemophilia and one of them (donor of case 5) showed relatively low coagulation activity, but none of the donors experienced abnormal bleeding requiring coagulation factor administration. The donor of case 5 experienced transient decrease in factor IX activity after liver resection. However, the value of coagulation activity recovered without supplementation.

DISCUSSION

Recurrence of hepatitis C is the most important problem in treating HCV-positive hemophiliac patients. Recent reports indicate that HIV/HCV-coinfected liver recipients have a relatively lower survival rate than HCV-monoinfected liver recipients, although the difference is not significant. In our series, two of three deaths were related to recurrent HCV, and two patients experienced fibrosing cholestatic hepatitis. Cholestatic hepatitis is characterized by a high rate of HCV replication and a paucity of inflammatory activity, and the risk might increase in LDLT recipients (*24*, *25*). In our center, IFN therapy is usually introduced preemptively as soon as possible. In our series, two cases infected with non-1b virus achieved SVR, whereas others did not achieve SVR. A report demonstrated the effectiveness of maintenance therapy with

pegylated (PEG)-IFN plus ribavirin (*26*), but this efficacy was not apparent in our series. Combination antiviral therapy with protease and polymerase inhibitors may improve the treatment results in the future.

With regard to HIV infection, when to restart antiretroviral therapy after LDLT has remained a question. Hemophiliacs often have a long-term treatment history. Five of six cases had a multiple history of treatment failure, and as a result, only one or two reliable antiretroviral combinations were available to each patient in that era. Protease inhibitors, key drugs for successful HIV suppression in such cases have a potential risk of liver toxicity, especially in those with HCV coinfection (*27*). Unlike whole liver transplantation, the initial graft size is relatively small in LDLT. The graft gradually increases its volume within several weeks after transplantation, and an unfavorable effect of antiretroviral treatment on graft growth during this period is a concern. Moreover, unintended treatment interruption due to early phase complications may result in further accumulation of resistance-associated mutations. Taking these issues into account, we delayed starting antiretroviral therapy until at least 4 weeks after LDLT. It is obvious, however, that earlier antiretroviral reintroduction has more benefit toward reducing opportunistic infections and improving the result of anti-HCV therapy after LDLT. The effectiveness and safety of a new class antiretrovirals, raltegravir (*28*), and enfuvirtide (*29*), were recently reported, and these compounds may play an important role in the management of HIV-infected split-graft recipients.

In our series, the immunosuppressant trough level was targeted to the same level as that in HIV-negative cases. It is not known, however, whether HIV-infected patients, particularly those with a relatively lower CD4 cell count, need the same blood level of immunosuppressants. Moreover, the CD4 cell count, may not act as accurate surrogate marker for immune function in those taking an immunosuppressant or steroid. In case 2, recurrent bleeding from CMV intestinal ulcer eventually led to death after immunosuppression was intensified to treat severe graft rejection. In this case, antiretroviral therapy could not be reintroduced because of severe liver damage, which might enhance excess immunosuppression. A more precise indicator than CD4 count and immunosuppressant level is needed. Dose modification of immunosuppressive drugs using an immune function assay (*30*) may contribute to more precise management, especially in HIV-coinfected patients.

A considerable number of HIV/HCV-coinfected patients are suffering from decompensated cirrhosis or HCC (*8*), and some of them are potential candidates for future liver transplantation. The shortage of deceased donor liver grafts is a major problem worldwide. LDLT can overcome such a problem. Clearly, regenerative medicine will have an important role in this field in the future. Those patients who are already in a cirrhotic state, however, cannot wait for such an innovative modality to be established. In our series, all patients who tolerated antiretroviral therapy achieved good HIV control, and those who cleared HCV survived long. Clinical cure of hemophilia after successful transplantation drastically improved the patients' quality of life. Cure of hemophilia also lead to considerable cost reduction. LDLT continues to have an important role in HIV-infected hemophiliacs.

MATERIALS AND METHODS

From April 2001 to October 2004, nine HIV/HCV-coinfected patients were referred to the University of Tokyo hospital for LDLT. The indication was HCV-related end-stage liver disease.

HIV-positive patients should meet the same standard criteria for liver transplantation as HIV-negative patients. The criteria for accepting candidates for LDLT were absolute CD4 T lymphocyte count more than 200/μL, or more than 14% CD4 proportion to total lymphocytes when hypersplenism-related leukocytopenia was considered the cause of an apparent decrease in the CD4 count. Undetectable HIV RNA was not required as long as effective HIV suppression was expected after transplantation. Exclusion criteria related to HIV infection were active AIDS-defining diseases except for esophageal candidiasis. All cases were approved by the ethics committee at the University of Tokyo. Donor was selected from those with spontaneous will and within the third-degree consanguinity of the patient. Those with abnormal coagulation values were excluded from candidate for the donor.

Two patients did not meet the criteria (one with concomitant uncontrollable fungal infection and one without appropriate donor). One patient retracted consent before operation. Finally, six HIV/HCV-coinfected hemophiliacs underwent LDLT. Two patients were transplanted emergently (within 2 weeks after referral) because of progressive hepatic encephalopathy and hepatorenal syndrome. None of the patients had concomitant active hepatitis B, HCC, or other malignancies. The patient characteristics are summarized in Table 1.

The appropriate type of concentrated coagulation factor was administered during the perioperative period. Concentrated coagulation factor was administered as a bolus just before the operation to achieve 100% coagulating

TABLE 1. Patient characteristics at LDLT and outcome

Case	Age/ sex	Type of hemophilia	HCV genotype	HCV-RNA at LDLT (KIU/mL)	HIV load (copy/mL)	MELD at LDLT	CCr	HTN/ DM	BMI	Graft	Graft size (%SLV)	ACR	CMV	Survival (mo)	Donor
1	41M	B	2a	3	UD	23	24	N/N	19.1	Right	66	0	1	Alive (115)	Brother
2	28M	A	2a, 2b	1410	6.2×10^4	15	76	N/N	23.4	Right	57	2	2	Died (3)	Mother
3	30M	A	1b, 3a	740	3.2×10^4	15	78	N/N	21.5	Right	42	1	2	Alive (96)	Mother
4	38M	A	1b, 3a	200	UD	34	69	N/N	20.0	Right	47	1	1	Alive (82)	Sister
5	31M	B	1a	747	2.6×10^4	18	72	N/N	24.3	Right	47	2	3	Died (5)	Mother
6	32M	B	1a, 1b	41	UD	48	62	N/N	25.2	Right	63	0	0	Died (50)	Father

HCV, hepatitis C virus; LDLT, living donor liver transplantation; HIV, human immunodeficiency virus; MELD, model for end-stage liver disease; CCr, creatine clearance; HTN, hypertension; DM, diabetes mellitus; BMI, body mass index; SLV, standard liver volume; ACR, acute cellular rejection; CMV, cytomegalovirus; UD, undetectable.

factor activity, followed by continuous infusion to maintain greater than 80% activity during the operation. Fresh-frozen plasma was also replaced. Initial dosage of the coagulation factor was calculated based on the results of preoperative pharmacokinetic studies, and the rate of continuous infusion was adjusted as necessary by periodical monitoring of coagulation factor activity.

Tacrolimus and steroids based immunosuppression was planned as previously described (*31*). The target tacrolimus trough level was same as that for the HIV-negative population. Moderate to severe rejection was treated with pulse steroids±mycophenolate mofetil.

The preoperative HCV-RNA value was positive in all subjects. The HCV genotype is listed in Table 1. All patients underwent concomitant splenectomy (*32*). Preemptive anti-HCV therapy with IFN (standard or pegylated form) plus ribavirin was planned after LDLT (*33*). Postoperative CMV reactivation was monitored using a pp65 antigen detecting method (CMV antigenemia), and a positive result was preemptively treated with ganciclovir (*34*) or valganciclovir.

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Original Article

Hepatocellular Carcinoma With Extrahepatic Metastasis

Clinical Features and Prognostic Factors

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BACKGROUND: Despite significant advances in the treatment of intrahepatic lesions, the prognosis for patients with hepatocellular carcinoma (HCC) who have extrahepatic metastasis remains poor. The objective of this study was to further elucidate the clinical course and prognostic determinants of patients with this disease. **METHODS:** In total, 342 patients who had HCC with extrahepatic metastasis were enrolled. The metastases were diagnosed at initial presentation with HCC in 28 patients and during follow-up in the remaining patients. The authors analyzed clinical features, prognoses, and treatments and established a scoring system to predict prognosis using a split-sample method with a testing set and a training set. **RESULTS:** The most frequent site of extrahepatic metastasis was the lung followed by lymph nodes, bone, and adrenal glands. These metastases were related directly to death in only 23 patients (7.6%). The median survival after diagnosis of extrahepatic metastasis was 8.1 months (range, 0.03-108.7 months). In univariate analysis of the training set (n = 171), performance status, Child-Pugh classification, the number and size of intrahepatic lesions, macroscopic vascular invasion, symptomatic extrahepatic metastases, α-fetoprotein levels, and complete responses to treatment were associated significantly with prognosis. On the basis of multivariate analysis, a scoring system was developed to predict prognosis that assessed uncontrollable intrahepatic lesions, extent of vascular invasion, and performance status. This scoring system was validated in the testing set (n = 171) and produced a concordance index of 0.73. **CONCLUSIONS:** The controllability of intrahepatic lesions and performance status were identified as important prognostic factors in patients with advanced HCC who had extrahepatic metastasis. ***Cancer*** **2011;117:4475-83.** © *2011 American Cancer Society.*

KEYWORDS: hepatocellular carcinoma, extrahepatic metastasis, clinical course, prognosis.

