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Genotypic and Phenotypic Analyses of Hepatitis C Virus from Patients Treated with JTK-853 in a Three-Day Monotherapy

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JTK-853, a palm site-binding NS5B nonnucleoside polymerase inhibitor, shows antiviral activity *in vitro* and in hepatitis C virus (HCV)-infected patients. Here, we report the results of genotypic and phenotypic analyses of resistant variants in 24 HCV genotype 1-infected patients who received JTK-853 (800, 1,200, or 1,600 mg twice daily or 1,200 mg three times daily) in a 3-day monotherapy. Viral resistance in NS5B was investigated using HCV RNA isolated from serum specimens from the patients. At the end of treatment (EOT) with JTK-853, the amino acid substitutions M414T (methionine [M] in position 414 at baseline was replaced with threonine [T] at EOT), C445R (cysteine [C] in position 445 at baseline was replaced with arginine [R] at EOT), Y448C/H (tyrosine [Y] in position 448 at baseline was replaced with cysteine [C] or histidine [H] at EOT), and L466F (leucine [L] in position 466 at baseline was replaced with phenylalanine [F] at EOT), which are known to be typical resistant variants of nonnucleoside polymerase inhibitors, were observed in a clonal sequencing analysis. These substitutions were also selected by a treatment with JTK-853 *in vitro*, and the 50% effective concentration of JTK-853 in the M414T-, C445F-, Y448H-, and L466V-harboring replicons attenuated the susceptibility by 44-, 5-, 6-, and 21-fold, respectively, compared with that in the wild-type replicon (Con1). These findings suggest that amino acid substitutions of M414T, C445R, Y448C/H, and L466F are thought to be viral resistance mutations in HCV-infected patients receiving JTK-853 in a 3-day monotherapy.

Hepatitis C virus (HCV) is the major cause of posttransfusion non-A non-B hepatitis. Approximately 130 million individuals worldwide are estimated to be infected with HCV (1). After the launch of direct-acting antivirals (e.g., telaprevir and boceprevir) for chronic hepatitis C in 2011, the achievement of a sustained virological response apparently increased (2–6). However, the treatment outcome needs to be further improved, and moreover, the emergence of drug-resistant HCV variants is inevitable for direct-acting antiviral-based therapies. Therefore, a new direct-acting antiviral showing a high genetic barrier to resistance development, in addition to potent antiviral activity, is strongly required for the treatment of chronic hepatitis C.

The NS5B protein is a viral RNA-dependent RNA polymerase that is responsible for the replication of HCV (7, 8). Owing to its apparent sequence and structural differences from human DNA and RNA polymerases, this HCV RNA polymerase is an attractive target for antiviral drugs. To date, a variety of nucleoside and nonnucleoside polymerase inhibitors have been reported. Nonnucleoside polymerase inhibitors interact with four distinct allosteric sites on HCV polymerase (9). We previously reported the discovery of thumb pocket-binding nonnucleoside HCV polymerase inhibitors with a benzimidazole and indole core (10–13).

JTK-853 is a novel selective palm site-binding nonnucleoside polymerase inhibitor that was discovered by high-throughput screening (14). JTK-853 shows inhibitory activity toward the replication of HCV replicons, and its activity remains unchanged even in the presence of human serum or human serum proteins. An *in vitro* resistance selection study identified C316Y, M414T, Y452H, and L466V to be the predominant mutations conferring resistance to JTK-853. Structural analyses demonstrated that JTK-853 associates with the palm I site of HCV polymerase (14, 15). Interestingly, its association was clearly distinct from that of other HCV polymerase palm site inhibitors because it bound to the β hairpin of the HCV polymerase. Furthermore, JTK-853 reduced the viral load of HCV genotype 1-infected patients by more than 1.0 $\log_{10}$ unit in a 3-day monotherapy. In addition, JTK-853 was well tolerated, with no reported discontinuations, serious adverse events, or deaths (16).

Here, we report the characterization of JTK-853-resistant *NS5B* variants in 24 HCV genotype 1-infected patients. Serum specimens were collected from the patients at the baseline (BL) and end of treatment (EOT) with JTK-853 and during the follow-up (FU) period with peginterferon alfa-2a (PEG-IFN) and ribavirin (RBV) treatments and subjected to genotypic analysis for *NS5B* and phenotypic analysis for JTK-853 susceptibility.

MATERIALS AND METHODS

Compound. The nonnucleoside polymerase inhibitor JTK-853 (patent WO 2007119889) was synthesized at Japan Tobacco Inc., Central Pharmaceutical Research Institute (Osaka, Japan) (14).

Patient population and study design. Twenty-eight patients were enrolled in a randomized, double-blind, placebo-controlled, 3-day, multiple-dose, phase 1b trial. All the patients had chronic infection with HCV genotype 1a or 1b with serum HCV RNA levels of ≥50,000 IU/ml and were negative for hepatitis B surface antigen and antibodies to hepatitis A virus and human immunodeficiency virus. The treatment regimens for

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the patients were as follows: 4 received placebo, 6 received JTK-853 at 800 mg twice daily (BID), 6 received JTK-853 at 1,200 mg BID, 6 received JTK-853 at 1,600 mg BID, and 6 received JTK-853 at 1,200 mg three times daily (TID). All the patients provided informed consent, and the study was approved by the Ethics Committee of the Central Pharmaceutical Research Institute of Japan Tobacco Inc. and the Institutional Review Board at Fundación de Investigación (FDI), Puerto Rico.

