Allele-Specific Real-Time PCR System for Detection of Subpopulations of Genotype 1a and 1b Hepatitis C NS5B Y448H Mutant Viruses in Clinical Samples[▽]

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The Y448H mutation in NS5B has been selected by GS-9190 as well as several benzothiadiazine hepatitis C virus (HCV) polymerase inhibitors *in vitro* and *in vivo*. However, the level and the evolution kinetics of this resistance mutation prior to and during treatment are poorly understood. In this study, we developed an allele-specific real-time PCR (AS-PCR) assay capable of detecting Y448H when it was present at a level down to 0.5% within an HCV population of genotype 1a or 1b. No Y448H mutation was detected above the assay cutoff of 0.5% in genotype 1b-infected Con-1 replicons prior to *in vitro* treatment. However, the proportion of replicons with the Y448H mutation rapidly increased in a dose-dependent manner upon treatment with GS-9190. After 3 days of treatment, 1.2%, 6.8%, and >50% of the replicon population expressed Y448H with the use of GS-9190 at 1, 10, and 20 times its 50% effective concentration, respectively. In addition, plasma from 65 treatment-naïve HCV-infected patients (42 and 23 with genotype 1a and 1b, respectively) was tested for the presence of Y448H by AS-PCR and population sequencing. As expected, all patient samples were wild type at NS5B Y448H by population sequencing. AS-PCR results were obtained for 62/65 samples tested, with low levels of Y448H ranging from 0.5% to 3.0% detected in 5/62 (8%) treatment-naïve patient samples. These findings suggest the need for combination therapy with HCV-specific inhibitors to avoid viral rebound of preexisting mutant HCV.

Hepatitis C virus (HCV) is a global health problem and infects more than 170 million individuals worldwide (15). The current standard treatment with pegylated interferon and ribavirin is complicated by frequent adverse reactions, and a sustained virologic response (SVR) can be achieved in only ~50% of patients infected with the most prevalent genotype, genotype 1. HCV NS5B polymerase is essential for viral replication, and a number of compounds that inhibit this enzyme (HCV polymerase inhibitors) have been discovered; some have advanced to phase I/II clinical trials and have demonstrated antiviral activity in HCV-infected patients in monotherapy (8). However, monotherapy with any specific HCV nonnucleoside inhibitors (NNIs) resulted in the rapid selection of resistance mutations in the replicon and HCV genotype 1-infected patients (19). Among the resistance mutations, Y448H in NS5B has been reported to be selected by GS-9190, as well as by the benzothiadiazine and acylpyrrolidine classes of NNIs (4, 13). The NS5B Y448H mutant remained sensitive to interferon, ribavirin, and inhibitors of HCV NS3 protease, NS5A, and NS5B (site II nonnucleoside and nucleoside) (4, 12).

HCV has extremely high genetic diversity and exists in HCV-infected individuals as a pool of closely related but distinct variants as quasispecies. These viral quasispecies may include drug-resistant variants existing within a predominantly wild-type virus population before treatment (2, 5, 7). The presence of drug-resistant variants at different low-level frequen-

cies in HCV-infected patients subsequently results in varying degrees of viral response and mutant enrichment upon treatment with NS5B inhibitors (6, 10). Thus, the determination of natural levels of low-frequency resistant variants at baseline offers the potential for the interpretation and prediction of the viral response to HCV inhibitors.

The most common method of detecting drug-resistant variants in HCV-infected patients involves extracting multiple viral genomes from plasma and reverse transcription (RT) and PCR amplification (RT-PCR), followed by population-based DNA sequencing. The method provides a composite of the major sequences present and is limited in detecting minor populations (<20 to 25%). Clonal analyses and single-genome sequencing (SGS) provide a greater capacity to detect a minor population but are highly labor-intensive. A simpler allele-specific real-time PCR (AS-PCR) method using the Multicode RTx real-time PCR technology (EraGen Biosciences) has been reported to quantify HIV-1 reverse transcriptase mutants down to a level of 0.01% in plasmid DNA mixtures and to 0.5% in preamplified PCR products (14, 20, 21). Taking advantage of this technology, we have developed a highly sensitive AS-PCR assay to detect low-frequency NS5B Y448H mutant variants in laboratory and clinical samples. The presence of the Y448H mutation was evaluated in replicon cells prior to and after treatment with GS-9190 and in 65 treatment-naïve HCV-infected patients.