Hepatocellular carcinoma (HCC) is a leading cause of cancer death, and its incidence is particularly high in Asian countries, including Japan.[1,2] HCC usually develops in a liver that already suffers from chronic disease, most notably because of hepatitis B virus (HBV) or hepatitis C virus (HCV) infection.[3] In the past, HCC often was diagnosed only at a far advanced stage, and this was accompanied by a very poor prognosis.[4] However, today, close surveillance with advanced diagnostic modalities on designated high-risk patients has facilitated the detection of HCC at a much early stage. Together with the considerable advances in treatment for HCC, such as surgical resection, percutaneous ablation, transcatheter arterial chemoembolization (TACE), and liver transplantation, the survival of HCC patients has improved much in recent years.[5-9]

Primary HCC lesions often can be removed completely when they are detected at an early stage. Although intrahepatic recurrence of HCC is very frequent, recurrent intrahepatic lesions can be treated successfully using modalities applicable to primary lesions. In particular, percutaneous ablation can be performed repeatedly on recurrent intrahepatic lesions even in patients with moderately impaired liver function. Thus, intrahepatic lesions can be kept under control, but extrahepatic metastasis still may arise.[10,11] Extrahepatic metastasis of HCC were once regarded as a terminal event,[12] and coexisting intrahepatic lesions usually are not treated by locoregional therapies like surgical resection or medical ablation.[13] Although systemic chemotherapies sometimes have been attempted, no standard protocols were established until

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recently.[14,15] In 2 recent, large, randomized controlled trials, it was demonstrated that the multikinase inhibitor sorafenib significantly prolonged survival in patients with advanced HCC, even when the primary lesion was accompanied by extrahepatic metastases; now, sorafenib is widely regarded as the standard treatment for such patients.[16,17] However, the clinical course for patients with extrahepatic metastasis has not yet been fully elucidated, and the prognostic factors remain unclear. This information will be vital when determining whether treatment with sorafenib or other such agents is indicated.

The prognosis for patients with HCC who had extrahepatic metastasis before the availability of sorafenib may represent the natural clinical course for affected patients, because no previous treatments had proven effective. In the current study, we retrospectively analyzed a cohort of these patients to further investigate the clinical features and prognostic factors for HCC with extrahepatic metastasis.

MATERIALS AND METHODS

Patients

This study was conducted according to the ethical guidelines for epidemiologic research designed by the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labor and Welfare, Japan. The study design was approved by the ethics committee of the host institution. Between 1990 and 2006, a total of 2386 patients with HCC were admitted to the University of Tokyo Hospital. A diagnosis of HCC was confirmed radiologically by hyperattenuation in the arterial phase and washout in the late phase using either contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI).[18] Ultrasound-guided tumor biopsies were performed when the diagnostic imaging results were inconclusive. In the current analysis, the follow-up period ended on the date of death or on December 31, 2008. Among the 2386 patients in the total HCC cohort in our hospital, extrahepatic metastases were noted in 28 patients at first hospitalization. In addition, extrahepatic metastases were detected in other 314 patients during follow-up observation. Therefore, we retrospectively analyzed 342 patients in our current study.

Diagnosis of Extrahepatic Metastasis and Evaluation of Intrahepatic Lesions

Screenings for extrahepatic metastases were not performed as part of the routine check-up. Most intra-abdominal metastases were detected on abdominal ultrasonography, CT, or MRI studies that were obtained every 3 to 4 months to evaluate intrahepatic lesions. Pulmonary lesions often were noted on chest x-rays, which were obtained routinely at each admission. Additional examinations, such as bone x-ray, bone scintigraphy, and brain CT or MRI studies, were indicated when symptoms attributable to extrahepatic metastasis appeared. These examinations also were undertaken when the HCC-specific tumor markers α-fetoprotein (AFP), lens culinaris agglutinin-reactive fraction of AFP (AFP-L3), or des-γ-carboxy prothrombin (DCP) were elevated and the elevation could not be accounted for by status of the intrahepatic lesion. A diagnosis of extrahepatic metastasis from HCC was based on the enhancement pattern observed on contrast-enhanced CT/MRI studies. Positron emission tomography/CT studies were not obtained routinely, because they were not covered by insurance in Japan. When tumor resections were performed, pathologic investigations also were undertaken. Extrahepatic metastasis detected only at autopsy was not considered an event in this study, because we focused primarily on the diagnosis and treatment of this condition in living patients.

We also evaluated viable intrahepatic lesions at the diagnosis of extrahepatic metastasis by using contrast-enhanced CT/MRI. Post-treatment lesions were not considered viable if they were not enhanced by contrast medium. In the current study, vascular invasion was diagnosed radiologically, indicating *macroscopic vascular invasion*. Vascular invasion included invasion to the portal vein, hepatic vein, inferior vena cava, and bile duct.

Treatment Responses in Patients With Extrahepatic Metastasis

In principle, treatment responses were evaluated according to Response Evaluation Criteria in Solid Tumors (RECIST) guidelines.[19] A complete response (CR) was defined as the disappearance of both intrahepatic lesion and extrahepatic metastasis. In addition, we defined a CR as the disappearance of all intratumoral arterial enhancement according to a recently proposed, modified RECIST assessment for HCC.[20] The evaluation was based on imaging results that were obtained at 2 months after the initiation of treatment. CR was confirmed by repeat assessments performed ≥4 weeks after the criteria for response first were met.

Statistical Procedures

Survival after diagnosis of extrahepatic metastases was defined as the interval from the date of diagnosis to the date of death from any cause or to the last visit before

December 31, 2008. The cumulative survival probability was calculated using the Kaplan-Meier method. The cause of death was investigated meticulously using medical records. To develop a scoring system as a prognostic predictor for patients with extrahepatic metastasis, a split-sample method was applied. Our 342 patient cohort was divided randomly into 2 groups: a training set (n = 171) and a testing set (n = 171). The clinical data obtained at the diagnosis of extrahepatic metastasis were assessed as predictors of survival using a Cox proportional hazards model in the training set. The following variables were included in this analysis: age, sex, Eastern Cooperative Oncology Group (ECOG) performance status,[21] hepatitis B surface antigen (HBsAg), HCV antibody, Child-Pugh classification, the size and number of intrahepatic lesion(s), the presence of macroscopic vascular invasion, the presence of symptoms of extrahepatic metastasis, HCC-specific tumor marker levels (AFP, AFP-L3, and DCP), and response to treatment. Each variable was assessed first in a univariate analysis, and the variables that reached a P value $< .05$ were evaluated in a multivariate analysis with stepwise variable selection using Akaike information criterion (AIC). Then, the ratio of regression coefficients of the final model was determined and was rounded to whole digits for convenience. This scoring system was validated in the test group using the chi-square trend test and the Harrell concordance index (c-index).[22] Data were expressed as the mean ± standard deviation unless specified otherwise. All P values $< .05$ were considered statistically significant. All analytical procedures were performed with S-plus (version 7.0; Insightful Corp., Seattle, Wash).

Table 1. Patient Characteristics at the Diagnosis of Extrahepatic Metastasis (n = 342)

Variable	No. of Patients (%)
Age: Mean ± SD, y	66.9 ± 9.0
Men	270 (78.9)
Performance status	
0-1	314 (91.8)
≥2	28 (8.2)
Viral infection	
HBsAg, positive	62 (18.1)
Anti HCVAb, positive	268 (78.4)
Both positive	15 (4.4)
Both negative	27 (7.9)
Child-Pugh class	
A	167 (48.8)
B	153 (44.7)
C	22 (6.4)
Status of intrahepatic lesions	
None	61 (17.8)
≤3 cm and 1-3 lesions	110 (32.2)
>3 cm or ≥4 lesions	171 (50)
Macroscopic vascular invasion, present	65 (19)
Site of extrahepatic metastasis[a]	
Lung	135 (39.5)
Lymph node	117 (34.2)
Bone	87 (25.4)
Adrenal gland	30 (8.8)
Brain	4 (1.2)
Spleen	2 (0.6)
Breast	1 (0.3)
Symptoms of extrahepatic metastasis, present	80 (23.4)
AFP>400 ng/mL	158 (46.2)
AFP-L3>15%[b]	169 (64.8)
DCP>100 mAU/mL	196 (57.3)

SD indicates standard deviation; HCVAb, hepatitis C virus antibody; AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP; DCP, des-gamma-carboxy prothrombin.
[a] Including overlap.
[b] Missing in 81 patients.

RESULTS

Patient Background Data

Table 1 indicates that the average age at diagnosis for patients with primary extrahepatic metastasis from HCC was 66.9 ± 9.0 years, and ratio of men to women was 4:1. The distribution of the metastases among patients was the lung in 135 patients (39.5%), lymph node in 117 patients (34.2%), bone in 87 patients (25.4%), adrenal in 30 patients (8.8%), brain in 4 patients (1.2%), spleen in 2 patients (0.6%), and breast in 1 patient (0.3%), for a total of 376 extrahepatic occurrences in 342 patients. Metastases that were detected within 2 weeks after diagnosis of the first metastasis were considered synchronous. Viable, coexisting intrahepatic HCC lesions were identified in 281 patients (82.2%) when the extrahepatic metastasis was diagnosed. Intrahepatic vascular tumor invasion was evident in 65 patients (19%) patients: Portal vein invasion was evident in 57 patients, hepatic vein and inferior vena cava invasion was evident in 13 patients, and invasion into the bile duct was evident in 4 patients. The ECOG performance status was 0 in 229 patients, 1 in 85 patients, 2 in 19 patients, 3 in 5 patients, and 4 in 4 patients. Eighty patients (23.4%) had symptoms caused by extrahepatic metastasis, including dyspnea caused by multiple lung metastases; bone fracture, nerve paralysis, and pain caused by bone metastasis; abdominal pain and obstructive jaundice caused by abdominal lymph node metastasis; and disturbance of consciousness caused by bleeding from brain metastasis.

Table 2. Treatments Received for Extrahepatic Metastasis in the Study Cohort[a]

		No. of Patients (%)					
Organ	Total No.	Resection	Ablation	TACE	Radiation	Chemotherapy	No Treatment
Lung	135	19 (14.1)	—	1 (0.7)	4 (3)	42 (31.1)	69 (51.1)
Lymph nodes	117	8 (6.8)	5 (4.3)	2 (1.7)	26 (22.2)	27 (23.1)	49 (41.9)
Bone	87	—	3 (3.4)	—	68 (78.2)	2 (2.3)	14 (16.1)
Adrenal gland	30	5 (16.7)	7 (23.3)	11 (36.7)	1 (3.3)	—	6 (20)
Brain	4	—	—	—	2 (50)	—	2 (50)
Spleen	2	1 (50)	—	—	—	—	1 (50)
Breast	1	—	—	—	1 (100)	—	—

TACE indicates transarterial chemoembolization
[a] Including overlap.