In this clinical study, JTK-853 effectively reduced the viral load in patients infected with genotype 1 HCV (16). Briefly, the mean reductions of the HCV RNA load were 0.9 $\log_{10}$ unit at 800 mg BID, 1.2 $\log_{10}$ units at 1,200 mg BID, 1.3 $\log_{10}$ units at 1,600 mg BID, and 0.9 $\log_{10}$ unit at 1,200 mg TID. Following the JTK-853 treatment as a 3-day monotherapy, the patients were offered a standard-of-care treatment, i.e., Pegasys (PEG-IFN) at 180 μg/week via a subcutaneous injection, along with Copegus (RBV; doses were dependent on body weight: 1,000 mg/day for subjects weighing ≤75 kg and 1,200 mg/day for subjects weighing >75 kg), for up to 48 weeks.

For the genotypic and phenotypic analyses described in this report, serum specimens were collected from the patients and stored at −70°C until use. The serum collection points were as follows: at BL (day 1) before dosing, at EOT (day 4), and during the FU period at weeks 4, 12, 24, and 48 (days 28, 84, 168, and 336, respectively) after withdrawal of JTK-853. Quantitative measurements of serum HCV RNA were performed with a Cobas TaqMan HCV test (Roche Diagnostics, Indianapolis, IN) on days 1, 4, 28, 84, 168, and 336. The lower limit of detection was defined to be 15 IU/ml.

Population sequencing analysis. Prior to conducting the genotypic analysis, the assay was validated in terms of accuracy, precision, reproducibility, amplification sensitivity, minor species detection, linearity, and specificity as described elsewhere (17).

Total RNA was extracted from the serum samples using a SepaGene RV-R device kit (EIDIA, Tokyo, Japan) or a High Pure viral RNA kit (Roche, Basel, Switzerland) according to each manufacturer's instructions. HCV cDNA was reverse transcribed from the total RNA using a SuperScript III first-strand synthesis system (Invitrogen, Carlsbad, CA). The *NS5B* gene was amplified from the HCV cDNA by nested PCR with genotype-specific primers (see Table S1 in the supplemental material). The first PCRs were performed using Blend *Taq*-Plus polymerase (Toyobo, Osaka, Japan) or LA *Taq* polymerase (TaKaRa Bio, Shiga, Japan). The first PCRs consisted of incubation at 94°C for 2 min, followed by 30 cycles of 98°C for 10 s, 55°C for 30 s, and 72°C for 2.5 min, or incubation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2.5 min. The second PCRs were performed using *KOD* FX polymerase (Toyobo) or LA *Taq* polymerase. The second PCRs consisted of incubation at 94°C for 2 min, followed by 40 cycles of 98°C for 10 s, 55°C for 30 s, and 68°C for 2 min, or incubation at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. The nucleotide sequence of *NS5B* was then determined using a BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3100 genetic analyzer (Applied Biosystems). The amino acid sequences were deduced from the nucleotide sequences using Vector NTI software (Invitrogen). The nucleotide and amino acid sequences at the EOT were compared with the corresponding nucleotide and amino acid sequences at the BL.

Clonal sequencing analysis. The amplified NS5B products were ligated into the pCR Blunt II TOPO vector using a Zero Blunt TOPO PCR cloning kit (Invitrogen) or an HCV replicon vector (18, 19) using an In-Fusion Advantage PCR cloning kit (Clontech, Mountain View, CA) according to each manufacturer's instructions. The subsequent procedures were performed as previously described (14). The *NS5B* genes in more than 10 clones for each patient were examined. The nucleotide and deduced amino acid sequences were compared with those of a reference strain of genotype 1a (H77; GenBank accession number AF009606) or genotype 1b (Con1; GenBank accession number AJ238799).

Phenotypic analysis. Prior to conducting the phenotypic analysis, the assay was validated in terms of accuracy, precision, reproducibility, assay modification, and virological cutoff as described elsewhere (17).