MATERIALS AND METHODS

Clinical isolates. Plasma samples were obtained from untreated genotype 1a and genotype 1b HCV-infected patients. All of the samples had patient consent for use and originated from the United States. Samples were obtained from three

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TABLE 1. cDNA synthesis, PCR, and AS-PCR real-time PCR primers

Genotype	Target or description	Primer name	Primer sequence (5'-3')
1a	NS5B cDNA	3'-9386	CTAAGAGGCCGGAGTGTTTAC
1b	NS5B cDNA	3'-9459	CCTATTGGCCTGGAGTGTTTAGCTC
1a	NS5B for PCR	5'-7380	GAATCAACCCTATCTACTGCCTTGGCCGAGC
1a	NS5B for PCR	3'-9386	CTAAGAGGCCGGAGTGTTTAC
1b	NS5B for PCR	5'-7595	TCGTACTCCTCCATGCCCCCCTTGA
1b	NS5B for PCR	3'-9459	CCTATTGGCCTGGAGTGTTTAGCTC
1a	448Y	MM2348	FAM-CCCTTGATTGCGAAATCTA
	448H	MM2350	HEX-CCCTTGATTGCGAAATCCA
1b set 1	448Y	MM2419	FAM-GGCCCTGTATTGTCAGATCT
1b set 1	448H	MM2420	HEX-GGCCCTGTATTGTCAGATCC
1b set 2	448Y	MM2417	FAM-GGCCCTGTATTGTCAAATCT
1b set 2	448H	MM2418	HEX-GGCCCTGTATTGTCAAATCC
1a/1b	Reverse primer	MM2351	AATGCGCTGAGGCCATGGAGTC

GS-9190-treated patients in a GS-196-0101 phase 1 study evaluating monotherapy with GS-9190 in treatment-naïve HCV-infected subjects (4).

Compounds and reagents. GS-9190 was synthesized at Gilead Sciences, Inc. (Foster City, CA). Dulbecco's modified Eagle medium (DMEM) and Geneticin (G418) were purchased from Gibco (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). The luciferase assay system was purchased from Promega (Madison, WI).

Replicon constructs and cell lines. HCV genotype 1b-PI-Rluc, a bicistronic replicon, and cured Huh7 cells (Lunet) were obtained from Ralf Bartenschlager (University of Heidelberg, Heidelberg, Germany). The genotype 1b-PI-Rluc construct contains a luciferase reporter gene driven by the poliovirus internal ribosome entry site (IRES) and the HCV nonstructural genes from genotype 1b Con-1 driven by the encephalomyocarditis virus IRES. Three adaptive mutations, T1280I and E1202G in NS3 and K1846T in NS4B, were introduced into this construct for efficient replication. Replicon cells were maintained in DMEM supplemented with 10% FBS and passaged twice a week before reaching confluent levels.

Site-directed mutagenesis and RNA transcription. Y448H mutations were introduced into the HCV replicon genotype 1b Con-1 and genotype 1a H77 plasmids using Stratagene's QuikChange II XL mutagenesis kit, following the manufacturer's instructions. Mutations were confirmed by sequencing. The transcripts of the HCV mutant or wild-type replicons were generated by using ScaI-linearized replicon plasmids and the Megascript T7 kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions, as previously described (17).

RNA extraction and cDNA synthesis. Total RNA was extracted from HCV replicon cells by using an RNeasy minikit (Qiagen, Valencia, CA) or from HCV-infected patient plasma samples with a QIAamp viral RNA minikit (Qiagen, Valencia, CA) according to the manufacturer's instructions. cDNA was synthesized from HCV RNA by using MonstScript reverse transcriptase (Epicentre, Madison, WI) and the HCV-specific primers 3'-9386 and 3'-9459 for NS5B of genotypes 1a and 1b, respectively. Briefly, 10 µl of the RNA isolated from 140 µl of plasma or replicon cells and 0.125 µM primer were incubated at 70°C for 4 min while they were chilled on ice. The reverse transcription reaction mixture was incubated at 50°C (genotype 1a) or 54°C (genotype 1b) for 10 min and then at 60°C for 40 min. Subsequently, the reverse transcriptase was heat inactivated at 90°C for 2 min. The cDNA was used as template for consecutive PCR amplification of the NS5B gene.

