simeprevir plus peginterferon and ribavirin provided SVR rates of only 34% or 38-51%, respectively.[3,7] The array of adverse events associated with peginterferon and ribavirin is well known; incremental toxicities associated with the addition of telaprevir to peginterferon and ribavirin included anemia, skin disorders and severe rash, and gastrointestinal-related disorders, while the addition of simeprevir is associated with hyperbilirubinemia due to inhibition of hepatic bilirubin transporters.[3] For patients who cannot tolerate or are not eligible for treatment with interferon-based therapy because of coexisting morbidities, treatment options are few to none. Clearly, the current treatment options are not adequate and an urgent unmet need remains for better treatment regimens for these patient populations.

Daclatasvir is a first-in-class, NS5A replication complex inhibitor with potent pan-genotypic antiviral activity *in vitro* (HCV genotypes 1-6).[8] Asunaprevir is a potent, selective NS3 protease inhibitor with antiviral activity against HCV genotypes 1, 4, 5, and 6 *in vitro*.[9] Both daclatasvir and asunaprevir have demonstrated robust antiviral activity, with no clinically meaningful pharmacokinetic interactions between them when coadministered.[8,10,11] Preliminary phase 2 studies showed potent antiviral effects using daclatasvir and asunaprevir as an all-oral therapy and in combination with a regimen of peginterferon/ribavirin in patients infected with HCV genotype 1 who had not responded to prior therapy.[12,13] We evaluated the safety and antiviral activity of interferon-free, ribavirin-free, all-oral therapy with daclatasvir and asunaprevir in a phase 3 trial involving Japanese patients infected with HCV genotype 1b who are interferon-ineligible/intolerant or nonresponders (null and partial) to interferon-based therapies.

Materials and Methods

Patients. A total of 259 patients were enrolled at 24 centers in Japan from January 5 2012 to March 30 2012. Eligible patients were men and women, 20 to 75 years of age, with chronic HCV genotype 1b infection, an HCV RNA level of 10^5 IU/mL or higher, with a body-mass index of 16 to 35 kg/m^2, and, in up to 10% of enrolled patients, evidence of compensated cirrhosis (Child-Pugh A), as documented either by liver biopsy or discriminated by a previously described algorithm.[14]

Key exclusion criteria included evidence of hepatocellular carcinoma, coinfection with hepatitis B virus or human immunodeficiency virus, or previous exposure to inhibitors of NS5A or NS3 protease. Patients with alanine aminotransferase (ALT) of more than 5 times the upper limit of normal range, total bilirubin of 2 mg/dL or higher, an international normalized ratio of 1.7 or higher, an albumin level 3.5 g/dL or below, and a platelet count of less than 50,000/mm^3 were also excluded.

Patients ineligible for interferon-based therapy, but potentially eligible for enrolment in this study, were treatment-naïve and considered poor candidates for interferon-based therapy because of medical complications including anemia, neutropenia, thrombocytopenia, depression, advanced age (≥65 years), or other conditions deemed not suitable for interferon-based therapy by the investigator, including hypertension, diabetes mellitus, autoimmune disease, and abnormal thyroid function. Patients intolerant to interferon-based therapy had received interferon-based therapy for less than 12 weeks and previously discontinued from therapy due to toxicities associated with interferon or ribavirin. Patients who were null or partial responders to previous peginterferon/ribavirin or interferon-beta/ribavirin therapy were defined as never having attained an undetectable HCV RNA level after at least 12 weeks of therapy. Null responders included patients who never attained at least a 2-$\log_{10}$ decrease from baseline in HCV RNA levels at week 12, and partial responders never achieved undetectable HCV RNA levels after 12 weeks of therapy.

Study Design. In this open-label, phase 3 study of two patient cohorts, interferon-ineligible/intolerant and

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nonresponder patients received daclatasvir and asunaprevir for 24 weeks. Patients were followed for an additional 24 weeks after treatment. Daclatasvir was administered orally at a dose of 60 mg once daily, and asunaprevir was administered orally at a dose of 100 mg twice daily. Host *IL28B* genotype was assayed for the rs12979860 single-nucleotide polymorphism by Monogram Biosciences using a real-time polymerase chain reaction (PCR) assay.

Nonresponder patients who met futility criteria, defined as an increase in viral load of at least 1 $\log_{10}$ or confirmed detectable HCV RNA of at least 15 IU/mL on or after week 8, were eligible for addition of peginterferon-alpha/ribavirin to continued treatment with daclatasvir and asunaprevir for an additional 24 weeks at the discretion of the investigator. Interferon-ineligible/intolerant patients were not candidates for interferon-based therapy; therefore, daclatasvir/asunaprevir dual therapy was stopped if futility criteria were met.

Study Oversight. This study was approved by the Institutional Review Board at each participating site and was conducted in compliance with the Declaration of Helsinki, Good Clinical Practice guidelines, and local regulatory requirements. All patients provided written informed consent.

Efficacy Assessments. HCV RNA levels were measured using the Roche COBAS Taqman test with a lower and an upper limit of quantitation of 15 IU/mL and 6.9 $\times$ 10^7 IU/mL, respectively. HCV RNA was measured at screening and at day 1, weeks 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24, and posttreatment at weeks 4, 8, 12, and 24.

Resistance Testing. Patient-derived HCV NS5A and NS3/4A sequence populations were PCR-amplified and sequenced. Patient samples selected for sequencing included all baseline samples and samples from patients with virologic failure.

Safety Assessments. Safety evaluations included reported adverse events and serious adverse events, clinical laboratory tests, physical examinations, and electrocardiograms.

Endpoints. The primary efficacy endpoint was the proportion of patients with HCV RNA <15 IU/mL (target detected [TD] or target not detected [TND]) at 24 weeks after completion of daclatasvir and asunaprevir treatment, including patients who discontinued treatment early. Key secondary endpoints included the proportion of patients with undetectable HCV RNA (TND) at weeks 4 and 12, at the end of treatment, and HCV RNA <15 IU/mL (TD or TND) at 12 weeks after the end of treatment. Safety endpoints included the frequency of serious adverse events, adverse events, discontinuations due to adverse events, and laboratory abnormalities.

Statistical Analysis. Analyses included all patients who received at least one dose of study medications. For virologic response, 2-sided 95% confidence intervals were calculated based on the normal approximation to the binomial distribution. Categorical variables were summarized using counts and percents. Continuous variables were summarized with univariate statistics. Patients with missing data or those who received additional peginterferon/ribavirin therapy were considered failures.

Role of the Funding Source. The study was designed and conducted by the sponsor (Bristol-Myers Squibb/Bristol-Myers KK) in collaboration with the principal investigators. The sponsor collected the data, monitored the study conduct, and performed the statistical analyses. All authors had access to the data and assume responsibility for the accuracy, integrity, and completeness of the reported data and for the fidelity of this report to the trial protocol. The article was prepared by authors employed by Bristol-Myers Squibb, with input from all authors and the assistance of a medical writer employed by Bristol-Myers Squibb. All authors made the decision to submit the article for publication.

Results

Patients. In all, 222 patients received treatment, 135 in the interferon-ineligible/intolerant group (100 medically ineligible for interferon, 35 intolerant to interferon) and 87 in the nonresponder group (48 null responders, 36 partial responders, 3 undetermined) (Fig. 1). Demographic baseline characteristics of patients are shown in Table 1. As expected, when compared with reported demographics from U.S. and European studies, patients were older and a larger proportion were female. Similar to the global population, however, there were more patients with *IL28B* CC genotype in the interferon-ineligible/intolerant population (69.6%) and more patients with *IL28B* non-CC genotype in the nonresponder population (81.6%). Overall, the rate of discontinuations from dual therapy was low (12.6%; 14 patients in each group), and was due primarily to adverse events (nine patients [6.7%] in the interferon-ineligible/intolerant group, two patients [2.3%] in the nonresponder group) and lack of efficacy (four patients [3.0%] in the interferon-ineligible/intolerant group, 11 patients [12.6%] in the nonresponder group).

Virologic Response. HCV RNA levels declined rapidly after initiation of treatment in both groups (Fig. 2). At week 2, the mean decrease in HCV RNA

Table 1. Demographic and Baseline Characteristics of Patients and Their Disease

Characteristic	Interferon-ineligible/intolerant n = 135	Nonresponder n = 87	Total N = 222
Age, years			
- Median	64.0	60.0	62.5
- Range	24-75	42-74	24-75
- ≥65 years, n (%)	62 (45.9)	27 (31.0)	89 (40.1)
Male sex, n (%)	38 (28.1)	39 (44.8)	77 (34.7)
IL28B rs12979860 genotype, n (%)			
- CC	94 (69.6)	16 (18.4)	110 (49.5)
- CT	40 (29.6)	66 (75.9)	106 (47.7)
- TT	1 (0.7)	5 (5.7)	6 (2.7)
HCV RNA			
- Mean $\log_{10}$ IU/mL ± SD	6.6 ± 0.58	6.8 ± 0.47	6.6 ± 0.55
- ≥800,000 IU/mL, n (%)	109 (80.7)	80 (92.0)	189 (85.1)
Cirrhosis, n (%)	11 (8.1)	11 (12.6)	22 (9.9)
Response to prior therapy (nonresponders), n (%)			
- Null	NA	48 (55.2)	48 (21.6)
- Partial	NA	36 (41.4)	36 (16.2)
- Other	NA	3 (3.4)*	3 (1.4)
Premedical status (interferon-ineligible/intolerant), n (%)			
- Ineligible-naïve	100 (74.1)	NA	100 (45.0)
• Depression	10 (10.0)	NA	10 (10.0)
• Anemia/neutropenia/thrombocytopenia	44 (44.0)	NA	44 (44.0)
• Other complications requiring medications†	34 (34.0)	NA	34 (34.0)
• Advanced age	12 (12.0)	NA	12 (12.0)
- Intolerant	35 (25.9)	NA	35 (15.8)

*Three patients had insufficient data to be classified as partial or null nonresponders.
†Other complications included hypertension, diabetes mellitus, autoimmune disease, abnormal thyroid function, insomnia, stroke, and psychological.
NA = not applicable.

from baseline was 5.2 $\log_{10}$ IU/mL. Overall, 167/222 patients (75.2%) had undetectable HCV RNA at week 4 during treatment, and 202 patients (91.0%) had undetectable HCV RNA at week 12 on treatment. At 12 weeks after the end of treatment period, 119 (88.1%) interferon-ineligible/intolerant and 70 (80.5%) nonresponder patients had achieved SVR_{12}; by 24 weeks after the end of treatment 118 (87.4%) interferon-ineligible/intolerant and 70 (80.5%) nonresponder patients had achieved SVR_{24} (Table 2). Patients with cirrhosis also achieved high rates of SVR_{24} (20/22, 90.9%). When analyzed by *IL28B* genotype, the response rates were similar for patients with *IL28B* CC genotype (84.5%) and *IL28B* non-CC genotypes (84.8%) (Table 2). Other baseline factors including gender, age, and baseline HCV RNA, did not appear to impact response rates (Table 2).