Among the *NS5B* sequences in more than 10 clones for each patient, the most dominant *NS5B* sequence selected from the clonal sequencing for each patient was employed to perform the phenotypic analysis. To construct each patient's *NS5B*-harboring replicon plasmids, the *NS5B* gene in a Con1 strain replicon plasmid was replaced with the patient's *NS5B* cDNA using the In-Fusion Advantage PCR cloning kit, and then an HCV replicon RNA was synthesized from the SpeI-digested HCV replicon plasmid by *in vitro* transcription using a MEGAscript T7 kit (Ambion, Austin, TX). Huh-7.5 cells (20) were used in the phenotypic analysis. The cells were propagated in high-glucose Dulbecco's modified Eagle's medium (Nikken BioMedical Laboratory, Kyoto, Japan) containing 10% fetal bovine serum (Moregate Biotech, Bulimba, Australia), 0.1 mM nonessential amino acids (Invitrogen), 100 U/ml of penicillin (Invitrogen), 100 μg/ml of streptomycin sulfate (Invitrogen), and 2 mM L-glutamine (Invitrogen). An HCV replicon RNA (5 or 15 μg) was electroporated into Huh-7.5 cells (8×10^6 cells) using a Gene Pulser II apparatus (Bio-Rad, Hercules, CA) at 960 μF and 270 V. Following the electroporation, aliquots of 2×10^4 cells were seeded to a 96-well plate and cultured for 48 h at 37°C under 5% CO_2. JTK-853 was dissolved in dimethyl sulfoxide (DMSO) and was added to the cell so that the final concentration of the DMSO in the culture medium was adjusted to be 0.5%, followed by culture for a further 48 h. HCV replication was determined by the amount of reporter gene luciferase activity (11). The luciferase activity at 96 h postelectroporation was adjusted by that at 4 h postelectroporation to normalize the transfection efficiency using a Steady-Glo luciferase assay system (Promega, Fitchburg, WI). The 50% effective concentration (EC_{50}) of JTK-853 was determined from the inhibitory activity against HCV replication. The cytotoxicity was measured at 96 h postelectroporation by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt (MTS), assay (CellTiter 96; Promega), and the 50% cytotoxic concentration (CC_{50}) of JTK-853 was determined.

To show the potential replication competence, the replication efficiencies (in percent) of a reference strain (Con1) and the patient's *NS5B*-harboring replicons were determined using the following equation: (luciferase activity at 96 h/luciferase activity at 4 h) ×100.

Furthermore, to determine the phenotypic assay window, the replication capacity (in percent) of the patient's *NS5B*-harboring replicons was expressed as follows: (replication efficiency of the patient's *NS5B*-harboring replicon/replication efficiency of the reference strain) × 100.

When the replication capacity was more than 0.03%, we defined that the inhibitory effect of JTK-853 in the patient's *NS5B*-harboring replicon could be appropriately evaluated.

Statistical regression analysis of the correlation. The correlation between the viral reduction in JTK-853-treated HCV-infected patients and the susceptibility to JTK-853 of the patient's *NS5B*-harboring replicon was expressed as the Pearson product-moment correlation coefficient by a linear regression analysis.

***In vitro* resistance analysis.** The procedures for determination of *in vitro* resistance to JTK-853 were performed as previously described (14).

RESULTS

Baseline characteristics of the patients. The baseline characteristics of all 28 patients enrolled in the study of JTK-853 in a 3-day monotherapy (16) are summarized in Table 1. Using serum samples from the JTK-853-treated patients, the genotypic analysis was performed for the BL, EOT, and FU periods and the phenotypic analysis was performed for the BL and EOT. For the placebo patients, the genotypic and phenotypic analyses were performed only for the BL.

Population sequencing analysis. During the FU period, the HCV RNA level in some JTK-853-treated patients became lower than the limit of detection (<15 IU/ml), as follows: patients 1205

TABLE 1 Baseline characteristics of the patients[a]

Treatment	Patient no.	HCV genotype	Time point	HCV RNA load (IU/ml)	Change in HCV RNA load from BL (log_{10})
800 mg BID	0402	1a	BL (day 1)	10,300,000	
			EOT (day 4)	595,000	−1.24
			FU (wk 4)	306	−4.53
			FU (wk 12)	<15	<−5.84
			FU (wk 24)	<15	<−5.84
			FU (wk 48)	<15	<−5.84
	0403	1a	BL (day 1)	2,080,000	
			EOT (day 4)	325,000	−0.81
			FU (wk 4)	15,700	−2.12
			FU (wk 12)	<15	<−5.14
			FU (wk 24)	<15	<−5.14
			FU (wk 48)	<15	<−5.14
	0404	1a	BL (day 1)	1,390,000	
			EOT (day 4)	1,370,000	−0.01
			FU (wk 4)	51,500	−1.43
			FU (wk 12)	<15	<−4.97
			FU (wk 24)	31,800	−1.64
			FU (wk 48)	1,200,000	0.06
	0405	1a	BL (day 1)	6,610,000	
			EOT (day 4)	90,200	−1.86
			FU (wk 4)	534	−4.09
			FU (wk 12)	<15	<−5.64
			FU (wk 24)	<15	<−5.64
	0406	1a	BL (day 1)	7,600,000	
			EOT (day 4)	537,000	−1.15
	0407	1a	BL (day 1)	580,000	
			EOT (day 4)	232,000	−0.40
1,200 mg BID	0101	1a	BL (day 1)	20,000,000	
			EOT (day 4)	25,100,000	0.10
	0102	1b	BL (day 1)	562,000	
			EOT (day 4)	16,700	−1.53
	0103	1b	BL (day 1)	4,860,000	
			EOT (day 4)	162,000	−1.48
			FU (wk 4)	4,920	−2.99
			FU (wk 12)	<15	<−5.51
			FU (wk 24)	<15	<−5.51
			FU (wk 48)	<15	<−5.51
	0104	1a	BL (day 1)	30,400,000	
			EOT (day 4)	149,000	−2.31
	0105	1a	BL (day 1)	1,560,000	
			EOT (day 4)	654,000	−0.38
	0106	1a	BL (day 1)	5,630,000	
			EOT (day 4)	95,900	−1.77
			FU (wk 4)	202,000	−1.45
			FU (wk 12)	<15	<−5.57
			FU (wk 24)	<15	<−5.57
			FU (wk 48)	<15	<−5.57
1,600 mg BID	0201	1a	BL (day 1)	4,830,000	
			EOT (day 4)	389,000	−1.09
			FU (wk 4)	95	−4.71
			FU (wk 24)	<15	<−5.51
			FU (wk 48)	3,110,000	0.19
	0203	1a	BL (day 1)	9,340,000	
			EOT (day 4)	1,090,000	−0.93
			FU (wk 4)	10,800	−2.94
			FU (wk 12)	<15	<−5.79
			FU (wk 24)	<15	<−5.79
			FU (wk 48)	<15	<−5.79
	0204	1a	BL (day 1)	4,590,000	
			EOT (day 4)	248,000	−1.27
			FU (wk 4)	780,000	−0.77
			FU (wk 12)	1,820,000	−0.40
	0206	1a	BL (day 1)	9,800,000	
			EOT (day 4)	1,820,000	−0.73