Treatment of Patients With Extrahepatic Metastasis

Retrospectively reviewed, the treatments for extrahepatic metastatic lesions in our study cohort were considered only in those patients who had Child-Pugh Class B or better liver function and an ECOG performance status ≥ 2 and when intrahepatic lesions, if any, generally were controlled or controllable. Patients also received treatment when they were suffering from symptoms caused by extrahepatic metastasis. Table 2 indicates that these treatments included resection, chemotherapy, irradiation, TACE, and percutaneous ablation.

Surgical resection was undergone by 19 patients who had a lung metastasis (including 13 patients who underwent video-assisted thoracoscopic surgery), 8 patients who had lymph node metastasis, 5 patients who had adrenal metastasis, and 1 patient who has a spleen metastasis. Percutaneous ablation, using either ethanol or radiofrequency, was undergone by 7 patients with adrenal metastasis, 5 patients with lymph node metastasis, and 3 patients with bone metastasis, and TACE was undergone by 11 patients, 2 patients, and 1 patient with of adrenal, lymph node, and lung metastasis, respectively. Irradiation was received by other patients with metastasis as follows: 68 patients with bone metastasis, 26 patients with lymph node metastasis, 4 patients with lung metastasis, 2 patients with brain metastasis, 1 patient with an adrenal metastasis, and 1 patient with a breast metastasis. Systemic chemotherapy was received by an additional 42 patients with lung metastasis, 27 patients with lymph node metastasis, and 2 patients with bone metastasis in our cohort. The most often used chemotherapeutic regimen was cis-diamminedichloroplatinum (CDDP) monotherapy (29 patients) followed by 5-fluorouracil (5-FU) plus interferon (IFN) (24 patients), TS-1 alone (7 patients), CDDP plus 5-FU (6 patients), etoposide alone (6 patients), and TSU-68 (5 patients).

Percutaneous ablation of the intrahepatic lesions, which was indicated only when any extrahepatic lesions had been completely resected or ablated or controlled by irradiation, was performed in 60 patients. TACE treatment of intrahepatic lesions was indicated for patients who had Child-Pugh Class A or B liver function and when the vast majority of the total tumor volume was located in the liver. By using a combination of systemic chemotherapy and/or locoregional therapy to treat intrahepatic lesions, 22 of the patients in our study group achieved a CR as evaluated by the overall response according to RECIST.

Prognosis After the Diagnosis of Extrahepatic Metastasis

In the current study, during the observation period, 301 patients died. The cause of death was related to HCC in 273 patients (90.7%) patients and to liver dysfunction in 15 patients (5%), and death was unrelated to the liver in another 13 patients (4.3%). Extrahepatic metastasis of HCC was related directly to death in 23 patients (7.6%) patients, including 17 deaths from respiratory failure because of a lung metastasis, 5 incidents of cerebral hemorrhage from a brain metastasis, and death in 1 patient who had a bone metastasis and suffered liver failure that caused by hemorrhaging from a bone fracture that was the result of this lesion.

Gastroesophageal varices rupture sometimes became a critical event at the terminal phase of advanced HCC. In the current study, gastroesophageal varices rupture occurred in 25 patients at the end of life. Portal hypertension in these patients was caused either by portal vein tumor thrombus or cirrhosis, which may often coexist and are difficult to discriminate accurately.

The cumulative survival rates at 1 year, 2 years, 3 years, and 5 years after the diagnosis of extrahepatic metastasis in our cohort were 39.3%, 15.3%, 7.4%, and 4%,

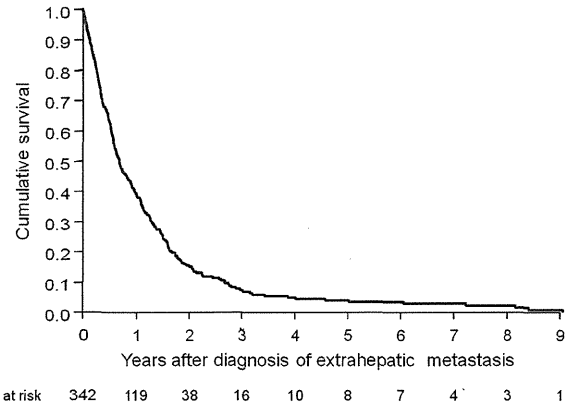

Figure 1. Cumulative survival is illustrated for patients with hepatocellular carcinoma who had a diagnosis of extrahepatic metastasis.

respectively (Fig. 1), and the median survival was 8.1 months (range, from 1 day to 108.7 months). The cumulative survival rates at 1 year, 2 year, and 3 years were 48.9%, 21.2%, and 10.6%, respectively, when the patients had received some treatment for extrahepatic metastasis; and the rates were 19%, 2.3%, and 0%, respectively, when no treatment had been indicated.

Predictors of Prognosis

Prognostic predictors after the diagnosis of extrahepatic metastasis were analyzed in the training set of 171 patients using a Cox proportional hazards model. These predictors were based on clinical factors that were recorded at diagnosis. In univariate analysis, the following factors were associated significantly with a poor prognosis: performance status, Child-Pugh classification, number and size of intrahepatic lesions, the presence of macroscopic vascular invasion, a symptomatic extrahepatic metastasis, AFP level, and CR to therapy (Table 3). Clinical factors that were statistically significant in univariate analysis were analyzed further in multivariate analysis with a stepwise selection of variables to minimize the AIC. To simplify the scoring system using multivariate analysis, intrahepatic tumor extension was categorized as none, a viable lesion without vascular invasion, or a viable lesion with vascular invasion. Only intrahepatic tumor extension at the diagnosis of extrahepatic metastasis and performance status were selected by a stepwise selection as factors in the final model (Table 4). Scores were assigned to each factor according to the estimated regression coefficient in the final model, and the prognosis score was defined as the sum of each score (Table 5). Our scoring system was validated using the testing set of 171 patients. A Kaplan-Meier plot was used to illustrate distinct survival curves according to the prognosis score (chi-square linear trend test: P thinsp;< .001) (Fig. 2). The c-index for the scoring system in the testing set was 0.73, thus reflecting good prognostic discrimination (Table 6).

Table 3. Predictors of Survival After a Diagnosis of Extrahepatic Metastasis: Univariate Analysis (n = 171)

Variable	β	HR (95% CI)	P
Age	0.02	1.02 (1.00-1.03)	.12
Men	0.07	1.08 (0.72-1.61)	.72
Performance status			
0		1.00	
1	0.36	1.44 (1.00-2.07)	.05
2	1.08	2.96 (1.29-6.79)	.01
3	2.61	13.5 (3.90-47.04)	<.0001
4	1.07	2.93 (0.40-21.26)	.29
HBsAg positive	−0.17	0.84 (0.53-1.33)	.46
Anti-HCVAb-positive	−0.27	0.76 (0.51-1.15)	.19
Child-Pugh class			
A		1.00	
B	0.37	1.44 (1.03-2.02)	.03
C	0.64	1.90 (0.97-3.69)	.06
Size of intrahepatic lesion, cm			
Absent		1.00	
≤3.0	0.71	2.04 (1.18-3.51)	.01
>3.0	1.41	4.12 (2.31-7.32)	<.0001
No. of intrahepatic lesion			
Absent		1.00	
1-3	0.67	1.96 (1.16-3.30)	.01
>3	0.93	2.52 (1.55-4.11)	.0002
Macroscopic vascular invasion, present	0.78	2.18 (1.46-3.25)	.0001
Symptom of extrahepatic metastasis, present	0.37	1.45 (1.01-2.09)	.047
AFP>400 ng/mL	0.54	1.71 (1.23-2.39)	.002
AFP-L3>15.0%	0.30	1.34 (0.92-1.96)	.12
DCP>100 mAU/mL	0.08	1.09 (0.78-1.51)	.62
Response to treatment, CR[a]	−0.77	0.46 (0.21-1.00)	.049

HR indicates hazard ratio; CI, confidence interval; HBsAg, hepatitis B surface antigen; HCVAb, hepatitis C virus antibody; AFP, alpha-fetoprotein; AFP-L3, lens culinaris agglutinin-reactive fraction of AFP; DCP, des-gamma-carboxy prothrombin; CR, complete response

[a] Response was evaluated using overall responses according to Response Evaluation Criteria in Solid Tumors (RECIST); treatments included locoregional therapy and systemic chemotherapy for both intrahepatic lesions and extrahepatic lesions.

DISCUSSION

The prognosis for patients with extrahepatic metastasis of HCC was poor in the current study, consistent with previous reports that the 1-year survival rate is approximately 40% for patients with this disease.[23-27] However, from our current analyses, we observed that extrahepatic

Table 4. Predictors of Survival After a Diagnosis of Extrahepatic Metastasis: Multivariate Analysis (n = 171)

Variable	β	HR (95% CI)	P
Intrahepatic viable lesion			
None		1.00	
Without macroscopic vascular invasion	0.67	1.96 (1.21-3.18)	.006
With macroscopic vascular invasion	1.31	3.70 (2.08-6.57)	<.0001
Performance status			
0		1.00	
1	0.30	1.36 (0.94-1.96)	.11
2	1.11	3.05 (1.32-7.06)	.009
3-4	1.78	5.94 (2.09-16.9)	.0008

HR indicates hazard ratio; CI, confidence interval.