PCR amplification of NS5B. PCR amplification was performed using genotype-specific primers to amplify the NS5B gene, which was then used as a template for AS-PCR and nested PCR for population sequencing. Genotype 1a was amplified using primers 5'-7380 (5'-GAA TCA ACC CTA TCT ACT GCC TTG GCC GAG C-3') and 3'-9386 (5'-CTA AGA GGC CGG AGT GTT TAC-3'). Genotype 1b was amplified using primers 5'-7595 (5'-TCG TAC TCC TCC ATG CCC CCC CTT GA-3') and 3'-9459 (5'-CCT ATT GGC CTG GAG TGT TTA GCT C-3'). PCR parameters were as follows for both genotypes: 94°C for 2 min and 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min. PCR was performed using an Applied Biosystems 9700 PCR instrument.

Population and single-genome sequencing. To obtain population-based nucleotide sequences, nested PCR was performed using PCR products amplified from the first-round PCR as described above. The following nested genotype-specific

primers were used to amplify the NS5B gene from products of first-round PCR of genotype 1a samples: 1aNS5b-5'-7419 (5'-TTTGGCAGCTCTCAACTTCC GG-3') and 1aNS5b-3'-9372 (5'-GAGTGTTTACCCCAACCTTCATCG-3'). Genotype 1b samples were amplified using primers 1bNS5b-5'-7637 (5'-GATC TCAGCGACGGGTCTTGTC-3') and 1bNS5b-3'-9424 (5'-TTGGGGAGCA GGTAGATGCCTAC-3') for nested PCR. PCR parameters were as follows for both genotypes: 94°C for 2 min and 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 2 min for nested PCR. An Applied Biosystems 9700 PCR instrument was used. All the nested PCRs were performed using Platinum *Taq* DNA polymerase (Invitrogen). PCR products were run on an agarose gel to detect amplification of the target product and then purified using a QIAquick PCR purification kit. The eluted purified PCR product was then diluted 1:20 for concentration calculation by spectroscopy at A_{260} . The cycle sequencing reaction used genotype-specific sequencing primers and the ABI BigDye Terminator sequencing technology (Applied Biosystems, Foster City, CA).

SGS was performed by amplification of serially diluted reverse-transcribed cDNA samples. The cDNA dilution that yielded PCR products in ~30% of the amplifications was assumed to contain a single cDNA copy template in about 80% of the reactions (16). After the optimal cDNA dilution was established, 95 reactions were amplified through 2 rounds of PCR to attain 20 to 30 (~30%) clones. These clones were then purified, sequenced using Applied Biosystems BigDye Terminator chemistry, sequenced using Applied Biosystems genetic analyzer technology, and analyzed as previously described (21).

Y448H allele-specific real-time PCR. PCR products were diluted to approximately 10^6 to 10^7 copies/µl prior to performing the AS-PCR. A single reverse primer (MM2351) was used with each set of genotype- and allele-specific forward-labeled primers. Two sets of genotype 1b forward-labeled primers were used, as determined by isolate population sequence. AS-PCR primers are listed in Table 1. AS-PCR was performed using a Roche LightCycler 480 real-time PCR instrument under the following conditions: 95°C for 2 min, one cycle of 48°C for 5 s, 72°C for 20 s, and 75 cycles of 95°C for 5 s, 60°C for 5 s, and 72°C for 20 s.

To generate a standard curve, wild-type 448Y and mutant 448H clonal DNAs were diluted to 10^6 copies/µl and mixed to prepare 100%, 50%, 10%, 5%, 1%, 0.5%, 0.1%, and 0% 448H mutant mixtures. Standard curve samples were PCR amplified using the same PCR conditions used for sample amplification to account for misincorporation of the background during PCR. Standard curve samples were run on the same plate along with clinical samples during AS-PCR and used to predict the percentage of Y448H mutants. Standard curves were prepared for genotypes 1a and 1b. The assay cutoffs for detection of the Y448H mutation in preamplified PCR products were set to 0.5% for the lower limit and 50% for the upper limit.