Virologic Failure. Thirty-four (15.3%) patients (17 each in the interferon-ineligible/intolerant group and nonresponder group) were considered virologic failures. Of patients with undetectable HCV RNA at the end of treatment, 11/129 (8.5%) interferon-ineligible/intolerant patients experienced viral relapse during posttreatment follow-up. Six of 76 patients (7.9%) in the nonresponder group with undetectable HCV RNA at the end of treatment had viral relapse. Two patients in the interferon-ineligible/intolerant group and one patient in the nonresponder group had detectable HCV RNA at the end of treatment. Virologic breakthrough occurred in 4 (3.0%) interferon-ineligible/intolerant patients and in 10 (11.5%) nonresponder patients. At the discretion of the investigators, 9 of the 10 nonresponder patients with virologic breakthrough had additional treatment with peginterferon/ribavirin according to protocol-defined criteria; all nine patients were declared treatment failures in the analysis of the primary endpoint. One of the nine patients who received additional peginterferon/ribavirin responded to treatment with no detectable HCV RNA at follow-up week 24, two patients had HCV RNA detectable at end of treatment, and six patients relapsed.

Of the 34 patients with virologic failure, 29 had resistance-associated substitutions to both daclatasvir (predominantly NS5A-L31M/V-Y93H) and asunaprevir (predominantly NS3-D168 variants) detected at failure. Twenty-two patients with virologic failure had NS5A polymorphisms L31M/V and/or Y93H prior to treatment (Supporting Table 1).

We also investigated the influence of pretreatment resistance-associated variants on efficacy in this study. Pretreatment L31M, Y93H, or linked L31V+Y93H NS5A polymorphisms were detected in 7, 29, and 1 of the 214 patients with available baseline NS5A

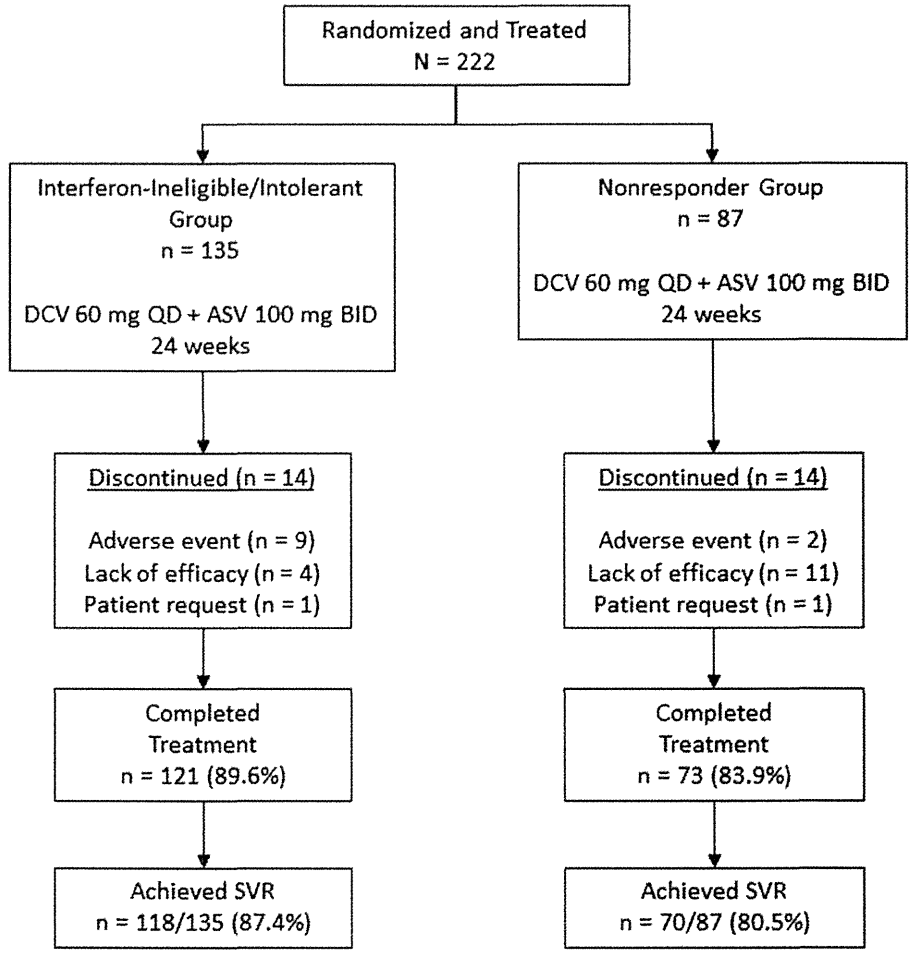

Fig. 1. Patient disposition.

sequences, respectively. Of the 37 patients with L31M/V and/or Y93H at baseline, 11/23 interferon-ineligible/intolerant patients and 4/14 nonresponder patients achieved SVR. The primary asunaprevir resistance-associated variant, NS3-D168E, was present in 2/221 patients with available baseline NS3 sequences; neither of these patients had concomitant NS5A resistance-associated variants. One of these patients achieved SVR; the other relapsed posttreatment.

In comparison with patients who achieved SVR, patients with virologic failure were more likely to have daclatasvir and asunaprevir trough concentrations below their respective median values but within the expected range (Supporting Fig. 1). Most patients with trough concentrations below median values achieved SVR. Treatment compliance, assessed by pill counts and interviews at each study visit, was 83.9% in prior nonresponders and 88.9% in interferon-ineligible/intolerant patients. Across both cohorts, patients with ≥95% compliance in dose and duration of treatment had an SVR_{24} rate of 92.7% (179/193), compared with a 31.0% (9/29) SVR_{24} rate in patients who were <95% compliant (15 out of the 29 patients were discontinued due to the lack of efficacy).

Safety. A total of 194 patients (87.4%) completed 24 weeks of therapy, 121 (89.6%) in the interferon-ineligible/intolerant group and 73 (83.9%) in the nonresponder group. No deaths occurred during the study period. Eleven patients (5.0%) discontinued after 4 to 23 weeks of treatment; 10 discontinued due to ALT and aspartate aminotransferase (AST) elevations and one patient discontinued due to myasthenia gravis, with subsequent detection of preexisting myasthenia gravis-related antibodies.

The most common adverse events were nasopharyngitis, increased ALT and AST, headache, diarrhea, and pyrexia (Table 3). Serious adverse events were reported in 13 (5.9%) patients during treatment. In nine (6.7%) interferon-ineligible/intolerant patients, these events included periarthritis, schizoaffective disorder, myasthenia gravis, myocardial infarction, pyrexia, appendicitis, pyelonephritis,

Table 2. Virologic Outcomes

Virologic Response, n (%) [95% CI]	Interferon-Ineligible/Intolerant n = 135	Nonresponder n = 87	Total N = 222
Week 4,*	114 (84.4)	53 (60.9)	167 (75.2)
	[78.3, 90.6]	[50.7, 71.2]	[69.5, 80.9]
Week 12.*	125 (92.6)	77 (88.5)	202 (91.0)
	[88.2, 97.0]	[81.8, 95.2]	[87.2, 94.8]
Weeks 4 and 12,*	106 (78.5)	48 (55.2)	154 (69.4)
	[71.6, 85.4]	[44.7, 65.6]	[63.3, 75.4]
End of treatment response*	129 (95.6)	76 (87.4)	205 (92.3)
	[92.1, 99.0]	[80.4, 94.3]	[88.8, 95.8]
Sustained virologic response	126 (93.3)	71 (81.6)	197 (88.7)
4 weeks after treatment (SVR_4)†	[89.1, 97.5]	[73.5, 89.7]	[84.6, 92.9]
Sustained virologic response	119 (88.1)	70 (80.5)	189 (85.1)
12 weeks after treatment (SVR_{12})†	[82.7, 93.6]	[72.1, 88.8]	[80.5, 89.8]
Sustained virologic response	118 (87.4)	70 (80.5)	188 (84.7)
24 weeks after treatment (SVR_{24})†	[81.8, 93.0]	[72.1, 88.8]	[79.9, 89.4]
SVR_{24} by subpopulations			
- Null responders	N/A	39/48 (81.3)	39/48 (81.3)
- Partial responders	N/A	28/36 (77.8)	28/36 (77.8)
- Undetermined	N/A	3/3 (100)	3/3 (100)
- Ineligible-naïve	85/100 (85.0)	N/A	85/100 (85.0)
- Intolerant	33/35 (94.3)	N/A	33/35 (94.3)
- Cirrhosis	10/11 (90.9)	10/11 (90.9)	20/22 (90.9)
- Noncirrhosis	108/124 (87.1)	60/76 (78.9)	168/200 (84.0)
- Male	32/38 (84.2)	32/39 (82.1)	64/77 (83.1)
- Female	86/97 (88.7)	38/48 (79.2)	124/145 (85.5)
- Age < 65 years	61/73 (83.6)	47/60 (78.3)	108/133 (81.2)
- Age ≥ 65 years	57/62 (91.9)	23/27 (85.2)	80/89 (89.9)
- HCV RNA < 800,000 IU/mL	25/26 (96.2)	6/7 (85.7)	31/33 (93.9)
- HCV RNA ≥ 800,000 IU/mL	93/109 (85.3)	64/80 (80.0)	157/189 (83.1)
SVR_{24} by *IL28B* genotype (rs12979860)			
- CC	79/94 (84.0)	14/16 (87.5)	93/110 (84.5)
- CT	38/40 (95.0)	52/66 (78.8)	90/106 (84.9)
- TT	1/1 (100)	4/5 (80)	5/6 (83.3)
Virologic failures			
- Virologic breakthrough	4 (3.0)	10 (11.5)‡	14 (6.3)
- End of treatment detectable	2 (1.5)	1 (1.1)	3 (1.4)
- Relapse (among patients undetectable at end of treatment)	11/129 (8.5)	6/76 (7.9)	17/205 (8.3)