Table 5. Scoring System to Predict Survival in Patients With HCC and Extrahepatic Metastasis

Variable	Score
Intrahepatic viable lesion	
None	0
Present without macroscopic vascular invasion	1
Present with macroscopic vascular invasion	2
Performance status	
0-1	0
2	2
3-4	3

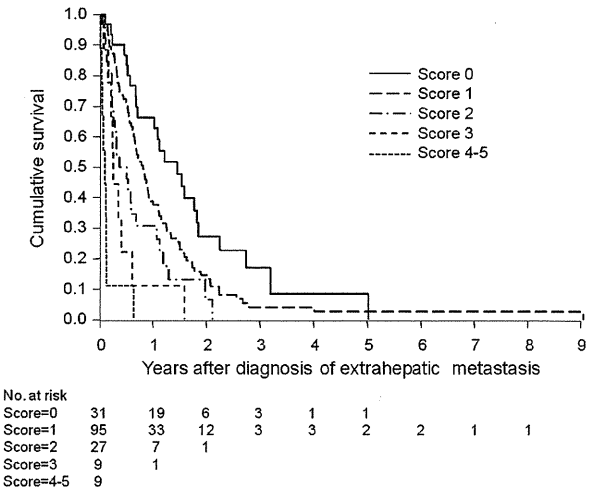

Figure 2. Stratified cumulative survival is illustrated for patients with hepatocellular carcinoma who had a diagnosis of extrahepatic metastasis based on prognostic scores. The prognosis for patients in the testing set could be stratified clearly by the scoring system based on an analysis of patients in the training set.

Table 6. Median Survival According to Prognostic Scores (n = 171)

Score	No. of Patients	Median Survival, mo
0	31	17.5
1	95	9.7
2	27	6.1
3	9	3.0
4-5	9	1.2

metastasis was not the direct cause of death in the majority of affected patients: the exceptions included respiratory failure from a bilateral lung metastasis and cerebral hemorrhage as a result of a brain metastasis, which accords with a previous report.[28] Hence, the presence of extrahepatic metastasis is an indicator of the aggressiveness of the primary HCC as a whole rather than an independent prognostic determinant.

In contrast to extrahepatic metastases, the progression of intrahepatic lesions was identified as the cause of death in 81% of patients in our current cohort, indicating the importance of controlling intrahepatic tumors in patients with HCC whenever possible. Repeated percutaneous ablations or TACE generally are considered for patients with HCC who develop an intrahepatic recurrence.[29,30] Intrahepatic arterial chemotherapy also reportedly is effective against advanced HCC with portal venous tumor invasion.[31] Thus, these locoregional treatments should be considered for intrahepatic lesions in selected patients who have extrahepatic metastasis, although the liver function reservoir should be evaluated cautiously in these patients.

In the current study cohort, patients received treatment for extrahepatic metastasis when their intrahepatic tumor was under control and liver function was maintained. Extrahepatic metastases also were treated when metastasis-related symptoms were strong or when further progression of the metastatic lesions was considered life-threatening. The prognosis was better among the current patients with HCC who received some treatment for their extrahepatic metastasis compared with those who were untreated. However, the contribution of these treatments to the overall prognosis remains unknown, because the patients who received them generally were in better condition. Nevertheless, our current findings indicate that treatments for extrahepatic metastases can be considered in patients who have hepatic lesions under control, because long-term survival was achieved only in those who had received such therapies.

Our current analyses indicated that resection of metastatic lesions produced a satisfactory local response, consistent with previous reports.[32-36] Locoregional therapy for extrahepatic metastasis also was discussed in earlier studies, including irradiation for bone,[37] lymph node,[38] brain,[39] and adrenal[40] metastases; TACE for adrenal metastasis[41]; and percutaneous ablation for adrenal[42] and bone metastases.[43] We also used these methods to treat some patients in our cohort. According to the conventional treatment strategy for solid tumors, the presence of metastatic disease is a contraindication for locoregional therapy, because it is believed that these tumor cells already have spread systemically. However, from the viewpoint of reducing tumor burden, locoregional therapy may be an adequate strategy when the target lesions account for the major portion of the total tumor volume. When resection and other locoregional therapies were contraindicated for extrahepatic metastasis, we sometimes used systemic chemotherapy. However, the overall response rate to conventional chemotherapy in the current study was only 25.4%. The establishment of an effective chemotherapeutic regimen still is needed for these patients, and molecular targeted agents, such as sorafenib,[16,17] are expected to improve their prognosis.

The scoring system we propose in the current study incorporates the presence of intrahepatic lesions, the extent of vascular invasion, and performance status. The progression of an intrahepatic lesion was the major cause of death among our patients, as described above. In patients who had extrahepatic metastases, evaluation of the size and number of intrahepatic lesions often is difficult because of disease progression. From the standpoint of these patients, the proposed scoring system is both simple and convenient. Vascular invasion is 1 of the most important prognostic factors for HCC.[12] Our current results demonstrated that macroscopic vascular invasion is significant even in patients who have extrahepatic metastasis. Performance status, which is an important biologic factor in clinical oncology, also is included in our scoring system.[13] Liver function no doubt is a prognostic determinant for patients with HCC; however, the Child-Pugh classification did not retain significance in our multivariate analysis. This may be because the Child-Pugh class is strongly correlated with performance status, which also includes other significant aspects of cancer biology.

Our current results indicate that the median survival of patients with HCC who have extrahepatic metastases varies widely from within 1 month to 1.5 years and can be discerned using the prognosis factors that were evaluated in this study. Patients who have a prognostic score ≥ 2, which indicates an estimated median survival ≥ 6 months, can be considered for intensive treatment, including surgical procedures. In addition, our scoring system may be used for the enrollment of patients into clinical trials of newly developed agents for which patients with extrahepatic metastasis or vascular invasion may be candidates, although further detailed research will be required to establish such use. We compared the prognosis of patients who were treated in the 1990s and the 2000s and observed no statistical difference between the 2 decades (data not shown). During the study period, newly developed agents, such as sorafenib and drug-eluting beads, were not available in Japan.

There were some limitations in this retrospective cohort study. First, a variety of treatments was provided for various intrahepatic and extrahepatic lesions. Substantial heterogeneity existed in patient background. Second, the proportion of patients who had vascular invasion in our cohort was relatively small despite the presence of extrahepatic metastasis, and this may indicate that the total tumor burden also was relatively small. This may have been because most extrahepatic metastasis in our cohort emerged while treatment for intrahepatic lesions was being repeated. Moreover, the proportion of patients with vascular invasion was not very high, even among the patients who had extrahepatic metastasis at initial presentation. Supposedly, this is because our hospital is a tertiary care center, and patients with an apparent indication for percutaneous ablation were referred to us selectively. Third, the number of patients who had prognostic scores of 3, 4, 5 was not large enough for confirmation, although the linearity of median survival (Table 6) suggests the relevance of the scoring system.

In conclusion, the major cause of death in patients with HCC who have extrahepatic metastases is progression of the intrahepatic HCC lesion. We contend that treatment of intrahepatic lesions should not be contraindicated merely because of the presence of an extrahepatic metastasis. Moreover, radical treatments for extrahepatic metastases may be considered when hepatic lesions are under reasonable control or if the metastasis is accompanied by severe symptoms.

CONFLICT OF INTEREST DISCLOSURES

The authors made no disclosures.

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The rs8099917 Polymorphism, When Determined by a Suitable Genotyping Method, Is a Better Predictor for Response to Pegylated Alpha Interferon/Ribavirin Therapy in Japanese Patients than Other Single Nucleotide Polymorphisms Associated with Interleukin-28B

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The rs8099917 Polymorphism, When Determined by a Suitable Genotyping Method, Is a Better Predictor for Response to Pegylated Alpha Interferon/Ribavirin Therapy in Japanese Patients than Other Single Nucleotide Polymorphisms Associated with Interleukin-28B▽†

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We focused on determining the most accurate and convenient genotyping methods and most appropriate single nucleotide polymorphism (SNP) among four such polymorphisms associated with interleukin-28B (IL-28B) in order to design tailor-made therapy for patients with chronic hepatitis C virus (HCV) patients. First, five different methods (direct sequencing, high-resolution melting analysis [HRM], hybridization probe [HP], the InvaderPlus assay [Invader], and the TaqMan SNP genotyping assay [TaqMan]) were developed for genotyping four SNPs (rs11881222, rs8103142, rs8099917, and rs12979860) associated with IL-28B, and their accuracies were compared for 292 Japanese patients. Next, the four SNPs associated with IL-28B were genotyped by Invader for 416 additional Japanese patients, and the response to pegylated interferon/ribavirin (PEG-IFN/RBV) treatment was evaluated when the four SNPs were not in linkage disequilibrium (LD). HRM failed to genotype one of the four SNPs in five patients. In 2 of 287 patients, the results of genotyping rs8099917 by direct sequencing differed from the results of the other three methods. The HP, TaqMan, and Invader methods were accurate for determination of the SNPs associated with IL-28B. In 10 of the 708 (1.4%) patients, the four SNPs were not in LD. Eight of nine (88.9%) patients whose rs8099917 was homozygous for the major allele were virological responders, even though one or more of the other SNPs were heterozygous. The HP, TaqMan, and Invader methods were suitable to determine the SNPs associated with IL-28B. The rs8099917 polymorphism should be the best predictor for the response to the PEG-IFN/RBV treatment among Japanese chronic hepatitis C patients.

Hepatitis C virus (HCV) infection is a global health problem, with worldwide estimates of 120 to 130 million carriers (7). Chronic HCV infection can lead to progressive liver disease, resulting in cirrhosis and complications, including decompensated liver disease and hepatocellular carcinoma (25). The current standard of care treatment for suitable patients with chronic HCV infection consists of pegylated alpha 2a or 2b interferon (PEG-IFN) given by injection in combination with oral ribavirin (RBV), for 24 or 48 weeks, dependent on HCV genotype. Large-scale treatment programs in the United States and Europe showed that 42 to 52% of patients with HCV genotype 1 achieved a sustained virological response (SVR) (3, 8, 13), and similar results were found in Japan. This treatment is associated with well-described side effects (such as a flu-like syndrome, hematologic abnormalities, and neuropsychiatric events) resulting in reduced compliance and fewer patients completing treatment (2). It is valuable to predict an individual's response before treatment with PEG-IFN/RBV to avoid these side effects, as well as to reduce the treatment cost. The HCV genotype, in particular, is used to predict the response: patients with HCV genotype 2 or 3 have a relatively high rate of SVR (70 to 80%) with 24 weeks of treatment, whereas those infected with genotype 1 have a much lower rate of SVR despite 48 weeks of treatment (8).

