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Viral Kinetics of the Y448H HCV NS5B Polymerase Mutant in Patients Treated with the HCV Inhibitor Tegobuvir (GS-9190)

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Abstract

Tegobuvir (GS-9190) is a novel hepatitis C virus (HCV) non-nucleoside NS5B polymerase inhibitor that effectively inhibits HCV replication in HCV-infected genotype (GT) 1 patients. The NS5B Y448H mutation was the most frequent mutation selected in patients receiving tegobuvir. In this study, we employed allele-specific PCR (AS-PCR) to monitor Y448H kinetics and estimate the pre-existing Y448H levels in HCV-infected patients and replicon cells. Y448H AS-PCR was developed with a 0.5% assay cut-off to test replicon cells treated with tegobuvir and samples from HCV-infected GT 1 patients who received 8-days of tegobuvir monotherapy. By population sequencing, Y448H was not detected at baseline in serum samples from any of the 65 patients enrolled in the study. Using the more sensitive AS-PCR, Y448H was assessed in 62/65 patients at baseline and detected at >0.5% in 5/62 patients. Longitudinal patient samples were tested to monitor the Y448H replication kinetics during the 8-day tegobuvir monotherapy. The replication kinetics of the mutant virus were used to extrapolate a median baseline Y448H frequency of 0.025% (-3.6 $\log_{10}$). For the in vitro selection of GT 1b tegobuvir-treated replicon cells, pre-existing Y448H levels were similarly estimated at 0.015% (-3.8 $\log_{10}$). Pre-existing levels of the drug-resistant variant Y448H suggest a maximal 3.6 $\log_{10}$ HCV RNA reduction during monotherapy with optimal doses of NS5B non-nucleoside polymerase inhibitors that select for Y448H. These results aid in the prediction of the patient viral response and study designs to best achieve maximal antiviral response.

Keywords: HCV; Allele-specific PCR; Y448H; Tegobuvir (GS-9190); NS5B non-nucleoside inhibitor

Abbreviations: AS-PCR: Allele-Specific PCR; BID: twice a day drug dose; DAA: Direct-Acting Antiviral; DRM: Drug-Resistant Mutation; EC_{50}: 50% Effective Concentration; GT: Genotype; HCV: Hepatitis C Virus; IFN-α: Interferon-alpha; Peg-IFN: Pegylated-Interferon alpha; RBV: Ribavirin; SOC: Standard of Care; SVR: Sustained Virologic Response; TGV: Tegobuvir (GS-9190)

Introduction

With the discovery and identification of HCV in 1989 [1,2], significant progress has been made in the research and treatment of HCV infection. It is estimated that 2-3% of the world's population is infected with HCV, which can lead to chronic hepatitis, resulting in cirrhosis, hepatocellular carcinoma and a need for liver transplantation [3-5]. HCV, an enveloped RNA virus, encodes a polyprotein of ~3,300 amino acids consisting of 4 structural proteins (Core, E1, E2 and p7) and 6 non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) [6]. HCV has been classified into six major genotypes (GT 1-6) and more than 100 subtypes based on the comparative analysis of the core, envelope, and NS5B nucleotide sequences [7]. In 2007, several isolates from HCV-infected patients were unable to be classified within the six genotypes by sequence analysis, leading to the proposal of GT 7 [8].

Since 2001, the standard of care (SOC) therapy for HCV-infected patients has been a 24-48 week course of weekly subcutaneous, pegylated-interferon alpha (Peg-IFN) injections with twice-daily, oral doses of ribavirin (RBV). Sustained virological response (SVR) was observed in 42-52%, 65-85% and 76-82% of individuals infected with HCV GT 1, GTs 4, 5 or 6 and GTs 2 or 3, respectively [9,10]. Adverse side effects of severe flu-like symptoms, depression, psychoses, and anemia have been reported, resulting in 20% of the patients discontinuing treatment [11,12]. Limited efficacy and side effects with the current SOC have fueled further research to develop direct-acting antivirals (DAAs), targeting genes involved with the virus life cycle. In 2011, the approval of the NS3 protease inhibitors, telaprevir and boceprevir, have led to a new SOC treatment with either telaprevir or boceprevir, Peg-IFN and RBV [13-15]. Now, several DAAs with demonstrated antiviral activity in vitro have advanced into clinical trials. However, monotherapy studies of most HCV DAAs, with the exception of NS5B nucleoside inhibitors such as sofosbuvir, resulted in the selection of drug-resistant mutants in patients and replicon systems [16-19].