Sequence alignment and analysis of data from population sequencing. The Sequencer program (version 4.0; Gene Codes Corporation, Ann Arbor, MI) was used to assemble and analyze the nucleotide sequence and translate the corresponding amino acid sequence. Sequence analysis of full-length NS5B was performed using Sequencer, and each sample was aligned against respective subtype reference (for genotype 1a, H77 [GenBank accession number AF009606]; for genotype 1b, Con-1 [GenBank accession number AJ238799]) to identify differences between patient and reference amino acid sequences. Analysis of the amino acid changes between two time points was conducted and the results are reported. Amino acid sequence analyses were performed for amino

FIG. 1. NS5B sequence alignment and Y448H AS-PCR primers. AS-PCR primers were designed by aligning NS5B population sequences of treatment-naïve patients infected with genotype 1a (A) and genotype 1b (B) in order to differentiate between the Y448H (CAC) mutant variant and the Y448Y (TAC) wild type (underscored). One set of a GT1a forward-labeled primer pair (MM2348_FAM/MM2350_HEX) and two sets of GT1b forward-labeled primer pairs (MM2417_FAM/MM2418_HEX and MM2419_FAM/MM2420_HEX) with a single reverse primer were designed. The sequences in this figure represent a subset of the 65 sequences analyzed in designing the AS-PCR primers.

Measurement of HCV RNA copy number and determination of inhibitor susceptibility. HCV RNA copy numbers were measured by using quantitative TaqMan real-time RT-PCR. The total amount of cellular RNA in each sample was determined using a NanoDrop ND-1000 apparatus (Thermo Scientific). Each sample was diluted and added into a mixture containing components from QuantiTect probe RT-PCR (Qiagen) and universal HCV RNA-specific primers. Serial dilutions of a synthesized HCV RNA standard were used to determine the HCV RNA copy numbers per sample. HCV RNA copy numbers were normalized by the total amount of RNA in each sample when appropriate. Quantitative TaqMan real-time RT-PCR was performed using a 7300 real-time PCR system (Applied Biosystems).

Development of Y448H AS-PCR assay. In order to ensure that the chosen primers covered the heterogeneity of patient HCV sequences, the nucleotide sequences of the NS5B gene covering ~100 bp upstream and downstream of the Y448 residue in viruses from 65 treatment-naïve genotype 1-infected patients were aligned and evaluated (Fig. 1). To differentiate between the Y448H mutant and Y448Y wild type, the two forward AS-PCR primers for genotype 1a differed at the penultimate nucleotide of the 3' end, matching either the mutant or the wild-type sequence. A T nucleotide at the penultimate nucleotide of the 3' end of the primer (TAC) amplifies the wild-type 448Y, whereas a C nucleotide at the penultimate nucleotide of the 3' end of the primer (CAC) amplifies the mutant 448H. One set of AS-PCR primers was designed for

genotype 1a to selectively amplify the Y448Y wild type or Y448H mutant. A similar strategy was used to design the forward allele-specific primers for genotype 1b. However, two sets of forward primers were designed for genotype 1b in order to cover the heterogeneity of the genotype 1b isolates. A sequence region at 47 bp downstream of Y448 was identified to be conserved among all isolates in both genotype 1b and genotype 1a. Therefore, a single reverse primer that was able to prime targets in both genotype 1a and genotype 1b was constructed. Fluorescent reporters were used to differentiate between the 448Y (TAC) wild type (6-carboxyfluorescein [FAM] reporter dye) and the 448H (CAC) mutant (hexachlorofluorescein [HEX] reporter dye). During the real-time PCR, the PCR cycle at which the fluorescence passes below a determined threshold correlates to the wild type and/or mutant. The cycle threshold (C_T), in which fluorescence passes the threshold per channel, is dependent on the copy number for each specified target. Standard curves constructed from C_T data from known concentrations of each target are used to determine concentrations for unknown isolates. The difference in cycle thresholds (ΔC_T s) between the two channels, calculated by subtracting the 448H mutant C_T from the 448Y wild-type C_T , was plotted against the standard curve to predict percent 448H mutant values in the samples.

To construct the standard curve, NS5B Y448Y wild-type and Y448H mutant clones from both genotype 1a and genotype 1b were quantified by SYBR green using a Roche LightCycler 480 instrument. Stocks of the wild type and mutant clones at 10^7 copies/ml were mixed to obtain standard curve mixtures at 100%, 50%, 10%, 5%, 1%, 0.5%, 0.1%, and 0% Y448H mutant mixes for both genotypes. These mixes were used directly in the MultiCode-RTx assay. Multiple measurements of the standard curve were performed to establish the assay background and detection limits of 0.5% for both genotypes 1a and 1b.