*HCV RNA <LLOQ (<15 IU/mL), target not detected.
†HCV RNA <LLOQ, target detected or target not detected.
‡9/10 patients received additional treatment with peginterferon/ribavirin according to protocol criteria.

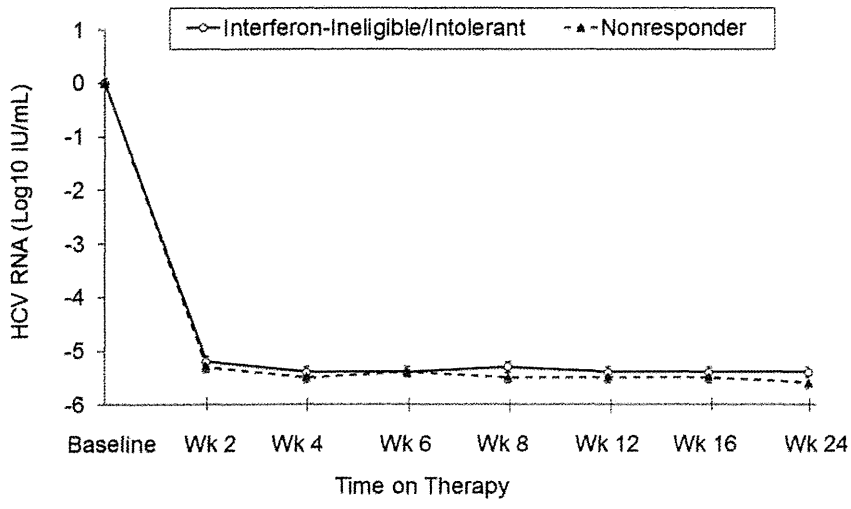

Fig. 2. Mean change in HCV RNA during treatment with daclatasvir and asunaprevir in interferon-ineligible/intolerant and nonresponder patients.

Table 3. Adverse Events and Grade 3-4 Laboratory Abnormalities During the Treatment Period

Event or Laboratory Abnormality, n (%)	Interferon-Ineligible/Intolerant n = 135	Nonresponder n = 87	Total N = 222
Serious adverse events (on treatment)	9 (6.7)	4 (4.6)	13 (5.9)
Adverse event*			
Nasopharyngitis	40 (29.6)	27 (31.0)	67 (30.2)
Increased ALT	24 (17.8)	11 (12.6)	35 (15.8)
Increased AST	18 (13.3)	10 (11.5)	28 (12.6)
Headache	18 (13.3)	17 (19.5)	35 (15.8)
Diarrhea	12 (8.9)	10 (11.5)	22 (9.9)
Pyrexia	12 (8.9)	15 (17.2)	27 (12.2)
Grade 3-4 laboratory abnormality			
Alanine aminotransferase	12 (8.9)	4 (4.6)	16 (7.2)
Aspartate aminotransferase	10 (7.4)	2 (2.3)	12 (5.4)
Hemoglobin	6 (4.4)	1 (1.1)	7 (3.2)
Lymphocytes	5 (3.7)	1 (1.1)	6 (2.7)
Platelets	2 (1.5)	2 (2.3)	4 (1.8)
Bilirubin, total	1 (0.7)	1 (1.1)	2 (0.9)
Neutrophils	0	1 (1.1)	1 (0.5)
Creatinine	1 (0.7)	0	1 (0.5)
Lipase, total	1 (0.7)	0	1 (0.5)

*Adverse events that occurred in more than 10% of patients in any group.

basal cell carcinoma, and hepatocellular carcinoma, respectively; events in four (4.6%) nonresponder patients included second-degree burn, increased liver enzymes, esophageal variceal hemorrhage, and herpes zoster.

ALT and AST elevations were the most frequent adverse events and grade 3/4 laboratory abnormalities (Table 3) and were the basis for 10 of the 11 discontinuations due to adverse events. Two of these 10 patients also had grade 3/4 total bilirubin elevations, but no patient experienced hepatic decompensation. Eight of the 10 patients who discontinued due to ALT/AST elevations (80%) subsequently achieved SVR. For the 16 patients who had grade 3/4 ALT elevations on-treatment, the median time to elevation was ~10 weeks (range 4 to 23 weeks), with rapid reversal in ~2.5 weeks after discontinuation. Most patients with baseline ALT and AST elevations experienced rapid improvement during the first 2 to 4 weeks of treatment, including all patients with grade 3/4 elevations at baseline, with mean decreases at 4 weeks of 43.7 U/L and 35.1 U/L, respectively.

Discussion

Treatment with interferon-based therapy is not an option for many patients with chronic HCV. The findings from this phase 3 study evaluating interferon-free, ribavirin-free, all-oral treatment with daclatasvir and asunaprevir demonstrated high rates of SVR in Japanese patients infected with HCV genotype 1b. Both interferon-ineligible/intolerant and previously treated nonresponder patient groups experienced a rapid reduction in HCV RNA by week 2. The primary endpoint, SVR_{24}, was achieved in 87.4% of patients who were ineligible or intolerant to interferon-based therapies and in 80.5% of patients who had not responded to treatment previously. These high rates of SVR obtained with daclatasvir and asunaprevir represent a significant improvement of cure rates in patient populations typically associated with poor responses to other therapies or with limited therapeutic options. Other factors typically associated with a poor response to therapy, including male gender, high baseline HCV RNA, advanced age, non-CC *IL28B* genotype, and cirrhosis, did not appear to impact response rates, although the number of patients in these subgroups was small.

The response rates in this study were higher than those observed in a phase 3 study evaluating triple therapy with telaprevir and peginterferon/ribavirin in Japanese patients infected with HCV genotype 1 with no response to prior treatment. The SVR rate of nonresponder patients in that study was 34.4%, and safety issues included anemia, severe rash, renal toxicity, and gastrointestinal-related disorders.[7] In a global phase 3 trial, SVR rates ranged from 54% to 59% in partial-responder and 29% to 33% in null-responder patients receiving telaprevir combined with peginterferon/ribavirin.[4] Simeprevir in combination with peginterferon/ribavirin achieved an SVR rate of 38-51% in Japanese nonresponder patients. In the present study, partial-responder and null-responder patients achieved better outcomes (77.8% and 81.3%, respectively), with a much more favorable safety profile. The response rate observed in the ineligible/intolerant group in this study was also notable, especially when considering these patients had no option for curative treatment.

This study was limited to Japanese patients; an ongoing phase 3 study in a similar patient population in the U.S. and Europe will determine whether region-related differences in patient characteristics influence outcomes with this regimen. The results from this phase 3 trial are consistent with the results of a small phase 2a study of Japanese patients treated with daclatasvir and asunaprevir; SVR rates were 64% in peginterferon/ribavirin-ineligible or intolerant patients and 91% in null responder patients.[15] A phase 2b trial combining NS3 (faldaprevir) and NS5B (deleobuvir) inhibitors showed only a 57% SVR in previously untreated patients with HCV genotype-1b infection.[16] In addition, other all-oral regimens earlier in clinical development may provide greater efficacy: in a phase 2

study, SVR rates of 95% to 100% were achieved in treatment-naïve and experienced genotype 1-infected patients treated with sofosbuvir (NS5B inhibitor) in combination with ledipasvir (NS5A inhibitor), with or without ribavirin.[17] The more complex combination of NS3 (ABT-450, plus ritonavir to improve drug exposure), NS5A (ABT-267), and NS5B (ABT-333) inhibitors, with or without ribavirin, achieved SVR rates of 88-96% in treatment-naïve patients and prior null responders with genotype 1 infection. Recent press reports indicate similar results in phase 3 studies with both of these regimens, although full study details are not yet available.[19,20] The combination of daclatasvir and sofosbuvir achieved SVR rates of 88-100% in treatment-naïve patients with genotype 1, 2, or 3 infection, and 95-100% in treatment-experienced patients with genotype 1 infection.[21] However, none of these studies involved patient populations directly comparable to those reported in the present study. Previous experience with HCV regimens indicates that both treatment eligibility and outcomes can vary in relation to variables such as disease stage, patient ethnicity, concomitant medical conditions, and other factors.[3] Further studies of all-oral combinations may provide the evidence needed for optimizing regimen selection on the basis of virologic and patient characteristics.