Characteristics of HCV may explain the quick emergence of drug-resistant variants upon treatment. Based on the mathematical modeling of viral dynamics during interferon-alpha treatment of HCV-infected patients, the virus has been estimated to produce 10^{12} virion particles per day [20,21]. The RNA-dependent polymerase is error prone without a proofreading ability. Within an infected individual, HCV diversifies into a genetically diverse population of variants known as quasispecies. The high genetic variability and rapid replication rate allows HCV to adapt to the selection pressures of antiviral treatment [22]. Different low-level frequencies of drug-resistant variants have been reported in patients treated with NS5B inhibitors, which resulted in differing viral response and mutant enrichment [23-26]. Determination of low-level drug-resistant variants at baseline through sensitive detection methods, such as allele-specific PCR (AS-PCR), would aid in the prediction of patient viral response to some HCV inhibitors.

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Tegobuvir (GS-9190, TGV) is a novel NS5B non-nucleoside inhibitor of the imidazopyridine class, targeting HCV replication in the replicon system and patients infected with HCV GT 1 [27]. TGV has a novel mechanism of action in which CYP1A-mediated oxidative metabolic activation and assistance from glutathione results in TGV binding covalently and interacting specifically to NS5B, inhibiting viral replication [28]. Population sequencing results of a TGV resistance selection in GT 1b replicon cells identified not only the NS5B mutation of Y448H, but also other NS5B mutations such as C316Y, C445F, and Y452H [27]. Population sequencing can only detect minor populations present at 20-25% of the population. As previously reported, a Y448H AS-PCR assay for HCV GT 1a and 1b has been developed with a 0.5% sensitivity [29]. In this study, we used this highly-sensitive detection method of AS-PCR to monitor the kinetics of the NS5B Y448H mutant in HCV-infected GT 1 patients and TGV-treated replicon cells. Extrapolation of the observed Y448H replication kinetics allowed for the estimation of the pre-existing Y448H levels in HCV-infected patients and replicon cells.

Material and Methods

Clinical study overview and sample selection for allele-specific PCR analyses

GS-US-196-0101 is a double-blind, placebo-controlled, dose-escalation study to investigate the antiviral activity of single and multiple doses of TGV in treatment-naïve patients with chronic GT 1 HCV infection [30]. Longitudinal plasma samples during and after treatment was obtained daily from 20 patients enrolled in the cohorts receiving 40 or 120 mg BID TGV or placebo for 8 consecutive days. All samples had patient consent and originated from the USA.

Replicon constructs and cell lines

The plasmid pFK-rep PI-luc/5.1 encodes a GT 1b (Con1) strain and firefly luciferase reporter; this plasmid was obtained from ReBLikon GMBH [31]. This plasmid consists of two HCV-IRES variants (nucleotides (nt) 1 to 377 or 1 to 389), the neomycin phosphotransferase gene, the encephalomyocarditis virus IRES, which directs translation of HCV sequences from NS2 or NS3 up to NS5B, and the 3' NTR [31]. For efficient replication, the three mutations of T1280I and E1202G in NS3 and K1846T in NS4B were introduced into this plasmid by site-directed mutagenesis [32-34]. This plasmid was transfected into Huh-7, a human hepatoma cell line, and the replicon was maintained by culturing in the presence of Geneticin (G418). Replicon cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with Geneticin (G418), penicillin/streptomycin and non-essential amino acids (Life Technologies, Carlsbad, CA) and fetal bovine serum (FBS) (Hyclone, Logan, UT). Cells were passaged every 3-4 days to maintain 90-95% subconfluent levels.

RNA extraction, cDNA synthesis and NS5B PCR amplification

Total RNA was extracted from HCV-infected patient plasma samples with the QIAamp viral RNA minikit (Qiagen, Valencia, CA) and HCV replicon cells with the RNeasy minikit (Qiagen, Valencia, CA) following manufacturer's instructions. cDNA synthesis was performed with the MonsterScript reverse transcriptase (Epicentre, Madison, WI) and the HCV-specific primers 1aNS5B-3'9386-5′ CTA AGA GGC CGG AGT GTT TAC-3′ and 1bNS5B-3'9459 -5′ CCT ATT GGC CTG GAG TGT TTA GCT C-3′ for genotype 1a and 1b respectively [29]. cDNA was used as a template for consecutive PCR amplification of the NS5B gene using cycling conditions previously described [29].