(Continued on following page)

TABLE 1 (Continued)

Treatment	Patient no.	HCV genotype	Time point	HCV RNA load (IU/ml)	Change in HCV RNA load from BL (log_{10})
			FU (wk 4)	539,000	−1.26
			FU (wk 12)	414	−4.37
			FU (wk 24)	<15	<−5.82
			FU (wk 48)	<15	<−5.82
	0207	1b	BL (day 1)	5,620,000	
			EOT (day 4)	11,700	−2.68
			FU (wk 4)	78,100	−1.86
	1205	1a	BL (day 1)	116,000	
			EOT (day 4)	471	−2.39
			FU (wk 4)	<15	<−3.89
			FU (wk 12)	<15	<−3.89
			FU (wk 24)	<15	<−3.89
			FU (wk 48)	<15	<−3.89
1,200 mg TID	0301	1a	BL (day 1)	20,000,000	
			EOT (day 4)	1,800,000	−1.05
			FU (wk 4)	172,000	−2.07
			FU (wk 12)	<15	<−6.12
			FU (wk 24)	<15	<−6.12
			FU (wk 48)	<15	<−6.12
	0302	1a	BL (day 1)	405,000	
			EOT (day 4)	193,000	−0.32
			FU (wk 4)	34,100	−1.07
			FU (wk 12)	<15	<−4.43
			FU (wk 24)	<15	<−4.43
			FU (wk 48)	<15	<−4.43
	0303	1a	BL (day 1)	82,500	
			EOT (day 4)	2,390	−1.54
			FU (wk 4)	<15	<−3.74
			FU (wk 12)	<15	<−3.74
	0305	1a	BL (day 1)	4,420,000	
			EOT (day 4)	1,260,000	−0.55
			FU (wk 4)	270,000	−1.21
			FU (wk 12)	1,170	−3.58
			FU (wk 24)	<15	<−5.47
			FU (wk 48)	<15	<−5.47
	0306	1a	BL (day 1)	1,060,000	
			EOT (day 4)	12,400	−1.93
			FU (wk 4)	13,100	−1.91
			FU (wk 12)	<15	<−4.85
			FU (wk 24)	<15	<−4.85
			FU (wk 48)	<15	<−4.85
	0307	1a	BL (day 1)	1,450,000	
			EOT (day 4)	934,000	−0.19
			FU (wk 4)	29,600	−1.69
			FU (wk 12)	<15	<−4.99
			FU (wk 24)	407,000	−0.55
			FU (wk 48)	2,620,000	−0.26
Placebo	0107	1a	BL (day 1)	18,100,000	
			EOT (day 4)	16,100,000	−0.05
	0202	1a	BL (day 1)	2,350,000	
			EOT (day 4)	1,450,000	−0.21
	0304	1b	BL (day 1)	2,040,000	
			EOT (day 4)	2,970,000	0.16
	0401	1b	BL (day 1)	39,500	
			EOT (day 4)	67,400	0.23

[a] BL, baseline; EOT, end of treatment; FU, follow-up period; BID, twice daily; TID, three times daily.

and 0303 at week 4; patients 0103, 0106, 0203, 1205, 0301, 0302, 0303, 0306, 0307, 0402, 0403, 0404, and 0405 at week 12; patients 0103, 0106, 0201, 0203, 0206, 1205, 0301, 0302, 0305, 0306, 0402, 0403, and 0405 at week 24; and patients 0103, 0106, 0203, 0206, 1205, 0301, 0302, 0305, 0306, 0402, and 0403 at week 48. Subsequently, the genotypic analysis was not performed for these patients.