Response rates at on-treatment week 4 were somewhat higher in the ineligible/intolerant group than in prior nonresponders (84.4% versus 60.9%), but this difference diminished as treatment continued. The early difference in response rates may reflect a reduced contribution of endogenous interferon response in prior nonresponders; the ultimate achievement of an 80.5% SVR rate in this group suggests that such nonresponsiveness can be largely overcome with a potent antiviral regimen.

All-oral treatment with daclatasvir and asunaprevir generally suppressed the enrichment/selection of NS5A and NS3 resistance-associated variants. Virologic failure occurred in 17 patients in each group. Both NS5A and NS3 resistance-associated variants were detected in most patients with virologic failure. There was no apparent association between preexisting NS3 resistance-associated polymorphisms and subsequent virologic outcome. Although more patients with NS5A L31M/V and/or Y93H resistance-associated variants experienced virologic failure, 15/37 of patients with these baseline variants achieved SVR. Thus, pretreatment resistance-associated variants were not absolutely predictive of virologic outcome. Moreover, factors other than resistance, such as lower drug exposure and suboptimal compliance to treatment, likely contributed to treatment failure. The patients with daclatasvir and asunaprevir trough plasma concentrations below median values appeared to be at increased risk of virologic failure (Supporting Fig. 1). Given that patients with $\geq$95% compliance had an SVR_{24} rate of 92.7%, the maintenance of higher compliance is essential for optimizing treatment outcomes.

The rate of premature discontinuation of treatment with daclatasvir and asunaprevir due to adverse events was low. Despite early discontinuation that occurred between weeks 4 and 23, 8 of the 10 patients who discontinued because of elevated levels of ALT and AST achieved SVR_{24}, with rapid reversal of transaminase elevations posttreatment. Although small in number, six patients who achieved SVR were on treatment for 12 weeks or less, suggesting that a shorter treatment period may be possible in some patients. Additionally, baseline elevations of ALT and AST corrected rapidly in most patients after 2 to 4 weeks on treatment, as would be expected with the rapid reduction in HCV RNA levels. The rate of serious adverse events was low and varied among patients, with no consistent pattern of events. The frequency of adverse events was also low, especially compared with historical data in patients receiving a triple regimen with telaprevir and peginterferon/ribavirin that showed a high rate of anemia (91%), pyrexia (85%), and skin disorders (82%).[7]

In conclusion, our findings suggest that 24-week treatment with daclatasvir and asunaprevir provides a highly effective option for patients who currently have no effective treatment options (ineligible or intolerant to interferon-based therapy) and for those patients who did not achieve SVR with prior treatment.

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Contributors: HK, EH, HI, AD, and HM designed the study; EH was the medical lead. HK, YS, KI, JT, YKar, KC, YKaw, AI, KY, KT, NI, KK, TT, NK, and MK recruited patients and obtained the data. HM, HI, EH, and AD analyzed the data. TE and FM provided pharmacokinetic and resistance analyses, respectively. HK, YS, KI, JT, YKar, KC, YKaw, AI, KY, KT, NI, KK, TT, NK, MS, HM, TE, FM, AD,

HI, and EH interpreted study findings. All authors participated in writing the report.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website.

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Cancer Immunology Research

Priority Brief

Quantitative Effect of Natural Killer–Cell Licensing on Hepatocellular Carcinoma Recurrence after Curative Hepatectomy

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Abstract

Natural killer (NK) cells have a potential role in immune surveillance of hepatocellular carcinoma (HCC). Self-recognition of human leukocyte antigens (HLA) through killer immunoglobulin-like receptors (KIR) confers competence to NK cells—a process termed "licensing." We investigated the effect of NK-cell licensing on the susceptibility of patients to HCC recurrence. A total of 170 Japanese patients with HCC who underwent primary curative hepatectomy between 1996 and 2010 were enrolled in this study. The median follow-up period was 5.4 years. We analyzed their KIR-HLA genotypes with sequence-specific polymorphism-based typing and estimated their susceptibility to HCC recurrence by performing propensity score–matching analyses. The presence of KIR2DL1-C2, KIR2DL2-C1, KIR3DL1-BW4, or KIR3DL2-A3/11, functional compound genotypes that intrinsically license NK cells, did not markedly affect HCC recurrence. However, the multiplicity of those compound KIR-HLA genotypes was significantly associated with the HCC recurrence rate, i.e., the cumulative risk of recurrence in patients with at least three compound genotypes was significantly lower than that in patients with one or two compound genotypes, suggesting that the effect of NK-cell licensing on HCC recurrence is quantitative. Patients at high risk of HCC recurrence after curative hepatectomy could be identified by KIR-HLA genotyping. *Cancer Immunol Res; 2(12); 1142–7. ©2014 AACR.*

Introduction

Hepatocellular carcinoma (HCC) frequently recurs despite curative resection (1). Because augmented cytolytic activities of natural killer (NK) cells in the liver are thought to be critical for HCC immune surveillance (2, 3), functional NK-cell competence potentially affects HCC recurrence and prognosis.

NK-cell activation is dependent upon inhibitory-activating receptor equilibrium, among which killer immunoglobulin-like receptors (KIR) are the most polymorphic. KIRs contribute to receptor–ligand interactions that determine NK-cell responses by recognizing specific human leukocyte antigen (HLA) class I allotype ligands (4). Self-specific inhibitory KIR and cognate HLA ligand interactions are fundamental to "licensing" (5), a process in which NK cells expressing inhibitory KIRs for self-HLA have a higher resting response capacity (6). Ligand specificities for five inhibitory KIRs have been defined: KIR2DL1 for the HLA-C Lys80 (C2) group of alleles, KIR2DL2 and KIR2DL3 for the HLA-C Asn80 (C1) group, KIR3DL1 for the Bw4 group of HLA-B (and some A) alleles, and KIR3DL2 for the HLA-A3/11 alleles (7). The genes for KIR and their cognate HLA ligands display extensive polymorphism and generate diverse immune responses to neoplastic cells. Here, we show that the multiplicity of functional compound KIR-HLA genotypes influences posthepatectomy recurrence.

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Patients and Methods

Patients and outcomes

A total of 170 Japanese patients with HCC who underwent primary hepatectomy at Hiroshima University between 1996 and 2010 were enrolled in this study based on the following inclusion criteria: Presence of histologically confirmed HCC by an expert pathologist; preserved preoperative liver function, i.e., Child-Pugh grade A; no residual tumor after surgery; no evidence of comorbid malignant tumor; and written informed consent. None of the patients received adjuvant HCC therapy. This study was approved by the Hiroshima University Research Ethics Committee, and informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Clinicopathologic and follow-up data were collected for 5 years after primary hepatectomy. After hepatectomy, patients were followed up by using ultrasound sonography, contrast-enhanced computed tomography, or magnetic resonance,

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combined with evaluation of serum α-fetoprotein and Des-γ carboxyprothrombin levels at 3-month intervals for up to 3 years. Thereafter, follow-up was performed at 6-month intervals for up to 5 years. HCC recurrence was defined as the appearance of a new focal liver lesion with typical characteristics: lymph node enlargement in the liver hilum or suspected extrahepatic lesions. The diagnosis was histologically confirmed if necessary. Cumulative risk of recurrence was defined as the time from the surgery date to the first tumor recurrence date. Overall survival (OS) was defined as the time from the surgery date to the date of death from any cause.

KIR and HLA genotyping

Genomic DNA was extracted from peripheral blood mononuclear cells derived from patients by using a QIAamp DNA Blood Mini Kit (Qiagen). KIR allele genotyping for KIR2DL1/2DL2/2DL3/3DL1/3DL2 was performed by sequencing KIR transcripts and detected by the reverse sequence-specific polymorphism-polymerase chain reaction (SSP-PCR)–Luminex typing method using a KIR genotyping SSO kit (One Lambda). HLA-A, HLA-B, and HLA-C alleles were identified by SSP-PCR using a WAKFLow HLA typing kit (Wakunaga). The presence of the HLA ligand for KIR was determined according to HLA genotypes, as previously described (8, 9).

Statistical analysis

A comparison of categorical and continuous variables was performed using the χ^2 test and the Wilcoxon test, respectively. To adjust for differences in baseline characteristics, one-to-two or two-to-one propensity score models were constructed on the basis of each patient's estimated propensity score. Variables used included age, sex, etiology [hepatitis B virus/hepatitis C virus (HCV)/others], number of tumors (1, 2, 3, or ≥4), maximum tumor diameter (≤20 mm, >20 mm and ≤50 mm, or >50 mm), histologic type (G1 or G2/G3), vascular invasion (negative/positive), satellite lesions (negative/positive), and surgical margin (<5 mm/≥5 mm). Propensity score matching was performed using IBM SPSS Statistics 18 (SPSS Inc.) and R statistical software version R2.10.0 (R Foundation for Statistical Computing; ref. 10). One-to-two or two-to-one nearest-neighbor matching were performed using a noncaliper.

We considered the 5-year cumulative risk of recurrence as a primary outcome. Cumulative risk of recurrence and OS were estimated and compared using Kaplan–Meier and log-rank statistics. The Cox proportional hazards model was used to calculate the hazard ratio (HR) and 95% confidence intervals (CI). Statistical analyses, except propensity score matching, were performed using JMP10 for Windows (SAS Institute). *P* values of <0.05 were considered statistically significant.