Y448H allele-specific real-time PCR

NS5B PCR products were diluted in M1E01 buffer (EraGen Biosciences, Madison, WI) to approximately 10^7 copies per AS-PCR reaction. A single reverse primer (MM2351) was used with each set of genotype and allele-specific forward labeled primers. For GT 1a samples, forward primers MM2348-FAM-CCCTTGATTGCGAAATCTA and MM2350-HEX-CCCTTGATTGCGAAATCCA were used for the assay. For GT 1b samples, there were two available forward primer sets for the assay. The first primer set is MM2419-FAM-GGCCCTGTATTGTCAGATCT and MM2420-HEX-GGCCCTGTATTGTCAGATCC. The second primer set is MM2417-FAM-GGCCCTGTATTGTCAAATCT and MM2418-HEX-GGCCCTGTATTGTCAAATCC. AS-PCR was performed on a LightCycler 480 Real-Time PCR Instrument (Roche Applied Science, Indianapolis, IN) using cycling conditions previously described [29].

Standard curves of diluted GT 1a and 1b wild type 448Y and mutant 448H clonal DNA at 10^6 copies/µl were prepared by mixing 100%, 50%, 10%, 5%, 1%, 0.5%, 0.1% and 0% 448H mutant. Standard curve samples were amplified under the same PCR conditions as the patient samples to account for PCR disincorporation background. For each AS-PCR assay run, both standard curve and patient samples were tested on the same plate to estimate the Y448H subpopulation. The assay range for Y448H mutant detection in pre-amplified PCR products was 0.5% to 50%.

Viral kinetics of HCV mutants and back-calculation of pre-treatment Y448H levels

Y448H AS-PCR was performed to quantify the percentage of Y448H mutant at each available time point. For each available sample per patient, 2-3 independent AS-PCR runs were performed to get an average Y448H percentage. This percentage was converted to IU per ml by multiplying it against the total HCV RNA viral load to evaluate Y448H mutant viral kinetics. To back-calculate the pre-existing levels of the Y448H mutant, a linear regression line was fitted to the mutant viral load at the available time points during the 8-day TGV monotherapy for all tested patients. Using the linear regression slope as the number of Y448H copies ($\log_{10}$) at baseline, the Y448H baseline frequency was calculated by dividing the baseline Y448H copies by the baseline total viral load.

Treatment of replicon cells with Tegobuvir (GS-9190)

10^6 GT 1b replicon cells were treated with 10X EC_{50} and 20X EC_{50} TGV over 10 days in a T75 flask and incubated at 37°C in 5% CO_2. Once the cells reached 90-95% confluence, 10^6 cells were placed into a new T75 and 10^6 cells were collected for RNA extraction as described above at day 0, 3, 6 and 10.

Results

Y448H detection in patient samples

In clinical study GS-US-196-0101, a total of 65 treatment-naïve GT 1a and 1b HCV-infected patients were tested by AS-PCR (Figure 1). At baseline, Y448H AS-PCR was successfully performed on 62/65 patients with 3/65 baseline samples failing due to primer mismatches. In 5/62 patients, Y448H was detected above the 0.5% assay background and ranged from 0.5% to 3.0% Y448H; 57/62 patients had <0.5% Y448H [29]. A total of 36 patients received TGV 40 or 120 mg BID over 8 days. For longitudinal AS-PCR analyses, 19/36 patients were selected on the basis of having detectable Y448H by population sequencing during