Levels of Y448H mutants in replicon cells upon treatment with HCV polymerase inhibitor GS-9190. To test and validate the utility of this novel AS-PCR assay, genotype 1b-infected Con-1 replicon cells were passaged in the presence of GS-9190 at levels $5\times$, $10\times$, or $20\times$ the 50% effective concentration (EC_{50}) in the absence of G418 for four passages. Replicon HCV RNA copy numbers were measured by TaqMan quantitative real-time PCR. At passage 3, replicon HCV copy numbers were reduced by 1.5 to 1.9 $\log_{10}$ units upon treatment with GS-9190 at $5\times$, $10\times$, and $20\times$ the EC_{50} (Fig. 2A). The RNA from passage 3 cells was extracted and initially analyzed by population sequencing. By population sequencing, replicons treated with GS-9190 at $5\times$ and $10\times$ EC_{50} were Y448 wild type and the replicons treated with GS-9190 at $20\times$ EC_{50} harbored a Y448Y/H mixture (Fig. 2B, top row). To assess if minor populations of Y448H were selected by GS-9190, the proportion of the Y448H mutant in the replicon cells at passage 3 was assessed by AS-PCR. As shown in Fig. 2, no detectable Y448H mutant ($<0.5\%$) was observed in replicon cells treated with dimethyl sulfoxide (DMSO). In contrast, treatment of replicon cells with GS-9190 increased the percentage of the Y448H mutant in the population in a dose-dependent manner, with a higher inhibitor concentration resulting in a higher prevalence of the Y448H mutant. At passage 3, subpopulations of Y448H mutants were present at 1.6%, 7.6%, and 19.2% following

treatment with GS-9190 at $5\times$, $10\times$, and $20\times$ EC_{50} , respectively.

The results obtained by AS-PCR were confirmed by clonal analysis. The RT-PCR product from passage 3 of the replicons treated with GS-9190 at $10\times$ EC_{50} were analyzed by TopoTA cloning. Subpopulations of the NS5B Y448H mutant were detected in 4 of 78 (5%) TopoTA clones analyzed (Fig. 2B, bottom row). Analyses of the RNA from virus treated with GS-9190 at $10\times$ EC_{50} by TopoTA and AS-PCR detected low levels of the Y448H mutant variants at 5% and 7.6%, respectively, which standard population sequencing had failed to detect.

Detection of preexisting Y448H mutant in plasma samples of HCV-infected patients. The assay for detection of the Y448H mutation was also performed on plasma samples from 65 treatment-naïve patients infected with HCV genotype 1 (genotype 1a, $n = 42$; genotype 1b, $n = 23$). AS-PCR results were unobtainable from three samples (3/65). The presence of low levels of the Y448H mutant was detected in 5 of the 62 successfully amplified samples. The frequency of the Y448H mutants among these 5 samples ranged from 0.5% to 3.0%, all above the limit of detection for the assay (Fig. 3). In the other 57/62 samples, Y448H mutant levels were below the limit of detection for the assay ($<0.5\%$). By population sequencing, the Y448H mutant was not detected in any of 65 HCV genotype 1-infected samples.

Detection of minor populations of Y448H mutant in HCV-infected patients following treatment with GS-9190 by both AS-PCR and SGS assays. As described above, the Y448H mutant population increased in replicon cells when cells were treated with GS-9190. It is possible that this mutant might also be selected in HCV-infected patients following treatment with GS-9190. To address this possibility and to further validate the AS-PCR assay, Y448H AS-PCR and SGS were performed on five patient plasma samples. These five samples were obtained from 3 HCV genotype 1-infected patients following 8 days of monotherapy with GS-9190, including 2 genotype 1a-infected patients at baseline and day 8 and 1 genotype 1b-infected patient at day 8. As shown in Table 2, HCV RNA from patients A and B was wild type by SGS and population sequencing and $<0.5\%$ had the Y448H mutation by AS-PCR at baseline. At day 8, patients A and B still had wild-type HCV RNA by population sequencing, while AS-PCR detected subpopulations with the Y448H mutation at 2.0% and 3.3% from patients A and B, respectively. Similar results were obtained with SGS, which identified the Y448H mutant from 1/16 (6.3%) and 5/51 (9.8%) clones from the day 8 samples from patients A and B, respectively. For patient C, population sequencing identified four possible amino acid mixtures at position Y448H/Y/C/R at day 8. AS-PCR detected $>50\%$ Y448H mutants and SGS detected 3 amino acids: Y (6/13), H (6/13), and N (1/13). Thus, AS-PCR and SGS showed similar quantification of the predominant Y448H mutant in this sample. The multiple amino acid calls of the population sequencing results are derivatives of all possible nucleotide combinations and do not necessarily indicate that they are present in the viral population.