Mutations at C316, M414, Y452, and L466 in NS5B attenuate the susceptibility to JTK-853 *in vitro* (14). The EC_{50}s of JTK-853 for the C316Y-, M414T-, C445F-, Y448H-, Y452H-, and L466V-harboring replicons attenuated the susceptibility by 58-fold, 44-fold, 5-fold, 6-fold, 44-fold, and 21-fold, respectively, compared with that in the wild-type replicon (Con1) (Table 2). To determine whether the mutations at these positions associated with

TABLE 2 *In vitro* resistance to JTK-853

Replicon	Fold change in EC_{50}[a]
WT	1
C316Y	58 ± 10[b]
M414T	44
C445F	5
Y448H	6
Y452H	44 ± 3[b]
L466V	21 ± 3[b]

[a] The *in vitro* antiviral activity of JTK-853 against HCV replicons bearing the resistance mutation is indicated as the fold change in EC_{50} compared with that for the wild type (WT).
[b] Data represent mean ± standard error of three independent experiments.

viral resistance were detected in the JTK-853-treated HCV-infected patients, the amino acid sequences at the EOT in the 24 patients were compared with the corresponding sequences at the BL by a population sequencing analysis. This analysis revealed that no amino acid substitutions were detected at C316, C445, Y448, Y452, and L466 in any of the 24 patients at the BL and EOT, while the amino acid substitution M414T was observed from the BL to the EOT only in patient 0405 (infected with genotype 1a) (Table 3). These results suggest that an amino acid substitution of M414T is a mutation for resistance to JTK-853, although the viral reduction in patient 0405 reached nearly 2.0 log_{10} units at the EOT.

Phenotypic analysis. Prior to performing the phenotypic analysis, the replication capacity of each patient's *NS5B*-harboring replicon was compared with that of the Con1 reference strain. The replication capacity of each patient's *NS5B*-harboring replicon ranged from 0.01% to 99% of that of the reference strain, as shown in Table 3. To maintain an adequate assay window for JTK-853 in the phenotypic analysis, we defined that the phenotypic analysis could be appropriately performed when the replication capacity of the replicon was more than 0.03% of that for the reference strain. For this reason, the phenotypic analysis could not be performed for patients 0202, 0204, 1205, 0302, and 0306 at the BL and patients 0203, 0306, 0403, 0404, 0406, and 0407 at the EOT.

To investigate the *NS5B* gene with regard to susceptibility to JTK-853, the EC_{50}s of JTK-853 were determined using the patient's *NS5B*-harboring replicons. Among 23 patients, excluding patient 0405 (infected with genotype 1a), the EC_{50}s of JTK-853 at the BL ranged from 61 to 537 nM in 16 patients infected with

TABLE 3 Population sequencing and phenotypic susceptibility in JTK-853-treated patients[a]

Treatment	Patient no.	GT	Change in HCV RNA from BL to EOT (log_{10})	BL (day 1)			EOT (day 4)			Fold change in EC_{50} from BL
				Substitution	EC_{50} (nM)	RC (%)	Substitution	EC_{50} (nM)	RC (%)	
800 mg BID	0402	1a	−1.24	—	172	7.4	—	61	9.3	0.35
	0403	1a	−0.81	—	137	18	—	ND	0.01	ND
	0404	1a	−0.01	—	396	11	—	ND	0.02	ND
	0405	1a	−1.86	—	56	10	M414T	1,320	6.9	24
	0406	1a	−1.15	—	119	0.54	—	ND	0.01	ND
	0407	1a	−0.40	—	294	0.15	—	ND	0.01	ND
1,200 mg BID	0101	1a	0.10	—	537	2.2	—	310	13	0.58
	0102	1b	−1.53	—	33	99	—	37	88	1.1
	0103	1b	−1.48	—	62	8.3	—	33	80	0.53
	0104	1a	−2.31	—	61	0.62	—	273	8.9	4.5
	0105	1a	−0.38	—	234	5.3	—	243	1.6	1.0
	0106	1a	−1.77	—	222	8.2	—	333	0.10	1.5
1,600 mg BID	0201	1a	−1.09	—	213	0.36	—	360	4.7	1.7
	0203	1a	−0.93	—	380	12	—	ND	0.02	ND
	0204	1a	−1.27	—	ND	0.01	—	186	29	ND
	0206	1a	−0.73	—	189	20	—	318	26	1.7
	0207	1b	−2.68	—	32	79	—	23	83	0.72
	1205	1a	−2.39	—	ND	0.02	—	388	0.03	ND
1,200 mg TID	0301	1a	−1.05	—	199	1.3	—	639	0.08	3.2
	0302	1a	−0.32	—	ND	0.02	—	416	0.28	ND
	0303	1a	−1.54	—	292	45	—	147	0.03	0.50
	0305	1a	−0.55	—	359	4.6	—	424	40	1.2
	0306	1a	−1.93	—	ND	0.04	—	ND	0.01	ND
	0307	1a	−0.19	—	189	24	—	264	13	1.4
Placebo	0107	1a	−0.05	—	171	2.0				
	0202	1a	−0.21	—	ND	0.02				
	0304	1b	0.16	—	16	11				
	0401	1b	0.23	—	20	3.1				

[a] —, no amino acid substitution at residue 316, 414, 445, 448, 452, or 466. ND, not determined owing to a low replication capacity (<0.03%); GT, genotype; BL, baseline; EOT, end of treatment; BID, twice daily; TID, three times daily; RC, replication capacity. For the reference strain of Con1 (GenBank accession number AJ238799), the EC_{50} was 23 to 56 nM. Data for the EC_{50}s represent the results from one experiment. Replication efficiency = (luciferase activity at 96 h/luciferase activity at 4 h) × 100; replication capacity = (replication efficiency of a patient's *NS5B*-harboring replicon/replication efficiency of the reference strain) × 100.