Recently, we reported from genome-wide association stud-

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TABLE 1. Characteristics of the patients examined

Parameter	Result for:	
	1st stage (n = 292)	2nd stage (n = 416)
Age (yr)	57.2 ± 10.2	56.6 ± 10.9
No. of patients male/female	145/147	194/222
No. (%) of patients in institution[a]:		
1	18 (6.2)	0 (0)
2	178 (61.0)	0 (0)
3	57 (19.5)	0 (0)
4	39 (13.3)	0 (0)
5	0 (0)	249 (59.9)
6	0 (0)	94 (22.6)
7	0 (0)	52 (12.5)
8	0 (0)	21 (5.0)

[a] Institutions: 1, The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine; 2, The Center for Liver Diseases, Shin-Kokura Hospital, Kitakyushu; 3, Tonami General Hospital, Tonami; 4, Department of Internal Medicine, Virology and Liver Unit, Hokkaido University Graduate School of Medicine, Sapporo; 5, Clinical Research Center, NHO Nagasaki Medical Center, Nagasaki; 6, Nagoya City University Graduate School of Medical Sciences, Nagoya; 7, Department of Gastroenterology and Hepatology, Nagoya Daini Red Cross Hospital; and 8, Division of Gastroenterology, Department of Medicine, Kawasaki Medical School, Okayama.

ies (GWAS) that several highly correlated common single nucleotide polymorphisms (SNPs), located in the vicinity of the lambda 3 interferon (IFN-λ3), coded for by the interleukin-28B (IL-28B) gene on chromosome 19, are implicated in nonvirological response (NVR) to PEG-IFN/RBV among patients with HCV genotype 1 (21). At almost exactly the same time as our report, the association between response to PEG-IFN/RBV and SNPs associated with IL-28B was reported from the results of GWAS by two other groups (6, 19). Determination of these SNPs associated with IL-28B before PEG-IFN/RBV treatment will provide extremely valuable information, because the patients predicted as showing NVR to PEG-IFN/RBV treatment could avoid the treatment. There are two questions to be asked before using these SNPs in clinical practice: (i) which methods for genotyping these SNPs are efficient, and (ii) which SNP is most informative in cases where the SNPs are not in linkage disequilibrium (LD)? We have developed five different methods for detecting the SNPs associated with IL-28B and compared their accuracies to establish the most efficient genotyping method. The response to PEG-IFN/RBV treatment was evaluated, when the SNPs associated with IL-28B were not in LD, to determine the best SNP to predict the response to PEG-IFN/RBV treatment.

MATERIALS AND METHODS

Study population. Samples were obtained from 708 Japanese chronic hepatitis C patients and divided into groups of 292 patients (145 males and 147 females; mean age, 57.2 years) and 416 patients (194 males and 222 females; mean age, 56.6 years) for the first and second stages (Table 1). In the first stage, we focused on analyzing the effective methods for determining the genotypes of four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) associated with IL-28B (Fig. 1A). Figure 2 shows the locations of these four SNPs in chromosome 19; rs11881222 and rs8103142 are located in the IL-28B gene, and rs12979860 and rs8099917 are located downstream from the IL-28B gene. The results of genotyping the four SNPs by five different methods, described below, were compared and evaluated for consistency. For this first stage, the 292 chronic hepatitis C patients were recruited from the National Center for Global Health and Medicine, Hokkaido University Hospital, Tonami General Hospital, and Shin-Kokura Hospital in Japan (Table 1). From the results of the first stage, the InvaderPlus assay was chosen as one of the best methods to determine the genotypes of the four SNPs associated with IL-28B and was used for genotyping 416 patients (Fig.

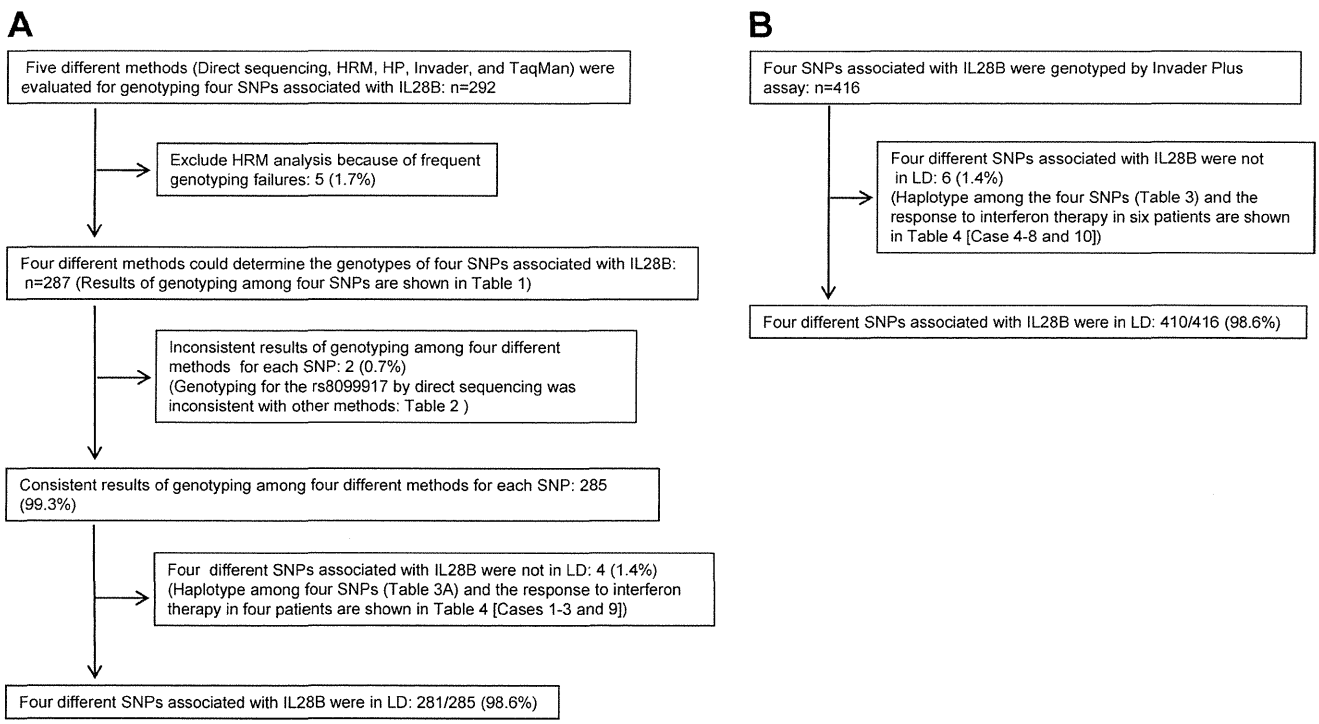

FIG. 1. Schema for the flowchart of the examinations.

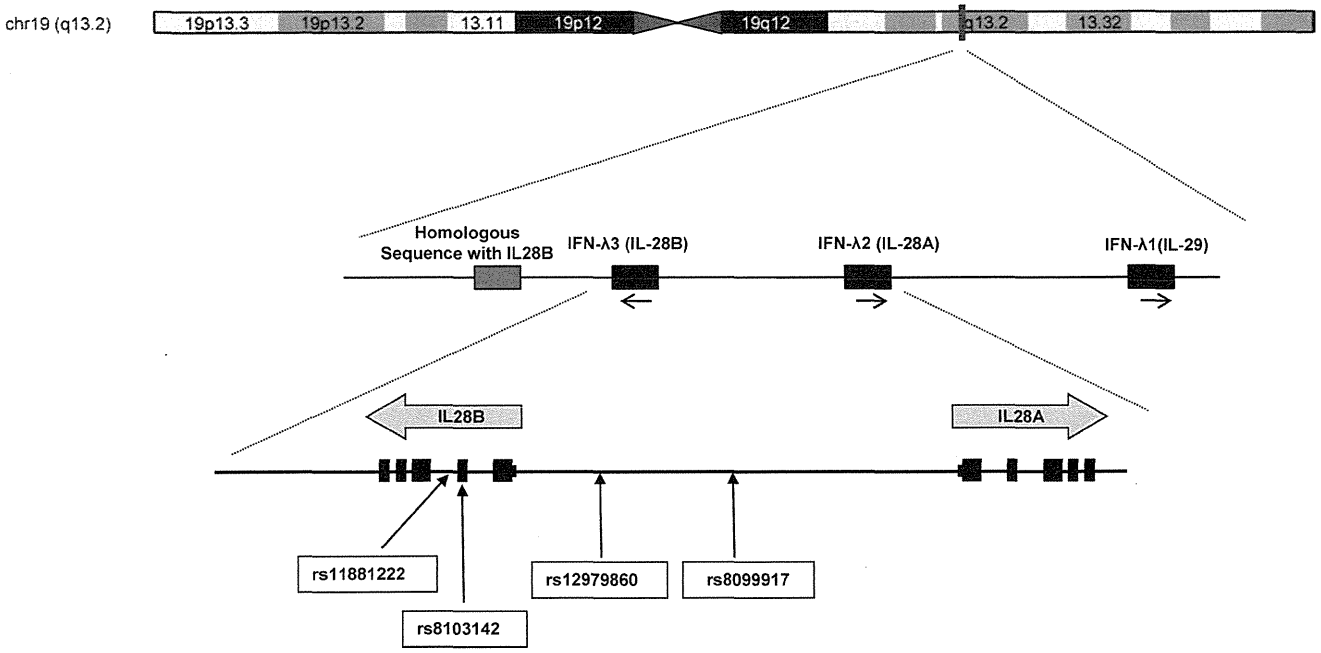

FIG. 2. Location of interferon lambda genes and the four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) associated with IL-28B. chr19, chromosome 19.