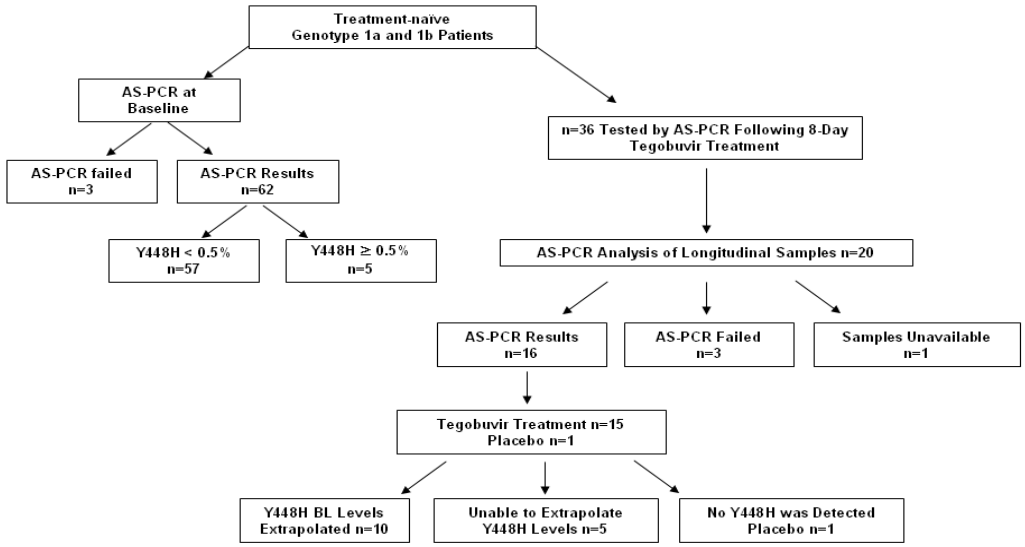


Figure 1: AS-PCR Results for Clinical Study GS-US-196-0101. Y448H AS-PCR was performed on baseline samples from 65 treatment naive HCV-infected genotype 1 patients. 62/65 patients had detectable Y448H at baseline. 20/36 patients in cohorts that received 8 day Tegobuvir (GS-9190) monotherapy underwent AS-PCR analysis of longitudinal samples. Since 3/20 patients failed the assay and 1/20 patient did not have samples available, AS-PCR results were obtained for 16/20 patients, including 1 placebo, to estimate pre-existing Y448H BL levels.

treatment (Figure 1). In addition to the 19 patients, 1 placebo patient was included as a negative control for the Y448H AS-PCR assay. With a 0.5% assay cut-off, results were obtained for 16/20 patients. There were 3 patients who failed the assay because RNA isolation was unsuccessful and one patient did not have available samples for analysis. There were a total of 9 GT 1a and 6 GT 1b TGV-treated patients. In GT 1a HCV-infected patients, AS-PCR detected >50% Y448H in 7/9 patients by day 8. For the remaining two patients, there was an average of 7.1% and 9.1% Y448H at day 8, with multiple earlier time points of <0.5% Y448H. In GT 1b HCV-infected patients by day 8, AS-PCR detected a >50% Y448H in 1/6 patients and <50% Y448H in 5/6 patients (4.5%, 11.7%, 14.6%, 20.2%, 40.4%, 48.9%). For the patient with >50% Y448H by day 8, there was an average of 92.9% Y448H at day 8. As expected, Y448H was not detected above the 0.5% assay cut-off at any available time point for the placebo patient. Overall during treatment, AS-PCR detected >50% Y448H in at least 1 time point in 8/15 HCV-infected GT 1 patients during TGV monotherapy.

Y448H viral replication kinetics in patients using AS-PCR

Longitudinal samples from 15 patients were evaluated to monitor Y448H replication during and after the 8-day TGV monotherapy. The average Y448H viral load change was plotted and compared to the viral load change of the wild-type virus (Figure 2). During TGV monotherapy, the wild-type viral load gradually decreased by an average of approximately -2 $\log_{10}$ IU/ml as the Y448H mutant viral load gradually increased by an average of approximately 2 $\log_{10}$ IU/ml. After monotherapy in the absence of TGV at the 240 hour time point, Y448H mutant viral load reached and remained at 3 $\log_{10}$ IU/ml, whereas the wild-type viral load decreased to 1.5 $\log_{10}$ IU/ml. There was an observed linear dependence of the Y448H mutant viral load versus time and an inverse relationship between the wild-type viral load and Y448H mutant viral load.