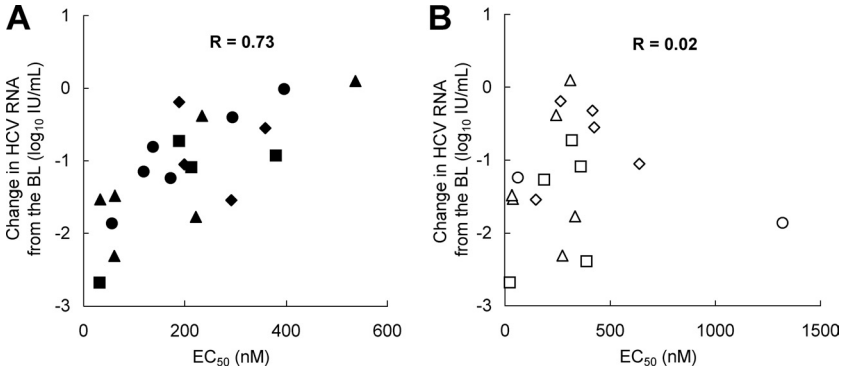

FIG 1 Correlations between the viral reduction in JTK-853-treated HCV-infected patients and the JTK-853 susceptibility in their *NS5B*-harboring replicon at the BL (A) and EOT (B). The *x* axis represents the EC_{50}, and the *y* axis represents the change in HCV RNA load from the BL to the EOT in a 3-day monotherapy. The correlation coefficient (*R*) is indicated in each panel. The symbols are as follows: circles, 800 mg BID; triangles, 1,200 mg BID; squares, 1,600 mg BID; diamonds, 1,200 mg TID.

genotype 1a and 32 to 62 nM in 3 patients infected with genotype 1b, while those at the EOT ranged from 61 to 639 nM in 14 patients infected with genotype 1a and 23 to 37 nM in 3 patients infected with genotype 1b, with JTK-853 not showing any cytotoxicity up to a concentration of 10 μM (Table 3). Regardless of the genotype, the change in the EC_{50} of JTK-853 at the EOT from that at the BL in each patient was within 5-fold. On the other hand, the EC_{50} of TK-853 at the BL was 56 nM and that at the EOT was 1,320 nM in patient 0405, who had the M414T mutation at the EOT. The fold change of the EC_{50} at the EOT from that at the BL was approximately 24-fold, which was much higher than the fold changes in the other patients. These findings also suggest that the emergence of M414T appeared to impair the responsiveness to JTK-853 in a 3-day monotherapy.

Notably, the phenotypic analysis also demonstrated that the EC_{50} of JTK-853 at the BL was well correlated with the viral reduction in JTK-853-treated HCV-infected patients with a correlation coefficient of 0.73, while that at the EOT was not (Fig. 1A and B). These findings suggest that in the phenotypic analysis the EC_{50} of JTK-853 at the BL can estimate the viral reduction in JTK-853-treated HCV-infected patients in a 3-day monotherapy.

Clonal sequencing analysis. To detect amino acid substitutions more precisely, we performed a clonal sequencing analysis for all of the JTK-853-treated HCV-infected patients. In the clonal sequencing analysis, we isolated more than 10 clones of the *NS5B* gene from each patient at the BL and EOT and determined the amino acid sequences.

At the BL, the amino acid substitutions detected were C445R in 2 patients and Y452H in 2 patients (Table 4). No other resistance-related substitutions of C316, M414, Y448, and L466 were detected at the BL. C445R was observed in patients 0302 (infected with genotype 1a) and 0307 (infected with genotype 1a), with incidences of 20% and 7%, respectively, while Y452H was observed in patients 0402 (infected with genotype 1a) and 0305 (infected with genotype 1a), with incidences of 9% and 18%, respectively. The incidences of the emergence of these amino acid substitutions were all low at ≤20%. In addition, all of these substitutions reverted to the reference strain (Con1) amino acid at the EOT, and the HCV RNA levels in these patients became lower than the limit of detection during the FU period. These findings suggest that these substitutions at the BL were not persistent.

At the EOT, the amino acid substitutions detected were M414T in 2 patients, C445R in 1 patient, Y448C/H in 2 patients, and L466F in 1 patient (Table 3). No other resistance-related substitutions of C316 and Y452 were detected at the EOT. M414T was observed in patients 0403 (infected with genotype 1a) and 0405 (infected with genotype 1a), with incidences of 18% and 100%, respectively. In addition, C445R, Y448H, Y448C, and L466F were detected in patients 0407 (infected with genotype 1a), 0406 (infected with genotype 1a), 0102 (infected with genotype 1b), and 0101 (infected with genotype 1a), with incidences of 7%, 10%, 9%, and 10%, respectively. In these patients, the incidences of emergence of the amino acid substitutions ranged from 7% to 100%, regardless of the viral reduction at the EOT with JTK-853. However, M414T, C445F, Y448H, and L466V were selected as mutations for resistance to JTK-853 *in vitro* and attenuated the susceptibility of JTK-853 by 5- to 44-fold compared with that of the reference strain (Con1), suggesting that M414T, C445R, Y448C/H, and L466F are likely to be mutations for resistance to JTK-853 in a 3-day monotherapy.

DISCUSSION

JTK-853 has demonstrated potential clinical utility in a phase 1b clinical study of treatment-naïve patients who are chronically infected with HCV genotype 1. In this report, we performed genotypic and phenotypic analyses of JTK-853-treated HCV-infected patients for the BL, EOT, and FU periods.