1B), recruited from NHO Nagasaki Medical Center, Nagoya City University Hospital, Nagoya Daini Red Cross Hospital, and Kawasaki Medical University Hospital in Japan, in the second stage (Table 1). We then focused on 10 patients whose four SNPs were found in the first and second stages not to be in LD and investigated the response to PEG-IFN/RBV treatment in detail for these patients. Informed consent was obtained from each patient who participated in the study. This study was conducted in accordance with provisions of the Declaration of Helsinki.

Definition of treatment responses. Nonvirological response (NVR) was defined as less than a 2-log-unit decline in the serum level of HCV RNA from the pretreatment baseline value within the first 12 weeks or detectable viremia 24 weeks after treatment. Virological response (VR) was defined in this study as the achievement of sustained VR (SVR) or transient VR (TVR); SVR was defined as undetectable HCV RNA in serum 6 months after the end of treatment, whereas TVR was defined as a reappearance of HCV RNA in serum after treatment was discontinued in a patient who had undetectable HCV RNA during the therapy or had achieved a more than 2-log-unit decline within the first 12 weeks after treatment.

DNA extraction. Whole blood was collected from all participants and centrifuged to separate the buffy coat. Genomic DNA was extracted from the buffy coat with Genomix (Talent SRL, Italy).

Five different genotyping methods. Four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) (Fig. 2) were determined in 292 patients by five different genotyping methods. We developed the five methods (direct sequencing, high-resolution melting analysis [HRM], hybridization probe (HP), InvaderPlus assay (Invader), and the TaqMan SNP genotyping assay (TaqMan) to determine the genotypes of the rs11881222 and rs8103142 polymorphisms. We also developed four different methods (direct sequencing, HRM, HP, and Invader) to determine the genotypes of the rs12979860 and rs8099917 polymorphisms. The genotype of rs12979860 was also determined by the TaqMan genotyping method developed by Duke University, and the genotype of rs8099917 was also determined with the TaqMan predesigned SNP genotyping assay. Figures 3,

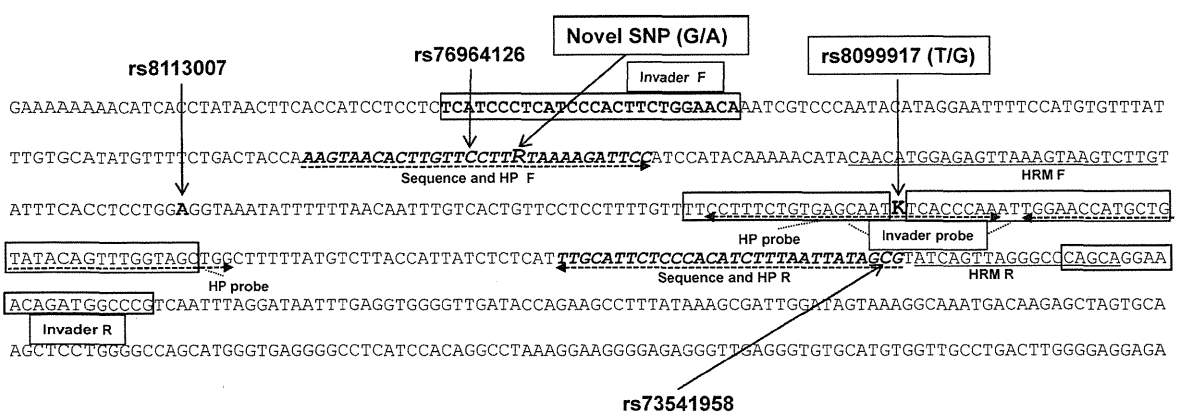

FIG. 3. The nucleotide sequence around rs8099917 is shown. Primers and probes for four different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay) to determine rs8099917 polymorphism are shown. F, forward primer; R, reverse primer.

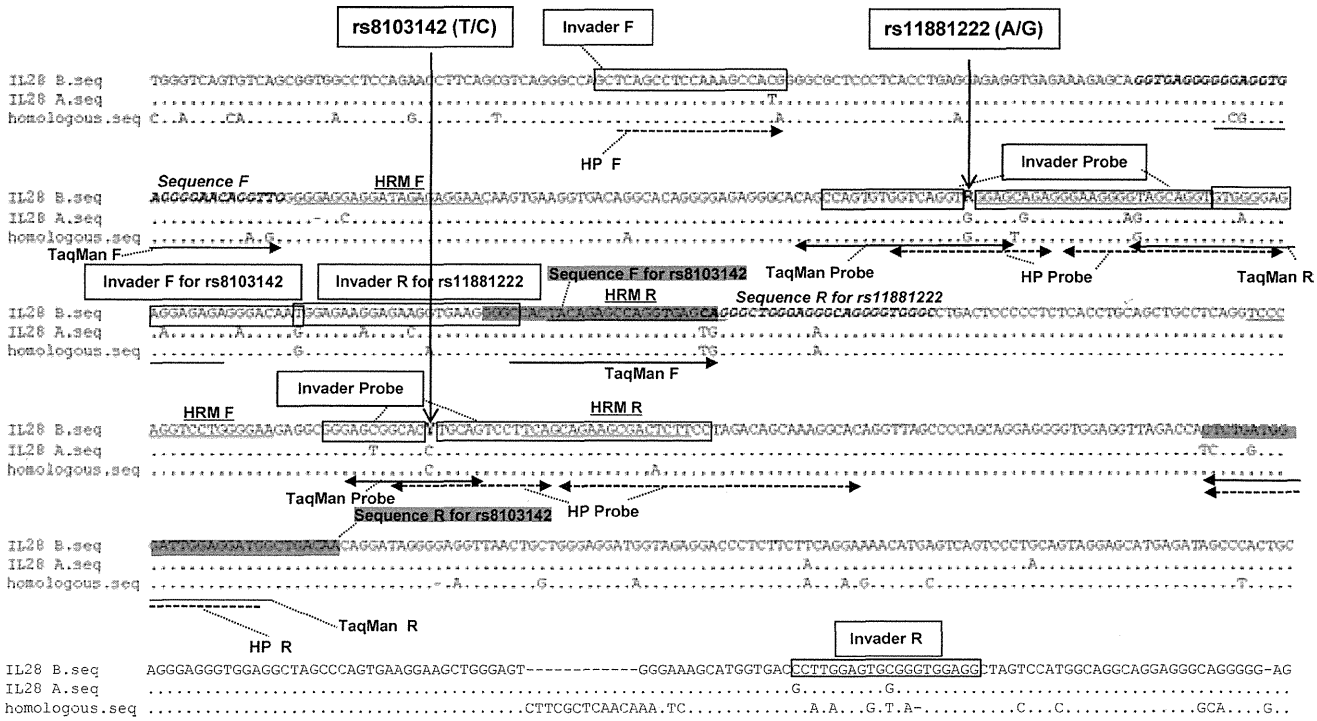

FIG. 4. The nucleotide sequence around rs11881222 and rs8103142 is shown. Primers and probes for five different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay; TaqMan, TaqMan assay) to determine rs11881222 and rs8103142 polymorphisms are shown. F, forward primer; R, reverse primer.

4, and 5 show the primers and probes for each genotyping method. Because the sequence of IL-28B is very similar to those of IL-28A, IL-29, and a homologous sequence upstream of IL-28B, we had to design the primers and probe for each method to distinguish IL-28B from the other sequences. First, primers were designed with Visual OMP Nucleic Acid software, and then we confirmed that the candidate primers should not amplify sequences other than the target region by using UCSC Genome Browser. Next, we confirmed that the amplicon was resolved as a single band, when the PCR products amplified by the primers under evaluation were electrophoresed. Finally, we had to optimize each set of primers and probe for each method (Fig. 3 to 5; see the table in the supplemental material).

Direct sequencing. PCR was carried out with 12.5 μl AmpliTaq Gold 360 master mix (Applied Biosystems), 10 pmol of each primer, and 10 ng of genomic DNA under the following thermal cycler conditions: stage 1, 94°C for 5 min; stage 2, 94°C for 30 s, 65°C for 30 s, 72°C for 45 s, for a total of 35 cycles; and stage 3, 72°C for 7 min. For sequencing, 1.0 μl of the PCR products was incubated with the use of a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). After ethanol purification, the reaction products were applied to the Applied Biosystems 3130xl DNA analyzer.

HRM analysis. HRM analysis was performed on a LightCycler 480 (LC480; Roche Diagnostics) as described previously (5, 15, 24). We designed pairs of primers flanking each SNP (Fig. 3 to 5) to amplify DNA fragments shorter than 200 bp. PCR was performed in a 20-μl volume containing 10 μl LightCycler 480 high-resolution melting master mix (Roche Applied Science), 4 pmol of each primer, and 10 ng genomic DNA. The cycling conditions were as follows: SYBR green I detection format, 1 cycle of 95°C for 10 min and 50 cycles of 95°C for 5 s, 60°C for 10s, and 72°C for 20 s, followed by an HRM step of 95°C for 1 min, 40°C for 1 min, and 74°C for 5 s and continuous acquisition to 90°C at 25 acquisitions per 1°C. HRM data were analyzed with Gene Scanning software (Roche Diagnostics).

Hybridization probe. We designed oligonucleotide primers and hybridization probes for the four SNPs (Fig. 3 to 5). All assays were performed with the LC480 as described previously (4, 18). The amplification mixture consisted of 4 μl of 5× reaction mixture (LightCycler 480 genotyping master; Roche Diagnostics), 5 pmol of each oligonucleotide primer, 3.2 pmol of each oligonucleotide probe, and 10 ng of template DNA in a final volume of 20 μl. Samples were amplified as follows: 45 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 10 s, and extension at 72°C for 20 s. The generation of target amplicons for each sample was monitored between the annealing and elongation steps at 610 and 640 nm. Samples positive for target genes were identified by the instrument at the cycle number where the fluorescence attributable to the target sequences exceeded that measured as background. Those scored as positive by the instrument were confirmed by visual inspection of the graphical plot (cycle number versus fluorescence value) generated by the instrument.