Results

In this study, 170 patients with HCC who underwent curative hepatectomy were enrolled. Because preoperative liver dysfunction is a risk factor for postoperative HCC recurrence (11, 12), patients with Child-Pugh grade A were included

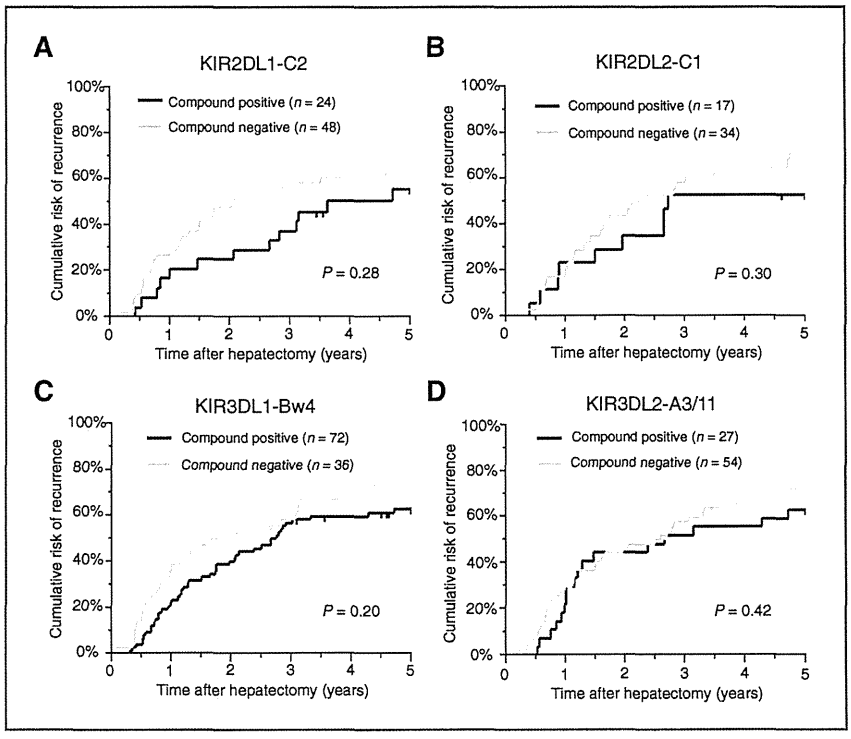

Figure 1. The presence of a single functional compound of KIR-HLA genotype did not affect HCC recurrence. The effect of each compound genotype (KIR2DL1-C2, KIR2DL2-C1, KIR3DL1-BW4, KIR3DL2-A3/11) on HCC recurrence was analyzed by propensity score–matched studies using nine variables (age, sex, etiology, tumor number, maximum diameter, histologic differentiation, vascular invasion, satellite lesion, and surgical margin). The 5-year cumulative risk of recurrence was compared between one-to-two or two-to-one patient pairs with or without each compound KIR-HLA genotype. A, KIR2DL1-C2; B, KIR2DL2-C1; C, KIR3DL1-Bw4; and D, KIR3DL2-A3/11.

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(Supplementary Table S1). The median follow-up period and median OS were 5.4 and 9.1 years, respectively.

The functional compound KIR-HLA genotypes KIR2DL1-C2, KIR2DL2-C1, KIR2DL3-C1, KIR3DL1-Bw4, and KIR3DL2-HLA A3/11, which intrinsically license NK cells, were found in 14.1%, 10.0%, 98.2%, 80.0%, and 15.9% of the cohort, respectively (Supplementary Table S2). The relatively low KIR and HLA genotype heterogeneity agreed with that previously reported (13).

We analyzed the effect of each compound genotype (KIR2DL1-C2, KIR2DL2-C1, KIR3DL1-BW4, and KIR3DL2-A3/11) on HCC recurrence. The KIR2DL3-C1 was present in nearly all patients. Propensity score–matched studies using nine variables (age, sex, etiology, tumor number, maximum diameter, histologic differentiation, vascular invasion, satellite lesion, and surgical margin), in which one-to-two or two-to-one patient pairs with or without each compound KIR-HLA genotype were created, minimized baseline characteristics bias (Supplementary Table S3). Those propensity score–matched analyses revealed that none of the compound KIR-HLA genotypes had a statistically significant effect on post-operative HCC recurrence and OS, although the recurrence rate in the group with each functional compound KIR-HLA genotype was lower than that in the group without that particular genotype (Fig. 1 and Supplementary Fig. S1).

Because NK cells expressing greater numbers of self-reactive inhibitory receptors have increased responsive potential (14), we questioned whether functional compound KIR-HLA genotype multiplicity influenced HCC recurrence. All patients had between one and four of the five functional compound KIR-HLA genotypes. Accordingly, the patients were divided into four groups for risk recurrence comparison. Compound KIR-HLA genotype multiplicity tended to be associated with the HCC recurrence rate and OS (Fig. 2A and B). In the propensity score–matched study using the same nine variables, in which one to two pairs of patients with at least three compound genotypes (the highly licensed NK group; $n = 46$) and patients with one or two compound genotypes (the poorly licensed NK group; $n = 92$) were created (Supplementary Table S4), it revealed that the cumulative recurrence risk in the highly licensed NK group was significantly lower than that in the poorly licensed NK group ($P = 0.018$; adjusted HR, 0.57; Fig. 2C). Likely because treatments against recurring HCC were persistently maintained, no statistical difference was found in OS between the two groups (Fig. 2D).

Subgroup analysis based on tumor–node–metastasis (TNM) classification (7th edition of Union for International Cancer Control) demonstrated that the difference in the cumulative risk of recurrence between the highly and poorly licensed NK groups was consistently recognized in stages I and II (Supplementary Fig. S2A–S2C). No difference was observed between the two groups in stage IIIA (Supplementary Fig. S2D), indicating that the surveillance function of NK cells is most critical in the early stages of HCC. Considering the possible effect of HCV infection on NK-cell activity, additional subgroup analyses were performed among patients with or without HCV. The lower cumulative risk of recurrence in the highly licensed NK group was statistically significant in the non–HCV-related cohort, but not in the HCV-related cohort ($P = 0.044$ and 0.17,

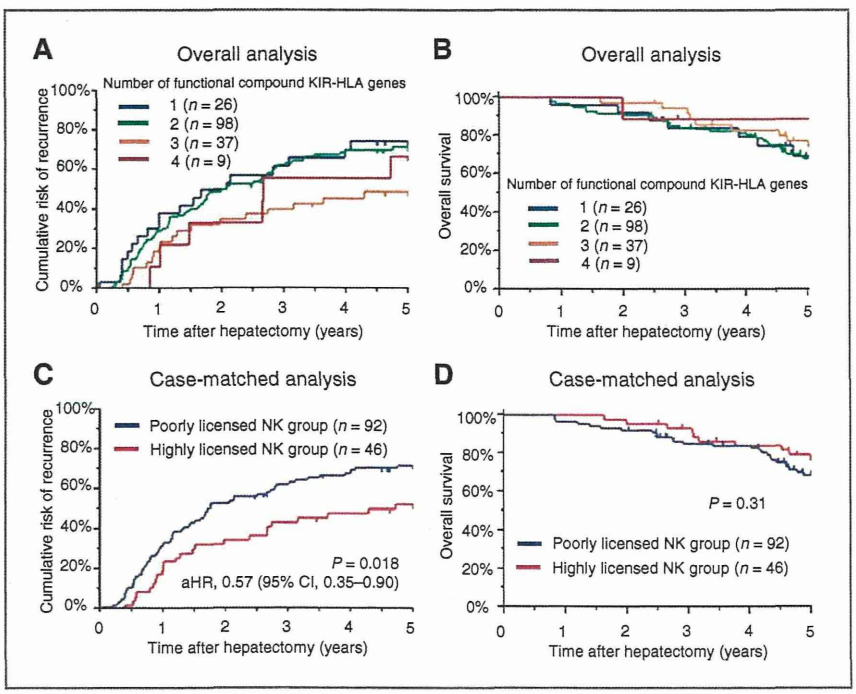

Figure 2. The multiplicity of compound KIR-HLA genotypes stratified the recurrence risk of HCC. Kaplan–Meier analyses of the 5-year cumulative risk of recurrence (A) and OS (B) for 170 patients were performed according to the number of functional compound KIR-HLA genotypes. Propensity score–matched analyses of the 5-year cumulative risk of recurrence (C) and OS (D) were also performed for patients with at least three compound genotypes (highly licensed NK group) and patients with one or two compound genotypes (poorly licensed NK group). The cumulative risk of recurrence in the highly licensed NK group was significantly lower than in the poorly licensed NK group. aHR, adjusted HR.