Back-calculation of pre-existing Y448H levels in patients

AS-PCR detected <0.5% Y448H for all baseline samples. Although

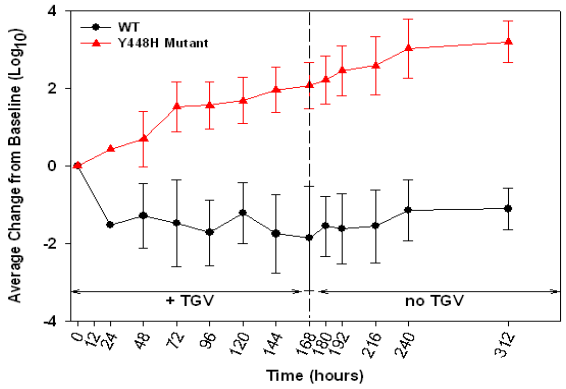
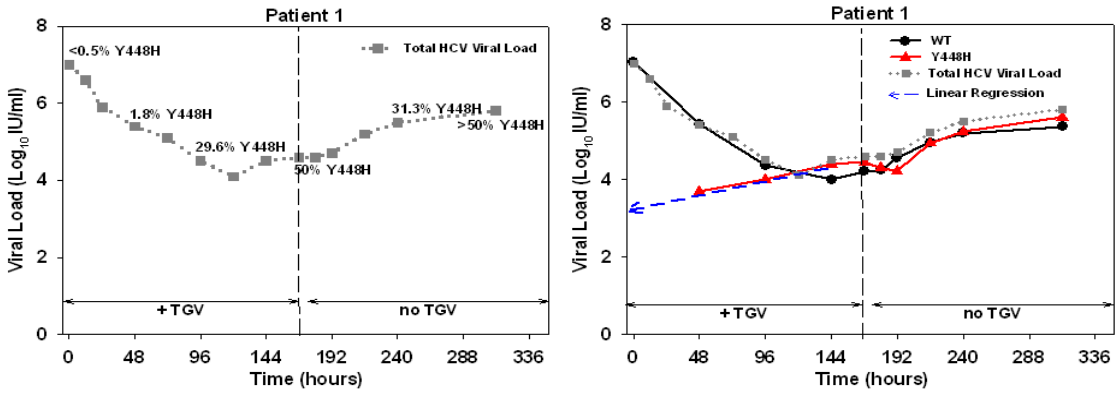


Figure 2: Average Replication Kinetics for Y448H and Wild-type Virus in HCV-infected Genotype 1 Patients. Using AS-PCR, the percentage of Y448H mutant was determined for each available time point for 16 patients. 8 day Tegobuvir (GS-9190) monotherapy suppressed the wild-type virus allowing for the gradual growth of the Y448H mutant. After monotherapy, Y448H viral load continued to increase while the wild-type viral load remained stable.

the baseline HCV viral load is known, the exact Y448H percentage could not be determined by AS-PCR because it is below the detection limit. A linear regression analysis was performed on the Y448H mutant viral load at available and earlier time points during TGV treatment to estimate by back-calculation the pre-existing Y448H levels. At least two time points from the 8-day monotherapy in which Y448H was detected above the 0.5% assay background were needed for the linear regression analysis. As shown for a representative patient, this calculation was based on the observation that there is a linear dependence of the Y448H mutant subpopulation with time (Figure 3). By fitting the line to the earliest time points of detectable Y448H, the pre-existing Y448H levels can be calculated and estimated because the slope is determined and the time variable is known (Figure 3). Since multiple, earlier time points from TGV monotherapy were unavailable for all selected patients, Y448H pre-existing levels could only be back-



Linear Regression Equation	Log_{10} Y448H Copies at Baseline	Y448H Copies at Baseline	Y448H Copies at Baseline/Total Viral Load at Baseline	Log_{10} (Y448H Copies at Baseline/Total Viral Load at Baseline)
Log_{10} viral load = 3.309 + (0.00739 x hours)	3.309	2037	0.00019	-3.72

Figure 3: Example of Back-Calculation of Pre-Existing Y448H Levels. AS-PCR detected and estimated Y448H levels at available time points during and after 8-day Tegobuvir monotherapy. Pre-existing Y448H levels were extrapolated by performing a linear regression analysis of at least two time points during monotherapy with Y448H.

calculated for 10/16 patients (Figure 4). As expected for the placebo patient, Y448H was not detected at any available time points above the assay background; therefore, no pre-existing Y448H level could be extrapolated. At baseline, all patients had >7 log_{10} IU/ml wild-type HCV viral load, which decreased during the 8-day monotherapy. After monotherapy, both wild-type and mutant Y448H viral load increased. Overall, the estimated median pre-existing Y448H levels was -3.61 log_{10} and ranged from -3.72 log_{10} to -3.42 log_{10} for the 25th to 75th percentile. The estimated median pre-existing Y448H frequency was 0.025% and ranged from 0.019% to 0.038% for the 25th to 75th percentile (Figure 4).