InvaderPlus assay. The InvaderPlus assay, which combines PCR and the Invader reaction (11, 12), was performed with the LC480. The enzymes used in InvaderPlus are native *Taq* polymerase (Promega Corporation, Madison, WI) and Cleavase enzyme (Third Wave Technologies, Madison, WI). The reaction is configured to use PCR primers with a melting temperature (T_m) of 72°C and Invader detection probe with a target-specific T_m of 63°C. The Invader oligonucleotide overlaps the probe by one nucleotide, forming at 63°C an overlap flap substrate for the Cleavase enzyme. The first step of InvaderPlus is PCR target amplification, in which the reaction is subjected to 18 cycles of a denaturation step (95°C for 15 s) and hybridization and extension steps (70°C for 1 min). At the end of PCR cycling, the reaction mixture is incubated at 99°C for 10 min to inactivate the *Taq* polymerase. Next, the reaction temperature is lowered to 63°C for 15 to 30 min to permit the hybridization of the probe oligonucleotide and the formation of the overlap flap structure. Data were analyzed by endpoint genotyping software (Roche Diagnostics).

TaqMan assay. The rs8099917 polymorphism was determined by using TaqMan predesigned SNP genotyping assays, as recommended by the manufacturer. The TaqMan assay for determination of the genotype of rs12979860 was kindly provided by David B. Goldstein at Duke University. We designed primers and probes for TaqMan genotyping assays for the other two SNPs. Each genomic DNA sample (20 ng) was amplified with TaqMan universal PCR master mix reagent (Applied Biosystems, Foster City, CA) combined with the specific TaqMan SNP genotyping assay mixture, corresponding to the SNP to be genotyped. The assays were carried out using the LC480 (Roche Applied Science) and the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were analyzed by endpoint genotyping software (Roche Diagnostics).

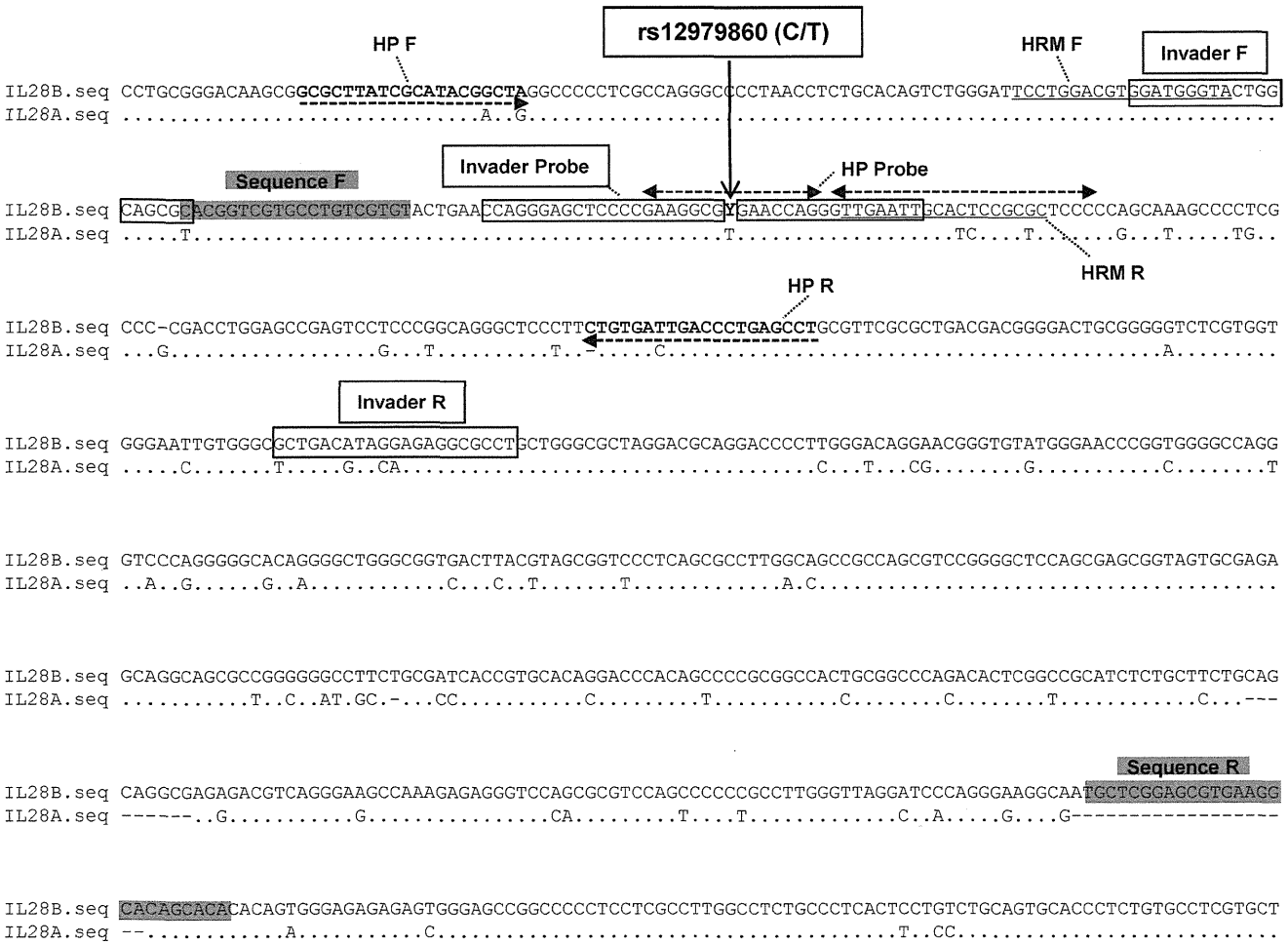

FIG. 5. The nucleotide sequence around rs12979860 is shown. Primers and probes for four different methods (Sequence, direct sequencing; HRM, high-resolution melting analysis; HP, hybridization probe; Invader, InvaderPlus assay) to determine rs12979860 are shown. F, forward primer; R, reverse primer.

RESULTS

Genotyping for four SNPs associated with IL-28B was unsuccessful by HRM in five cases. Figure 1A shows the patients' flowchart of the first stage. Genotyping of four SNPs (rs11881222, rs8103142, rs12979860, and rs8099917) was attempted by five different methods (direct sequencing, HRM, HP, Invader, and TaqMan) for 292 patients. In five cases, one of the four SNPs could not be genotyped by HRM. Therefore, we excluded the HRM method from further study. The genotyping failures by HRM involved two cases for rs11881222, two cases for rs8103142, and one case for rs8099917.

Consistencies of four different methods to determine genotypes for four SNPs associated with IL-28B. Consistencies among the results of genotyping by the remaining four methods were 100%, except for the results for rs8099917 (Table 2). For rs8099917, the results determined by direct sequencing were inconsistent with the other three methods in two cases (Tables 2 and 3). The HP, TaqMan, and Invader methods were accurate and reliable for genotyping the four SNPs associated with IL-28B. Invader was chosen for genotyping in the second stage, because the analysis time was the shortest and the sen-

TABLE 2. Determination of four SNPs associated with IL-28B by four different methods[a]

SNP	Genotype	No. (%) of cases with genotype by:			
		Direct sequencing	HP	Invader	TaqMan
rs11881222	AA	199 (69.3)	199 (69.3)	199 (69.3)	199 (69.3)
	AG	84 (29.3)	84 (29.3)	84 (29.3)	84 (29.3)
	GG	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
rs8103142	TT	199 (69.3)	199 (69.3)	199 (69.3)	199 (69.3)
	TC	84 (29.3)	84 (29.3)	84 (29.3)	84 (29.3)
	CC	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
rs12979860	CC	198 (69.0)	198 (69.0)	198 (69.0)	198 (69.0)
	CT	85 (29.6)	85 (29.6)	85 (29.6)	85 (29.6)
	TT	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)
rs8099917	TT	204 (71.1)	202 (70.4)	202 (70.4)	202 (70.4)
	TG	79 (27.5)	81 (28.2)	81 (28.2)	81 (28.2)
	GG	4 (1.4)	4 (1.4)	4 (1.4)	4 (1.4)

[a] There was 100% consistency for rs11881222, rs8103142, and rs12979860, and there was 99.3% consistency for rs8099917.

TABLE 3. Inconsistency in two cases between rs8099917 genotyping by direct sequencing and three other methods

Case no.	rs8099917 genotype by[a]:			
	Direct sequencing	HP	Invader	TaqMan
1	**T/T**	T/G	T/G	T/G
2	**T/T**	T/G	T/G	T/G

[a] Homozygous genotypes are highlighted in boldface.

sitivity was the greatest of the three methods (HP, TaqMan, and Invader), as reported previously (20).

Genotyping error for rs8099917 by direct sequencing due to novel SNP. In two cases, the results of genotyping for rs8099917 by direct sequencing were inconsistent with the results by the other methods (Table 3). Direct sequencing determined the genotype for rs8099917 as T/T in cases 1 and 2; however, the other three genotyping methods (HP, Invader, and TaqMan) determined the genotypes for rs8099917 as T/G in both cases. Further study using alternative primers for direct sequencing revealed that the correct genotypes were T/G and revealed a novel minor SNP present in the forward primer binding site in these two cases (data on file) and which interfered with the PCR amplification step (Fig. 3).