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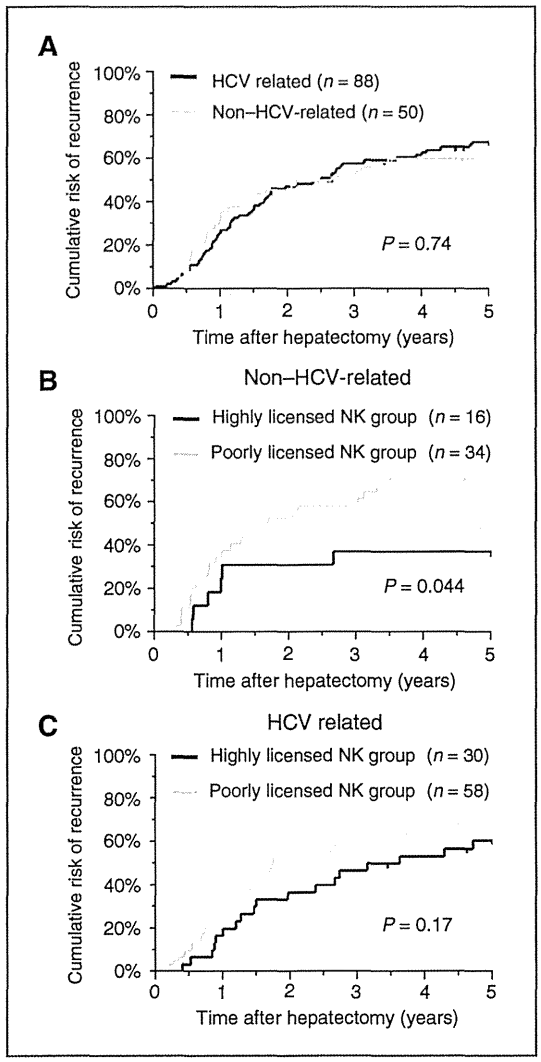

Figure 3. The lower cumulative risk of recurrence in the highly licensed NK group was significant among the non–HCV-related cohort. A, according to the presence or absence of HCV infection, Kaplan–Meier analyses of the 5-year cumulative risk of recurrence were performed for 138 matched patients belonging to the highly and poorly licensed NK groups. Further subgroup analyses were performed in non–HCV-related patients (B) and HCV-related patients (C).

respectively; Fig. 3). Consistently, on the log-rank and the Cox proportional hazards model analyses, the number of KIR-HLA genotype ($\leq$2) was defined as a significant risk factor for HCC recurrence in an unadjusted overall cohort, but was not so in the HCV-related cohort (Table 1).

Discussion

Postoperative recurrent HCC can be monocentric, leading to intrahepatic metastasis, or multicentric as a *de novo* carcinogenesis. To study the role of NK cells in intrahepatic metastasis, we previously investigated the effect of decreasing NK-cell functions on the engraftment susceptibility of intraportally injected HCC cells in a mouse model (15, 16). The anti-HCC activity of hepatic NK cells significantly decreased after partial hepatectomy, allowing intrahepatic metastasis growth in mice receiving HCC cells (16). Intravenous adoptive immunotherapy performed using activated NK cells extracted from normal livers markedly inhibited intrahepatic metastasis. NK-cell competence and ability to survey and eliminate *de novo* neoplastic cells may provide defense against both monocentric and multicentric recurrence.

The human liver contains an unusually high number of infiltrating immune cells; 30%–50% of lymphocytes are NK cells (2). Liver NK cells have unique properties, including TNF-related apoptosis-inducing ligand (TRAIL)–dependent cytotoxicity, high NKp46 and CD122 expression, and specific cytokine profiles (2, 3). TRAIL on NK cells binds to four receptors, including death-inducing receptors (DR4 and DR5) that signal apoptosis and decoy receptors (DcR1 and DcR2; refs. 17). Moderately/poorly differentiated HCC remarkably expresses DR4/DR5 but not DcR1/DcR2, increasing TRAIL-expressing NK cell–mediated cell killing susceptibility (2, 18). On the basis of those findings, we proposed a novel immunotherapy of intravenously injecting activated liver allograft–derived NK cells into liver transplant recipients to control HCC recurrence (19).

In addition to TRAIL, hepatic NK-cell roles in immune tumor surveillance are likely mediated by perforin, granzyme, and interferon-γ (20). Gene polymorphisms for KIR and its HLA ligands possibly contribute to the heterogeneous tumor-surveillance functions of NK cells and likely affect clinical HCC outcomes. Recently, a small cohort study of patients with HCV-related HCC who underwent curative treatment by either surgical resection or radiofrequency thermal ablation (RTA) showed that the compound KIR2DL2-C1 and KIR3DS1-Bw4T80 genotypes are associated with longer time to recurrence and worse OS, respectively (21). We also analyzed the impact of these genotypes in the present study, but did not observe consistent results (Table 1 and Supplementary Table S5). This discrepancy might be related to the fact that the time to recurrence was markedly longer in our study than that in the previous study (median time to recurrence = 29.7 vs. 17 months, respectively), which is likely due to the heterogeneity of the therapeutic modality used in the previous study (i.e., time to recurrence in patients treated with RTA was significantly shorter than that in patients treated by resection; ref. 21). Our propensity score–matched studies demonstrated that the presence of a single functional compound KIR-HLA genotype did not markedly affect HCC recurrence, but that compound KIR-HLA genotype multiplicity was associated with the HCC recurrence rate. Taken together, with this finding and the fact that the number and type of host MHC class I alleles quantitatively tune the responsiveness of individual NK-cell subsets expressing the corresponding KIR (14, 22), the effect of NK-cell licensing on HCC recurrence should be quantitative. This effect of NK-cell licensing on HCC recurrence reached statistical significance in the non–HCV-related cohort but not in the

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Table 1. Cumulative risk of recurrence and overall survival of patients with HCC according to clinicopathologic characteristics and compound KIR-HLA genotypes

	Total patients ($N = 170$)				HCV-related patients ($n = 97$)			
	Cumulative risk of recurrence		OS		Cumulative risk of recurrence		OS	
	P	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)
Age (≤65 vs. >65 years)	0.745	NA	0.362	NA	0.626	NA	0.592	NA
Sex (male vs. female)	0.078	1.54 (0.99–2.53)	0.966	NA	0.554	NA	0.216	NA
Etiology (HBV vs. HCV vs. others)	0.448	NA	0.117	NA	NA	NA	NA	NA
Tumor number (≥2 vs. 1)	0.010	1.57 (1.06–2.31)	0.039	1.64 (1.11– 2.39)	0.088	1.56 (0.92–2.51)	0.604	NA
Maximum diameter (≤50 mm vs. >50 mm)	0.222	NA	0.511	NA	0.749	NA	0.313	NA
Histologic differentiation (G1+G2 vs. G3)	0.253	NA	0.498	NA	0.866	NA	0.909	NA
Vascular invasion	0.733	NA	0.851	NA	0.612	NA	0.454	NA
Satellite lesion	0.657	NA	0.743	NA	0.451	NA	0.486	NA
Surgical margin (<5 mm vs. ≥5 mm)	0.126	NA	0.162	NA	0.039	1.88 (0.98–3.36)	0.003	1.88 (0.97 – 3.37)
KIR2DL1-C2	0.113	NA	0.754	NA	0.189	NA	0.964	NA
KIR2DL2-C1	0.253	NA	0.945	NA	0.456	NA	0.745	NA
KIR2DL3-C1	0.793	NA	0.283	NA	0.694	NA	0.105	NA
KIR3DL1-Bw4	0.269	NA	0.359	NA	0.572	NA	0.935	NA
KIR3DL2-A3/11	0.585	NA	0.570	NA	0.845	NA	0.986	NA
Number of KIR-HLA genotypes (≥3 vs. ≤2)	0.016	0.61 (0.38–0.94)	0.224	NA	0.130	NA	0.735	NA

NOTE: Cumulative risk of recurrence and OS were compared by log-rank statistics for univariate analysis. Cox proportional hazards model was conducted for multivariate survival analysis. Only variables presenting $P < 0.1$ in the univariate analysis were included in the multivariate model. $P < 0.05$ was considered statistically significant.
Abbreviation: NA, not assessed.

HCV-related cohort, which might be explained by the fact that hepatic NK cells exhibited reduced cytotoxicity and TRAIL expression in patients with chronic HCV infection (23).

We demonstrated that patients at high risk of HCC recurrence after curative hepatectomy could be identified by KIR-HLA genotyping. Licensed NK cells generally have higher resting capacity for responses including interferon-γ production and cytotoxicity than unlicensed NK cells, but both NK-cell types are highly activated by *in vitro* stimuli (24). Therefore, therapeutic strategies manipulating NK-cell activity either *in vivo* or *in vitro* could compensate for genetic susceptibility to HCC recurrence. This concept might also be supported by a previous randomized trial demonstrating that adoptive immunotherapy with autologous lymphocytes activated *in vitro* with recombinant IL2 and anti-CD3 Abs decreased the frequency of recurrence after HCC curative resection (25).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: N. Tanimine, Y. Tanaka, H. Ohdan
Development of methodology: N. Tanimine, Y. Tanaka
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Tanimine, T. Kobayashi, M. Imamura, H. Aikata, H. Ohdan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Tanimine, J. Tanaka, H. Ohdan
Writing, review, and/or revision of the manuscript: N. Tanimine, K. Chayama, H. Ohdan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Tanimine, D. Miki
Study supervision: H. Tashiro, J. Tanaka, H. Ohdan

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Cancer Immunology Research

Quantitative Effect of Natural Killer–Cell Licensing on Hepatocellular Carcinoma Recurrence after Curative Hepatectomy

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Emergence of resistant variants detected by ultra-deep sequencing after asunaprevir and daclatasvir combination therapy in patients infected with hepatitis C virus genotype 1

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SUMMARY. Daclatasvir (DCV) and asunaprevir (ASV) are NS5A and NS3 protease-targeted antivirals respectively, currently under development for the treatment of chronic hepatitis C virus (HCV) infection. We analysed the relationship between pre-existing drug-resistant variants and clinical outcome of the combination treatment with DCV and ASV. Ten patients with HCV genotype 1b were orally treated with a combination of ASV and DCV for 24 weeks. The frequencies of amino acid (aa) variants at NS3 aa positions 155, 156 and 168 and at NS5A aa31 and 93 before and after treatment were analysed by ultra-deep sequencing. We established a minimum variant frequency threshold of 0.3% based on plasmid sequencing. Sustained virological response (SVR) was achieved in 8 out of 10 patients (80%), and relapse of HCV RNA after cessation of the treatment and viral breakthrough occurred in the other two patients. Pre-existing DCV-resistant variants (L31V/M and/or Y93H; 0.9–99.4%) were detected in three out of eight patients who achieved SVR. Pre-existing DCV-resistant variants were detected in a relapsed patient (L31M, Y93H) and in a patient with viral breakthrough (Y93H); however, no ASV-resistant variants were detected. In these patients, HCV RNA rebounded with ASV- and DCV- double resistant variants (NS3 D168A/V plus NS5A L31M and Y93H). While pre-existing DCV-resistant variants might contribute to viral breakthrough in DCV and ASV combination therapy, the effectiveness of prediction of the outcome of therapy based on ultra-deep sequence analysis of pre-existing resistant variants appears limited.