In this study, the amino acid substitution M414T was observed in patient 0405 at the EOT in a population sequencing analysis as well as in a clonal sequencing analysis, with the incidence of resistance emergence being 100%. The EC_{50} of JTK-853 for patient 0405's *NS5B*-harboring replicon with M414T at the EOT was 1,320 nM, which was approximately 24-fold higher than that at the BL in this patient. In our previous study, the 3-fluoro-4-trifluoromethoxy benzene moiety of JTK-853 appeared to participate in a CH-π interaction with M414 and bound to the hydrophobic (leucine-tryptophan-phenylalanine) pocket of the HCV polymerase (14, 15). In addition, M414 is an amino acid residue that is located close to the site of JTK-853 association with the NS5B protein. Furthermore, the EC_{50} of JTK-853 for the M414T-harboring replicon (1.4 μM) was attenuated by 44-fold compared with that for the wild-type Con1 replicon (32 nM) *in vitro* (14). These data indicate that M414T is a resistance mutation for JTK-

TABLE 4 Clonal sequencing in JTK-853-treated patients[a]

Patient no.	Treatment	GT	Time point	Change in HCV RNA from BL (log_{10})	Residue (% of patients) in:					
					C316	M414	C445	Y448	Y452	L466
0402	800 mg BID	1a	BL (day 1)		—	—	—	—	H (9)	—
			EOT (day 4)	−1.24	—	—	—	—	—	—
			FU (wk 4)	−4.53	—	—	—	—	—	—
			FU (wk 12)	<−5.84	NT	NT	NT	NT	NT	NT
			FU (wk 24)	<−5.84	NT	NT	NT	NT	NT	NT
			FU (wk 48)	<−5.84	NT	NT	NT	NT	NT	NT
0403	800 mg BID	1a	BL (day 1)		—	—	—	—	—	—
			EOT (day 4)	−0.81	—	T (18)	—	—	—	—
			FU (wk 4)	−2.12	—	—	—	—	—	—
			FU (wk 12)	<−5.14	NT	NT	NT	NT	NT	NT
			FU (wk 24)	<−5.14	NT	NT	NT	NT	NT	NT
			FU (wk 48)	<−5.14	NT	NT	NT	NT	NT	NT
0405	800 mg BID	1a	BL (day 1)		—	—	—	—	—	—
			EOT (day 4)	−1.86	—	T (100)	—	—	—	—
			FU (wk 4)	−4.09	—	T (8)	—	—	H (8)	—
			FU (wk 12)	<−5.64	NT	NT	NT	NT	NT	NT
			FU (wk 24)	<−5.64	NT	NT	NT	NT	NT	NT
			FU (wk 48)	NS						
0406	800 mg BID	1a	BL (day 1)		—	—	—	—	—	—
			EOT (day 4)	−1.15	—	—	—	H (10)	—	—
			FU (wk 4)	NS						
			FU (wk 12)	NS						
			FU (wk 24)	NS						
			FU (wk 48)	NS						
0407	800 mg BID	1a	BL (day 1)		—	—	—	—	—	—
			EOT (day 4)	−0.40	—	—	R (7)	—	—	—
			FU (wk 4)	NS						
			FU (wk 12)	NS						
			FU (wk 24)	NS						
			FU (wk 48)	NS						
0101	1,200 mg BID	1a	BL (day 1)		—	—	—	—	—	—
			EOT (day 4)	0.10	—	—	—	—	—	F (10)
			FU (wk 4)	NS						
			FU (wk 12)	NS						
			FU (wk 24)	NS						
			FU (wk 48)	NS						
0102	1,200 mg BID	1b	BL (day 1)		—	—	—	—	—	—
			EOT (day 4)	−1.53	—	—	—	C (9)	—	—
			FU (wk 4)	NS						
			FU (wk 12)	NS						
			FU (wk 24)	NS						
			FU (wk 48)	NS						
0302	1,200 mg TID	1a	BL (day 1)		—	—	R (20)	—	—	—
			EOT (day 4)	−0.32	—	—	—	—	—	—
			FU (wk 4)	−1.07	—	—	—	—	—	—
			FU (wk 12)	<−4.43	NT	NT	NT	NT	NT	NT
			FU (wk 24)	<−4.43	NT	NT	NT	NT	NT	NT
			FU (wk 48)	<−4.43	NT	NT	NT	NT	NT	NT
0305	1,200 mg TID	1a	BL (day 1)		—	—	—	—	H (18)	—
			EOT (day 4)	−0.55	—	—	—	—	—	—
			FU (wk 4)	−1.21	—	—	—	—	—	—
			FU (wk 12)	−3.58	—	—	—	—	—	—
			FU (wk 24)	<−5.47	NT	NT	NT	NT	NT	NT
			FU (wk 48)	<−5.47	NT	NT	NT	NT	NT	NT

(Continued on following page)

TABLE 4 (Continued)