FIG. 2. Detection of subpopulations of the HCV NS5B Y448H mutant in GS-9190-treated replicon cells. RNA extracted from Huh-Luc cells expressing genotype 1b replicons treated with 5 $\times$, 10 $\times$, and 20 $\times$ EC₅₀ GS-9190 from passage 3 were analyzed for the Y448H mutation by AS-PCR and population sequencing. The 10 $\times$ EC₅₀ GS-9190-treated RNA replicons were also analyzed by TopoTA cloning. (A) HCV RNA reduction in Huh-Luc cells expressing GT1b replicons treated with GS-9190 and corresponding AS-PCR results. (B) Sequence alignment of NS5B amino acids 430 to 480 of the population sequencing of RNA from replicons treated with 5 $\times$, 10 $\times$, and 20 $\times$ GS-9190 EC₅₀ and TopoTA clonal sequencing of the RNA from the replicon treated with 10 $\times$ GS-9190 EC₅₀. Four of 78 clones analyzed contained Y448H (5%). The alignment represents a subset of 13/78 of TopoTA clones sequenced. The sequences of the clones treated with 10 $\times$ GS-9190 EC₅₀ not represented were all wild type at NS5B Y448.

DISCUSSION

The present study describes the development of a sensitive and quantitative real-time AS-PCR assay to detect the Y448H HCV NS5B mutation that confers resistance to GS-9190 and the benzothiadiazine HCV polymerase inhibitors. The AS-PCR assays developed here are capable of detecting the NS5B Y448H mutants in genotype 1a- or 1b-infected plasma samples at levels as low as 0.5%. A good correlation was observed between the AS-PCR results and those of both SGS and clonal analyses (Table 2 and Fig. 3); however, the TopoTA cloning and SGS analyses were more expensive and labor-intensive. Using this new assay, minor populations of the Y448H mutant were successfully detected in replicon cells and HCV genotype 1-infected patients following treatment with GS-9190. In addition, low levels of the Y448H mutant were detected in 5/62 (8%) treatment-naïve HCV genotype 1-infected individuals (i.e., the mutant existed prior to therapy). Compared to

traditional mutation detection assays, including population sequencing, clonal sequencing, or hybridization to allele-specific oligonucleotides, the real-time AS-PCR assay developed here is more sensitive, easy to use, and cost-effective and is therefore suitable for testing of large numbers of samples. However, the assay described here is limited to the detection of the specific mutation amplified by the allele-specific primer. For additional mutations, new assays would need to be developed. Additionally, AS-PCR assays do not allow determination of a linkage between mutations in the viral strain that could compensate for the reduced fitness of the resistant mutant or induce higher levels of resistance. The HCV genotype 1b-infected Con-1 replicons treated with DMSO had <0.5% Y448H mutant (below the assay cutoff) by AS-PCR. In contrast, treatment of the replicon cells with 5 $\times$, 10 $\times$, and 20 $\times$ the EC₅₀ of GS-9190 resulted in the selection of detectable subpopulations of the NS5B Y448H mutant at fre-

FIG. 3. Detection of Y448H mutant subpopulations in HCV genotype 1a-infected treatment-naïve patients. The average ΔC_T values of standard curves were calculated from each of the independent runs from which the patient values were generated (open bars). ΔC_T values for patients from treatment-naïve patients are shown as gray bars. Error bars represent standard deviation of the mean C_T values from 2 to 3 independent experiments. Data for the genotype 1b-infected patient are not shown. C_{mut} , C_T for the mutant; C_{wt} , C_T for the wild type. The dashed line represents an assay cutoff at 0.5%.