Keywords: antiviral resistance, asunaprevir, combination treatment, daclatasvir, deep sequencing.

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, such as cirrhosis and hepatocellular carcinoma [1,2]. A number of direct acting antivirals (DAAs) are currently under development. Telaprevir (TVR) has been approved for clinical use in several countries and has shown promising results when combined with peg-interferon (PEG-IFN) and ribavirin (RBV) [3]. However, this combination therapy has poor therapeutic effect in null responders, in which the sustained virological response (SVR) rate remains low at 37% in patients with HCV genotype 1b [4]. Moreover, PEG-IFN and RBV are associated with frequent side effects [5,6], and the addition of TVR results in elevated rates of anaemia and additional adverse events such as rash, pruritus and renal dysfunction [7–10].

Daclatasvir (DCV) and asunaprevir (ASV) are currently undergoing clinical development for treatment of HCV infection. DCV (BMS-790052) is a first-in-class, highly selective NS5A replication complex inhibitor with picomolar potency and broad genotypic coverage [11]. NS5A is an RNA binding multi-functional viral protein and is essential for viral proliferation by interacting with other HCV NS proteins and cellular proteins [12]. ASV (BMS-650032) is a selective inhibitor of NS3 protease with antiviral activity *in vitro* against genotypes 1 and 4 [13].

Abbreviations: aa, amino acid; ASV, asunaprevir; DAA, direct-acting antiviral agent; DCV, daclatasvir; HCV, hepatitis C virus; PEG-IFN, peg-interferon; RBV, ribavirin; SVR, sustained virological response; TVR, telaprevir; WT, wild type.

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Combinations of two DAAs may overcome interferon nonresponsiveness in null responders by increasing antiviral activity and reducing the risk of developing resistance-associated variants [14]. One recent PEG-IFN and RBV-sparing study of DCV plus ASV (AI447017) has examined the efficacy and safety of this combination for 24 weeks in a small cohort of ten genotype 1b null responders, in whom an SVR rate of 90% was observed [15]. The study was then expanded to include an additional cohort of null responders and a group of patients ineligible to receive, or intolerant of, PEG-IFN and RBV [16]. As with other antiviral agents, the efficacy of DCV and ASV can be compromised by the development of drug resistance. In this study, there were three viral breakthroughs and four relapsers out of 43 patients. Karino *et al.* [17] reported on the relationship between pre-existing drug-resistant variants by direct sequencing analysis and clinical antiviral responses to DCV and ASV combination treatment.

Recently, deep sequencing has been employed as a useful tool in the detection of viral variants and determining the mutational rate without cloning [18–21]. In this study, ultra-deep sequencing was performed using sera from 10 Japanese HCV genotype 1b patients who participated in a clinical phase 2a trial using ASV and DCV to analyse the relationship between the pre-existence of minor populations of ASV- and DCV-resistant variants and clinical antiviral responses.

MATERIALS AND METHODS

Study design

This study is a phase 2a clinical trial (clinicaltrials.gov identifier NCT01051414) to evaluate the antiviral activity and safety of DCV plus ASV against HCV genotype 1 in treatment-naïve patients and nonresponders to prior PEG-IFN and RBV combination therapy. Written informed consent was obtained from all patients. The study was approved by institutional review boards at each site and conducted in compliance with the Declaration of Helsinki, Good Clinical Practice Guidelines, and local regulatory requirements.

Patients

Ten patients who met the following inclusion and exclusion criteria participated in the clinical trial. Inclusion criteria for this clinical trial were as follows. (i) Patient age was between 20 to 75 years. (ii) Patients were infected with HCV genotype 1 for at least 6 months, and serum HCV-RNA level was more than 5 log IU/mL. (iii) Eligible patients had no evidence of cirrhosis as diagnosed by laparoscopy, imaging or liver biopsy within 2 years. (iv) Eligible patients consisted of two groups: a) treatment-naïve patients with no history of anti-HCV therapy, including interferon therapy; and b) nonresponders who failed to achieve a 2 log copy/mL of HCV-RNA decrease in prior IFN therapy lasting 12 weeks or longer. (v) Patients have no history of hepatocellular carcinoma, co-infection with hepatitis B virus or human immunodeficiency virus, other chronic liver disease, or evidence of hepatic decompensation. (vi) Patients were also excluded if they had other severe or unstable conditions or evidence of organ dysfunction in excess of that consistent with the age of the patient, were unable to tolerate interferon and oral medication or had conditions that could impact absorption of the study drug, or were exposed to any investigational drug within 4 weeks of study participation or had any previous exposure to inhibitors of NS5A. (vii) Laboratory findings that excluded participation were alanine aminotransferase >5 times the upper limit of normal (×ULN); total bilirubin ≥2 mg/dL; direct bilirubin >1.5 ×ULN; international normalized ratio of prothrombin time ≥1.7; albumin ≤3.5 g/dL; haemoglobin <9.0 g/dL; white blood cells <1500/mm^3; absolute neutrophil count <750/mm^3; platelets <50 000/mm^3; or creatinine >1.8 ×ULN.

Treatment protocol

All patients received combination therapy with DCV plus ASV for 24 weeks. Patients received 24 weeks of treatment with DCV 60 mg once daily (two 30 mg tablets), combined with ASV 200 mg twice daily, with 24 weeks of post-treatment follow-up. In the sentinel cohort of null responders, ASV was initially administered as three 200 mg tablets twice daily (600 mg BID), subsequently reduced to 200 mg BID during treatment following reports from another study of greater and more frequent aminotransferase elevations with the higher dose [22].

Determination of amino acid sequences in the HCV core region

Substitution at aa70 in the HCV core region was analysed by direct sequencing, as described previously [23]. Briefly, HCV RNA was extracted from 100 μl of stored serum samples by SepaGene RV-R (Sanko Junyaku Co., Ltd, Tokyo, Japan), and reverse transcription (RT) was performed with random primer (Takara Bio, Shiga, Japan) and M-MLV reverse transcriptase (Takara Shuzo, Tokyo, Japan). Then, the HCV core region was amplified using converted cDNA by nested PCR, and direct sequencing analysis was performed using an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Arginine was considered wild type for aa70 in the core region, and other amino acids were considered mutant type.

Determination of HCV and IL28B genotypes

IL28B SNP genotype (rs8099917) was determined using TaqMan Pre-Designed SNP Genotyping Assays as described previously [24].

Assessment of virological responses

Serum was collected at baseline and at fixed time points: Weeks 1, 2, 4, 6, 8, 12 and then every 4 weeks on-treatment. HCV RNA was determined at a central laboratory using the Roche COBAS TaqMan HCV Auto assay (Roche Diagnostics KK, Tokyo, Japan; LLOQ, 15 IU/mL). SVR occurred if HCV RNA became continuously undetectable by qualitative PCR assay for 24 weeks after the end of treatment. Viral breakthrough was defined as a confirmed ≥1 log IU/ml increase from nadir of HCV RNA, or HCV RNA ≥15 IU/ml after having been confirmed as undetectable during treatment. Post-treatment relapse was defined as confirmed HCV RNA ≥15 IU/ml during follow-up in patients with undetectable HCV RNA at the end of treatment.

Detection of drug-resistant substitutions by ultra-deep sequencing

Hepatitis C virus RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), and cDNA synthesis was performed using a random primer and M-MLV reverse transcriptase. Briefly, the NS3 and NS5A region in the HCV genome was amplified by nested PCR and the fragment distributions were assessed using the Agilent BioAnalyzer 2100 platform. The amplified fragments were modified by the Multiplexing Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequence analysis was performed by Illumina Genome Analyzer. Imaging analysis and base calling were performed using Illumina Pipeline software with default settings as in our previous report [25]. The N-terminal domain of NS3, which includes R155, A156 and D168, and NS5A, which includes L31 and Y93, were analysed. This technique revealed an average coverage depth of over 1000 sequence reads per base pair in the unique regions of the genome. Read mapping to the HCV-KT9 reference sequence was performed using BWA [26]. Because of the short 36 nucleotide read length, hypervariable regions with multiple closely spaced variants could prevent reads from mapping to the reference sequence. Therefore, unmapped reads were examined and alternative reference sequences were included based on direct sequencing to improve coverage in variable regions. Codon frequencies were calculated using a haplotype-aware custom walker.

RESULTS

Characteristics of patients and treatment efficacy

Baseline characteristics of the 10 patients are shown in Table 1. Five of the patients were prior nonresponders (Cases 1–5), and the other 5 patients were treatment-naïve (Cases 6–10). To compare dosing effects of ASV, two patients (Cases 1 and 2) were administered 1200 mg/day and the remaining eight patients were administered 400 mg/day of ASV. As shown in Table 1, subjects included two males and eight females with a median age of 62. All subjects were infected with HCV genotype 1b. The *IL28B* rs8099917 genotype was TT in four patients and TG in six patients, including two patients who were nonresponders to previous PEG-IFN plus RBV combination therapy. Substitutions at aa70 in the HCV core region were found in six patients. SVR was achieved in 8 of 10 patients (80%), whereas HCV RNA relapsed in a patient after cessation of the treatment (Case 9), and viral breakthrough occurred in one patient (Case 10). The SVR ratio was not associated with either *IL28B* genotype (TT: 75%, TG: 83%) nor Core70 type (Wild type: 75%, Mutant type: 83%).