Distribution of haplotypes among four SNPs associated with IL-28B. In the first stage, the four SNPs were in LD in 281 (98.6%) of 285 cases and not in LD in the remaining 4 (1.4%). The first stage revealed five different haplotypes (no. 1 to 5 in Table 4). In haplotypes 1 to 3, the four SNPs were in LD (haplotype 1, homozygous of the major allele among 4 SNPs; n = 198 [69.5%]; haplotype 2, heterozygous among 4 SNPs; n = 79 [27.7%]; and haplotype 3, homozygous of the minor allele among 4 SNPs; n = 4 [1.4%]). In haplotype 4 (3 cases) rs11881222, rs8103142, rs12979860, and rs8099917 were AG, TC, CT, and TT, respectively. In haplotype 5 (one case), rs11881222, rs8103142, rs12979860, and rs8099917 were AA, TT, CT, and TT, respectively. Genotyping by the Invader method of the four SNPs associated with IL-28B in 416 patients in the second stage revealed that the four SNPs were not in LD in 6 cases (1.4%) (Table 4). A total of 410 (98.6%) of 416 cases were in LD for the four different SNPs. The second stage showed six different haplotypes (haplotypes 1 to 4, 6, and 7). Haplotypes 1 to 4 were detected in the first stage, but haplotypes 6 and 7 were not. The distribution of haplotypes was such that haplotypes 1, 2, 3, and 4 were found in 294 (70.7%), 110 (26.5%), 6 (1.4%), and 4 (1.0%) cases, respectively. In haplotype 6 (one case), rs11881222, rs8103142, rs12979860, and rs8099917 were AG, TT, CC, and TT, respectively. In haplotype 7 (one case), rs11881222, rs8103142, rs12979860, and rs8099917 were AA, TT, CT, and TG, respectively.

Response to PEG-IFN/RBV treatment in 10 cases in which the four SNPs associated with IL-28B were not in LD. In 7 (cases 1 to 7 [70%]) of the 10 cases where the four SNPs were not in LD, the haplotype was such that rs11881222, rs8103142, rs12979860, and rs8099917 were AG, TC, CT, and TT, respectively (Table 5). In nine cases (cases 1 to 9), rs8099917 was homozygous for the major allele, while one or more of the other SNPs were heterozygous. Eight (cases 1 to 8) of these

TABLE 4. Distribution of haplotypes among four SNPs associated with IL-28B in stages 1 and 2

Stage	Haplotype no.	Genotype for SNP:				No. (%) of cases with haplotype shown
		rs11881222	rs8103142	rs12979860	rs8099917	
1	1	AA	TT	CC	TT	198 (69.5)
	2	AG	TC	CT	TG	79 (27.7)
	3	GG	CC	TT	GG	4 (1.4)
	4	AG	TC	CT	TT	3 (1.0)
	5	AA	TT	CT	TT	1 (0.4)
2	1	AA	TT	CC	TT	294 (70.7)
	2	AG	TC	CT	TG	110 (26.5)
	3	GG	CC	TT	GG	6 (1.4)
	4	AG	TC	CT	TT	4 (1.0)
	6	AG	TT	CC	TT	1 (0.2)
	7	AA	TT	CT	TG	1 (0.2)

nine cases were viral responders who met the following criteria: HCV had disappeared during therapy, or HCV RNA had decreased more than 2 log copies/ml before 12 weeks after beginning of therapy, although some cases were under treatment or before determination of the final response to PEG-IFN/RBV. Case 9 was NVR due to poor adherence of PEG-IFN (<50% dose), even though rs8099917 was homozygous of the major allele. The haplotype of case 9 showed that rs11881222, rs8103142, rs12979860, and rs8099917 were AA, TT, CT, and TG, respectively. NVR in case 10 was reasonable from the genotypes of rs8099917 and rs12979860, because they were heterozygous, although rs11881222 and rs8103142 were homozygous for the major allele.

DISCUSSION

The relationship between SNPs associated with IL-28B and the response to PEG-IFN/RBV therapy for chronic hepatitis C was found by SNP array, using GWAS technology, by three different groups throughout the world, including our own, in 2009 (6, 19, 21). Following these reports, many studies have confirmed the association between the response to PEG-IFN/RBV and SNPs associated with IL-28B (14, 16). Therefore, it is obvious that these SNPs may be valuable for predicting the response to PEG-IFN/RBV therapy. Recently, it was reported that various SNPs were associated with development of disease and response to therapy and correlated with adverse effects. Several SNPs, such as the UGT1A1 polymorphism for the treatment with irinotecan (1, 17), have already been exploited in clinical practice to avoid severe adverse effects. These tailor-made therapies are expected to become more common in clinical practice in the near future (9). The next step toward tailor-made therapy for PEG-IFN/RBV therapy against chronic hepatitis C involved the development of simple, accurate, and inexpensive methods to determine the genotype of SNPs and determination of the best SNP where the four SNPs associated with IL-28B were not in LD, so that they may be applied in clinical practice.

Genotyping of IL-28B SNPs is quite different from other SNPs, because the sequence of IL-28B is very similar to those of IL-28A, IL-29, and an additional homologous sequence upstream of IL-28B (Fig. 2). We had to design primers and probes for each method to distinguish IL-28B specifically. We

TABLE 5. Clinical characteristics of 10 cases in which the SNPs associated with IL-28B were not in LD

Case no.[a]	SNP of IL-28B[b]				Age (yr)	Gender	Genotype	Viral titer	Final response to PEG-IFN/RBV	VR or NVR	Period of disappearance of HCV
	rs11881222	rs8103142	rs12979860	rs8099917							
1	A/G	T/C	C/T	**T/T**	64	Female	1b	6.5	TR	VR	4 wk
2	A/G	T/C	C/T	**T/T**	72	Male	1b	2.9	SVR	VR	4 wk
3	A/G	T/C	C/T	**T/T**	64	Male	1b	7	ND[c]	VR	8 wk
4	A/G	T/C	C/T	**T/T**	51	Female	1b	7.2	Under treatment	VR	3.6 log units down after 12 wk
5	A/G	T/C	C/T	**T/T**	60	Female	2	5.8	Under treatment	VR	12 wk
6	A/G	T/C	C/T	**T/T**	56	Female	1b	5.9	Under treatment	VR	2.0 log units down after 2 wk
7	A/G	T/C	C/T	**T/T**	62	Male	1b	5.4	SVR	VR	4 wk
8	A/G	**T/T**	**C/C**	**T/T**	58	Male	1b	6.2	TR	VR	12 wk
9	**A/A**	**T/T**	C/T	**T/T**	68	Male	1b	7	NVR	NVR	—[d]
10	**A/A**	**T/T**	C/T	T/G	48	Female	1b	6	NVR	NVR	—

[a] All cases shown were treated with PEG-IFN/RBV.
[b] Homozygous genotypes are highlighted in boldface.
[c] ND, not determined. The final response to PEG-IFN/RBV was not determined in this patient because 6 months had not passed after the end of treatment.
[d] —, HCV did not disappear.

think that the results in this paper are especially applicable to IL-28B genotyping. In this study, only HRM failed to determine the genotype of SNPs associated with IL-28B. The reason HRM failed more frequently than the other genotyping methods is attributable to the characteristics of this specific method. Because HRM determines the genotype of each SNP by distinguishing the melting curve of an amplicon of around 200 bp, it may tend to be influenced by another SNP. As a matter of fact, minor SNPs around rs8099917 were found in cases of genotyping failure by HRM (data not shown). Although this specific characteristic of the HRM method is useful for detecting novel mutations or SNPs, it is not suitable for determination of the genotype of SNPs associated with IL-28B.

Direct sequencing erroneously reported the T/G genotype as T/T for the rs8099917 polymorphism. We found that the cause of this genotyping error was a novel rare SNP in the forward primer binding site used for amplification and direct sequencing (data on file). Because this novel SNP was not registered as an SNP in the NCBI database, the primer was designed at this site. Since the novel SNP correlated with the rs8099917 polymorphism in LD, adenine for the novel SNP is present on the same allele as guanine in the rs8099917 polymorphism. Therefore, the forward PCR primer (AAGTAACACTTGTTCCTT GTAAAAGATTCC) could not anneal to the binding site, which was changed from guanine (G) to adenine (A) at the underlined nucleotide position: only the allele which has T at the rs8099917 was amplified, the genotype was determined as T/T. Rare sequence variations not registered in the database, might be present in the primer binding sites for amplification and might be the cause of erroneous direct sequencing. Ikegawa et al. reported that annealing efficiency in direct sequencing led to the mistyping of an SNP (10). Although our results in this paper are especially applicable to IL-28B genotyping, it should be recognized that allele-dependent PCR amplification and erroneous typing can occur when SNPs are genotyped by a PCR-based approach. Should SNPs associated with IL-28B be found not to be in LD, it would be preferable to confirm the genotype by another method.

In 10 cases, four SNPs associated with IL-28B were not in LD. In seven (70%) of the 10 cases, the haplotype showed that rs11881222, rs8103142, rs12979860, and rs8099917 were AG, TC, CT, and TT, respectively. Only the rs8099917 polymorphism differed frequently from the other three SNPs. The reason for the high frequency of this haplotype is thought to be attributable to the location of these SNPs. The location of rs8099917 is downstream and quite far from the two SNPs (rs11881222 and rs8103142) in the IL-28B gene (Fig. 2). The SNPs rs11881222 and rs8103142 were almost perfectly in LD, because they are located close to each other.

It is well described that homozygosity for the major allele of SNPs associated with IL-28B is correlated with a better response to PEG-IFN/RBV treatment, and minor allele-positive patients are poor responders. However, the response to PEG-IFN/RBV remains unknown when several SNPs associated with IL-28B are not in LD. Because cases in which the SNPs are not in LD are quite rare, it was thought to be difficult to study such cases. In this study, 10 (1.4%) of 708 patients showed haplotypes in which the four SNPs were not in LD. We focused on the response to PEG-IFN/RBV therapy in these 10 cases (Table 5). We evaluated the response to PEG-IFN/RBV treatment from the viewpoint of virological response, because some patients had not completed their PEG-IFN/RBV treatment. (Case 3 was before determination for the final response after finishing the treatment, and cases 4 to 6 were under treatment.)

Thomas et al. reported that allele frequencies for rs12979860 varied among racial and ethnic groups (23). Indeed, the observation that the major allele is less frequent among individuals of African descent than those of European descent might explain the observed discrepancy in the frequencies of viral clearance in these two ethnic groups, where clearance occurs in 36.4% of HCV infections in individuals of non-African ancestry, but in only 9.3% of infections in individuals of African ancestry (22). We have recruited only Japanese chronic hepatitis C patients for this study. Since the distribution of haplotype and response to PEG-IFN/RBV treatment should vary among populations, further study will be necessary for any other populations except Japanese.

We have shown that the rs8099917 polymorphism determined by Invader assay should be the best predictor of the