quencies of 1.6%, 7.6%, and 19.2%, respectively. The detection of the Y448H mutant in replicon cells treated with 5 $\times$ and 10 $\times$ the EC_{50} of GS-9190 was associated with a 1.5 to 1.9 $\log_{10}$ reduction in replicon HCV RNA. Assuming that the growth of the Y448H mutant was negligible in these few days of treatment, we can estimate that the frequency of the Y448H mutant in the replicon cells prior to treatment was between 0.051% and 0.096%. This estimate is in agreement with the results from other studies for HCV inhibitors. For example, 0.18% of a preexisting M414T NS5B mutant was found in treatment-naïve genotype 1b-infected N replicon cells by AS-PCRs (11). In addition, the frequency of preexisting drug-resistant mutants was estimated by selecting resistant colonies from cell populations in the presence of both HCV inhibitors and G418 for 3 to 4 weeks under conditions in which cells do not undergo splitting.

Using this method, the drug resistance mutation was found to preexist at an estimated frequency ranging from 0.05% to 0.62% in HCV genotype 1b-infected replicons resistant to HCV NS5B nonnucleoside inhibitors or protease inhibitors.

The level of this preexisting resistance limits the degree of the maximal HCV RNA reduction to the 2.5 to 4 $\log_{10}$ units observed in HCV inhibitor monotherapy (1, 18). However, combination therapy could result in the clearance of HCV mutants that are resistant to one drug but are sensitive to other classes of compounds in the regimen, consequently producing a greater reduction in HCV RNA (3, 9, 22).

The levels of the Y448H mutant virus were variable among the HCV-infected patients at baseline in the present study. This variation may indicate the influence of genetic background on the fitness of the mutant, which may be one of the important determinants for the prevalence of a specific mutation. A higher level of drug-resistant variants at baseline could result in a poorer treatment response. Therefore, the determination of the prevalence of key preexisting resistance mutations may be meaningful for predicting the treatment response. For example, naturally preexisting Y448H variants were present at <0.5% by AS-PCR in the majority of the treatment-naïve patients. Therefore, it could be expected that monotherapy with an optimal dose of GS-9190 or benzothiadiazine NNIs would produce a >2.5- $\log_{10}$ -unit reduction of HCV RNA in the majority of patients. However, AS-PCR detected Y448H mutants at levels that ranged from 0.5% to 3% in 5/62 treatment-naïve patients infected with HCV genotype 1, suggesting that the maximal response in these patients may be limited to approximately 2.3 $\log_{10}$ units or less during monotherapy with GS-9190 or benzothiadiazine NNIs. These results are similar to previous findings, in which low levels of preexisting M414T mutants ranging from 0.11 to 0.60% by AS-PCR were detected in 6 of 15 (40%) plasma samples from treatment-naïve HCV-infected patients (11).

In conclusion, novel sensitive real-time AS-PCR assays for quantitatively detecting a key HCV NS5B mutation, Y448H, have been developed and characterized. The assays would be useful to monitor the selection and evolution of Y448H mutants during clinical studies with GS-9190 and other NS5B inhibitors that select for the resistant Y448H mutant. In addition, our results indicate that resistant Y448H mutants preexist at 0.05% to 0.1% levels in replicon cells and are selected rapidly upon *in vitro* treatment with GS-9190. In addition, low levels of Y448H (0.5 to 3%) mutants were detected in 5/62 (8%) treatment-naïve HCV genotype 1-infected patients, and mutant development was observed within 8 days of monotherapy with GS-9190 (4), suggesting the need for combination therapy for the effective treatment of HCV infection.

TABLE 2. Y448H results of patient samples by population sequencing, AS-PCR, and single-genome sequencing methods before and after treatment with GS-9190

Patient	Time point	Genotype	Population	SGS		% isolates with Y448H by AS-PCR
				Residue	No. of isolates with indicated residue/total no. tested (%)	
A	BL ^a	1a	WT ^b	H	0/31	<0.5
A	Day 8	1a	WT	H	1/16 (6.3)	2.0
B	BL	1a	WT	H	0/39	<0.5
B	Day 8	1a	WT	H	5/51 (9.8)	3.3
C	Day 8	1b	H/R/C/Y	H	6/13 (46.2)	>50
				N	1/13	

^a BL, baseline.

^b WT, wild type.

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