Table 1 Clinical characteristics of 10 patients with chronic hepatitis C virus (HCV) genotype 1b infection treated with asunaprevir (ASV) and daclatasvir (DCV) combination therapy for 24 weeks

Case	Age (years)	Sex	Prior IFN treatment	*IL28B*	HCV RNA (log copy/ml)	Core aa70	ASV (mg/day)	DCV (mg/day)	Efficacy
1	63	F	Naive	TG	7.1	Mutant	1200	60	SVR
2	59	F	Naive	TG	6.4	Mutant	1200	60	SVR
3	58	F	Naïve	TG	6.6	Mutant	400	60	SVR
4	67	F	Naïve	TG	7.0	Wild	400	60	SVR
5	48	M	Naïve	TT	5.7	Mutant	400	60	SVR
6	75	F	NR	TG	6.7	Mutant	400	60	SVR
7	70	F	NR	TT	6.1	Wild	400	60	SVR
8	61	M	NR	TT	6.5	Wild	400	60	SVR
9	75	F	NR	TT	6.8	Wild	400	60	Relapse
10	48	F	NR	TG	7.0	Mutant	400	60	Breakthrough

IFN, interferon; *IL28B*, rs8099917 genotype; NR, nonresponder to prior peg-interferon plus ribavirin therapy; SVR, sustained virological response; Core aa70, presence of wild type or mutant amino acid at position 70 of the HCV core protein.

Detection of drug-resistant hepatitis C virus variants prior to therapy

We conducted ultra-deep sequencing analysis for these 10 patients prior to therapy to determine whether or not HCV strains with naturally occurring DCV- and ASV- resistant variants were present prior to exposure. We obtained between 11 575 and 2 711 250 total reads for each patient. To estimate the error rate due to PCR errors and limitations of the sequencing platform, we sequenced an HCV-expressing plasmid as a control. Nucleotide substitutions by position varied from 0.09 to 0.14 with a median of 0.08 (Table S1). The nucleotide substitution rate was not correlated with depth of coverage (Fig. S1). To determine a threshold for detecting rare variants, we compared the frequency of synonymous and non synonymous substitutions at each codon position using a haplotype-aware custom walker and selected a minimum frequency threshold of 0.3% (Tables 2, S1 and S2). Although a candidate A156V variant was found within the error threshold in Case 2, no ASV-resistant variants with a frequency above 0.3% were detected (Tables 3, S3 and S4). In the NS5A region, DCV-resistant variants were detected in five patients: L31V/M in Cases 3 and 9, and Y93H in Cases 1, 3, 7, 9 and 10 (Tables 4, S3 and S4).

Table 2 Determination of the minimum variant frequency based on ultra-deep sequencing of NS3 amino acid 155/156/168 and NS5A amino acid 31/93 determined by sequencing a wild type hepatitis C virus-expressing plasmid as a control. The per-nucleotide substitution rate for these positions ranged from 0.09 to 0.14 with a median of 0.08. To account for PCR and sequencing errors, the minimum threshold for detecting an amino acid substitution was set at 0.3% of the aligned reads for that position based on analysis of nonsynonymous substitutions. This value is above the 0.23% NS3 aa156 and the 0.18% NS3 aa155 nonsynonymous substitution rate and well above the substitution rates for NS3 aa168 and NS5A aa31 and aa93, which were each <0.1

Position	Aligned reads	Frequency (%)	Nonsynonymous substitution rate, %
NS3 aa155	1 405 486	R (98.82), L (0.09), W/Q/P/G/H (0.11)	0.18
NS3 aa156	1 301 281	A (99.77), V (0.11), T/D/S/P/G (0.12)	0.23
NS3 aa168	4 053 122	D (99.91), G (0.03), V/Y/N/E/A (0.06)	0.09
NS5A aa31	1 571 297	L (99.97), S (0.02), F/I/V (0.01)	0.03
NS5A aa93	723 377	Y (99.94), H (0.02), C/F/S/N/D (0.05)	0.06

Substituted amino acids are shown by standard single-letter codes; Aligned reads: the number of reads that overlap the given codon position in the reference alignment; WT, wild type.

Table 3 Ultra-deep sequence analysis of NS3 amino acid 155, 156 and 168 in 10 patients prior to the start of asunaprevir plus daclatasvir combination therapy

	aa155			aa156			aa168		
Case	Aligned reads	WT (R) (%)	Variant (%)	Aligned reads	WT (A) (%)	Variant (%)	Aligned reads	WT (D) (%)	Variant (%)
1	264 515	99.9	–	272 154	99.9	–	711 021	100	–
2	420 927	99.9	–	411 290	99.8	–	1 077 727	99.9	–
3	50 236	100	–	58 614	100	–	580 892	99.9	–
4	147 312	99.7		146 496	99.9	–	554 264	99.9	–
5	403 055	99.8	–	402 456	99.9	–	576 881	99.9	–
6	357 934	100	–	363 838	100	–	861 707	99.9	–
7	37 512	100	–	29 481	100	–	575 327	99.9	–
8	24 352	100	–	12 868	100	–	599 928	99.9	–
9	29 925	100	–	11 575	100	–	807 927	100	–
10	48 605	99.9	–	36 712	99.9	–	446 494	100	–

Substituted amino acids are shown by standard single-letter codes. Dashes indicate amino acid substitutions with a frequency less than 0.3% of the aligned reads at that position; Aligned reads: the number of reads that overlap the given codon position in the reference alignment; WT, wild type.

Virological response

Between weeks 1 and 8 of treatment, serum HCV-RNA titres decreased below the limit of detection in all 10 patients. HCV-RNA titres in 9 of the 10 patients remained undetectable until the end of the treatment, and eight out of nine patients achieved SVR (Fig. 1). In one patient (Case 9), serum HCV-RNA titre rebounded at week 36 (12 weeks after cessation of the treatment) and returned to pretreatment levels. In this case, ultra-deep sequencing showed that 100% of the total reads were the wild type sequence at NS3 aa168, indicating that this amino acid was completely replaced with the ASV-resistant variant D168A by week 40 (16 weeks after cessation of the treatment) (Fig. 2a). 99.1% and 67.5% of the aligned reads showed wild type sequences in NS5A aa31 and 93 before treatment, respectively. These aa were predominantly replaced by DCV-resistant variants; 99.1% of L31M and 96.6% of Y93H.

In Case 10, serum HCV-RNA titre decreased below the detectable limit at week 2 of treatment. However, serum HCV-RNA titre rebounded at week 10, and the treatment was stopped at week 16 (Fig. 1). In this case, ASV-resistant variants were not detected prior to treatment; however, NS3 D168V was enriched at week 10 and increased to 45.7% at week 16 (Fig. 2b). In this case, 99.4% of NS5A Y93H was detected before treatment, and the variants persisted at high frequency during the course of therapy. The L31M variant was not detected before treatment but was detected in up to 99.6% of the sequences at week 10. By week 32 (16 weeks after cessation of the treatment), NS3 aa168 had been completely replaced by wild type, whereas NS5A aa31 was completely replaced by the DCV-resistant variant.

DISCUSSION

Drug resistance has been shown to emerge with different classes of DAA regimens. However, the reason why treatment fails in some patients remains unclear. Amino acid substitutions in HCV related proteins that confer resistance

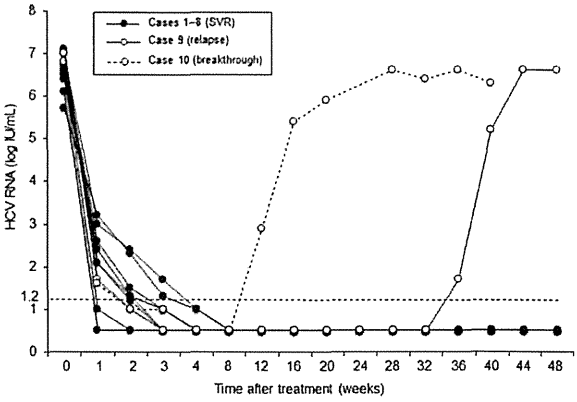

Fig. 1 Hepatitis C virus (HCV) RNA levels over time for patients treated with daclatasvir and asunaprevir. Serum HCV RNA titres decreased below the detectable limit 4 weeks after the beginning of treatment in all patients. Serum HCV RNA rebounded at 12 weeks after cessation of the treatment in Case 9 (relapse) and at week 12 of the treatment in Case 10 (breakthrough).

Table 4 Ultra-deep sequence analysis of NS5A amino acid 31 and 93 in 10 patients prior to the start of the combination therapy with asunaprevir and daclatasvir

	aa31			aa93		
Case	Aligned reads	WT (L) (%)	Variant (%)	Aligned reads	WT (Y) (%)	Variant (%)
1	821 229	99.9	–	75 800	28.7	H (71.2)
2	851 400	100	–	217 011	99.7	–
3	1 137 359	99.0	V (1.0)	175 520	0.5	H (99.4)
4	80 535	100	–	451 140	99.8	–
5	2 711 250	99.9	–	469 945	99.8	–
6	757 927	99.9	–	198 881	99.8	–
7	790 076	99.9	–	456 161	99.1	H (0.9)
8	670 404	99.9	–	527 879	99.9	–
9	588 331	99.1	M (0.7)	300 666	67.5	H (32.4)
10	1 436 741	99.7	–	451 138	0.6	H (99.4)

Substituted amino acids are shown by standard single-letter codes. Dashes indicate amino acid substitutions with a frequency less than 0.3% of the aligned reads at that position; Aligned reads: the number of reads that overlap the given codon position in the reference alignment; WT, wild type.