Patient no.	Treatment	GT	Time point	Change in HCV RNA from BL (log_{10})	Residue (% of patients) in:					
					C316	M414	C445	Y448	Y452	L466
0307	1,200 mg TID	1a	BL (day 1)		—	—	R (7)	—	—	—
			EOT (day 4)	−0.19	—	—	—	—	—	—
			FU (wk 4)	−1.69	—	—	—	—	—	—
			FU (wk 12)	<−4.99	NT	NT	NT	NT	NT	NT
			FU (wk 24)	−0.55	—	—	—	—	—	—
			FU (wk 48)	−0.26	—	—	—	—	—	—

[a] More than 10 clones of the *NS5B* gene were examined for each patient and time point. —, no amino acid substitution at residue 316, 414, 445, 448, 452, or 466; NS, no sample; NT, not tested, as genotypic analysis was not performed because the serum HCV RNA was lower than the limit of detection (<15 IU/ml); GT, genotype, BL, baseline, EOT, end of treatment; FU, follow-up period; BID, twice daily; TID, three times daily.

853 *in vitro*. Actually, M414T was selected after treatment with certain palm site-binding nonnucleoside polymerase inhibitors in HCV-infected patients and HCV replicons (e.g., benzothiadiazine derivatives of ABT-333, ANA-598, and A-782759 and an acylpyrrolidine derivative of GSK625433) (21–24). Moreover, patient 0405 responded to JTK-853 with less than a 2.0-log_{10}-unit viral load reduction at the EOT because the patient might have had suboptimal exposure to JTK-853 or JTK-853 might have shown suboptimal antiviral activity in patient 0405. These findings suggest that M414T is a resistance mutation for JTK-853 in a 3-day monotherapy. On the other hand, we detected some other amino acid substitutions within NS5B outside the region reported to have mutations associated with viral resistance that arose during the JTK-853 treatment phase in a population sequencing analysis (see Table S2 in the supplemental material). However, not only were those substitutions not selected *in vitro*, but also, those amino acids did not participate in an association with HCV polymerase by a cocrystal structure analysis (14, 15). Therefore, these findings suggest that these mutations appear to have no relation to viral resistance and are sporadic.

The clonal sequencing analysis at the EOT also revealed that the amino acid substitutions M414T, C445R, Y448H, Y448C, and L466F in patients 0403, 0407, 0406, 0102, and 0101 occurred with incidences of 18%, 7%, 10%, 9%, and 10%, respectively. The EC_{50}s of JTK-853 for the M414T-, C445F-, Y448H-, and L466V-harboring replicons were attenuated by 44-fold, 5-fold, 6-fold, and 21-fold, respectively, *in vitro* compared with the EC_{50} for the wild-type replicon (Con1 strain), as shown in Table 2. Patients 0406 and 0102 showed viral reductions of more than 1.0 log_{10} unit with JTK-853 treatment, while patients 0403, 0407, and 0101 showed reductions of less than 1.0 log_{10} unit. The rates of emergence of these substitutions were as low as less than 20%, and they were detected as mixtures with the wild-type virus sequence. However, this seems to be due to a suboptimal exposure to JTK-853 or suboptimal JTK-853 antiviral activity in these patients. These findings suggest that these substitutions are also resistance mutations in a 3-day monotherapy.

The clonal sequencing analysis at the BL showed that four JTK-853-treated patients had amino acid substitutions of C445R (patients 0302 and 0307) and Y452H (patients 0402 and 0305) with incidences of 20%, 7%, 9%, and 18% in patients 0302, 0307, 0402, and 0305, respectively. Patient 0402 showed a viral reduction of more than 1.0 log_{10} unit with JTK-853 treatment, while patients 0302, 0305, and 0307 showed reductions of less than 1.0 log_{10} unit. In addition, at 24 weeks after withdrawal of JTK-853, patient 0307 had a viral rebound with PEG-IFN plus RBV treatment during the FU period. However, all of these amino acid substitutions at the BL completely reverted to the reference strain amino acids at the EOT, regardless of the viral reduction with JTK-853 treatment, suggesting that the emergence of the substitutions at the BL did not appear to be related to the viral reductions or to be persistent. Regarding the insufficient effectiveness of JTK-853 in these patients, we may need to take longer treatment periods, better exposure, and certain host factors into consideration.

It was previously reported that the Y448H substitution was selected and persistent after treatment with GS-9190 *in vitro* and in HCV-infected patients (25, 26). Importantly, despite the fact that JTK-853 belongs to the same class as GS-9190, Y448H was detected in only one patient with an incidence of 10%, and moreover, JTK-853 treatment showed a viral reduction of more than 1.0 log_{10} unit in this patient. These findings strongly suggest that JTK-853 shows a high genetic barrier to the emergence of the persistent resistance mutation Y448H during a 3-day monotherapy.

In conclusion, the amino acid substitutions M414T, C445R, Y448H, Y448C, and L466F were detected in 6 HCV-infected patients at the EOT with JTK-853 in a clonal sequencing analysis, and JTK-853 did not show meaningful viral reductions (<2.0 log_{10}) in these patients due to a suboptimal exposure to JTK-853 or suboptimal JTK-853 antiviral activity. In addition, M414T, C445F, Y448H, and L466V attenuated JTK-853 susceptibility by 5- to 44-fold. These findings suggest that M414T, C445R, Y448H, Y448C, and L466F might be viral resistance mutations in HCV-infected patients receiving JTK-853 in a 3-day monotherapy.

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