Estimation of pre-existing Y448H levels in replicon cells

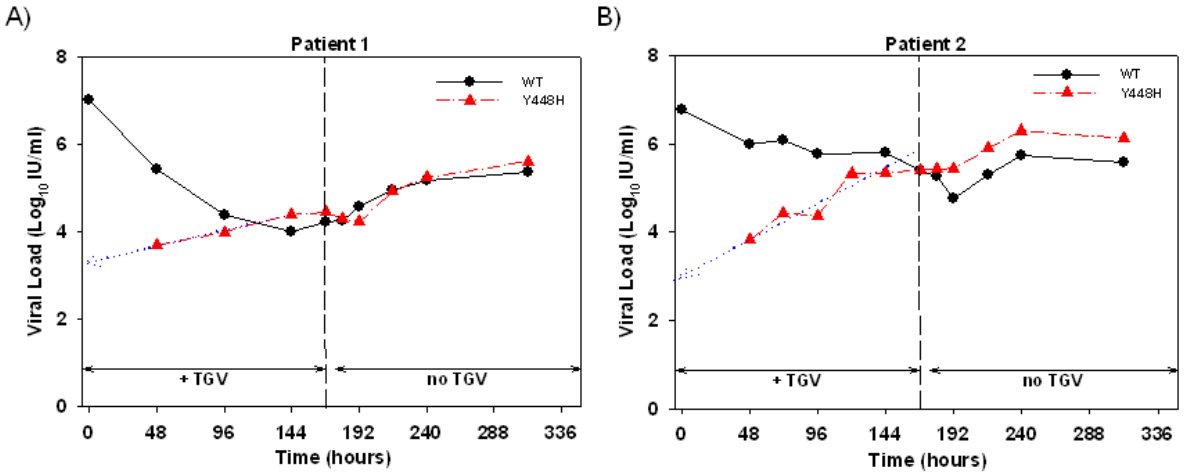
For two independent TGV selections with GT 1b replicon cells, samples from all 4 passages for each 10-day selection were tested with AS-PCR to monitor the Y448H viral replication kinetics. The Y448H mutant subpopulation was detected above the 0.5% assay background level for 10X EC_{50} and 20X EC_{50} TGV treatments at only passage 3 (day 10). Using AS-PCR detection, the Y448H mutant subpopulation ranged from 0.6% to 5.0% and averaged 2.4% for passage 3 for all 10X EC_{50} and 20X EC_{50} selections. After passage 3 (day 10), Y448H was only detected at 1.1% for the 10X EC_{50} TGV treatment of the second selection. Pre-existing Y448H levels were calculated by multiplying the percentage of the passage 3 (day 10) Y448H mutant population by the ratio of the passage 3 mutant viral load to the baseline viral load. Pre-existing Y448H levels ranged from 0.002% to 0.040% and averaged 0.015% (-3.8 log_{10}).

Discussion

Using population-based DNA sequencing, the Y448H NS5B mutant has been detected in some HCV-infected patients that received TGV and in HCV replicon cells treated with TGV [27,29]. At baseline, population sequencing did not detect Y448H in any of the 65 treatment-naïve patients enrolled in clinical study GS-US-196-0101 [29]. However, population-based DNA sequencing can only detect variant populations present at >20% of the population. With the same baseline samples, the more sensitive method of AS-PCR detected the Y448H mutant subpopulation in 5/62 analyzed patients and ranged from 0.5% to 3.0% Y448H; 57/62 patients had <0.5% Y448H. In GT 1b replicon cells treated with TGV, the Y448H mutant subpopulation was only first detected at passage 3 (day 10) and averaged 2.4%. These results confirm that AS-PCR is a more sensitive detection method for detected mutant genomes, such as Y448H, in comparison to population sequencing. A limitation of AS-PCR is that allele-specific primers must be designed for each specific mutation and that the technique does not allow for mutation linkage analysis.

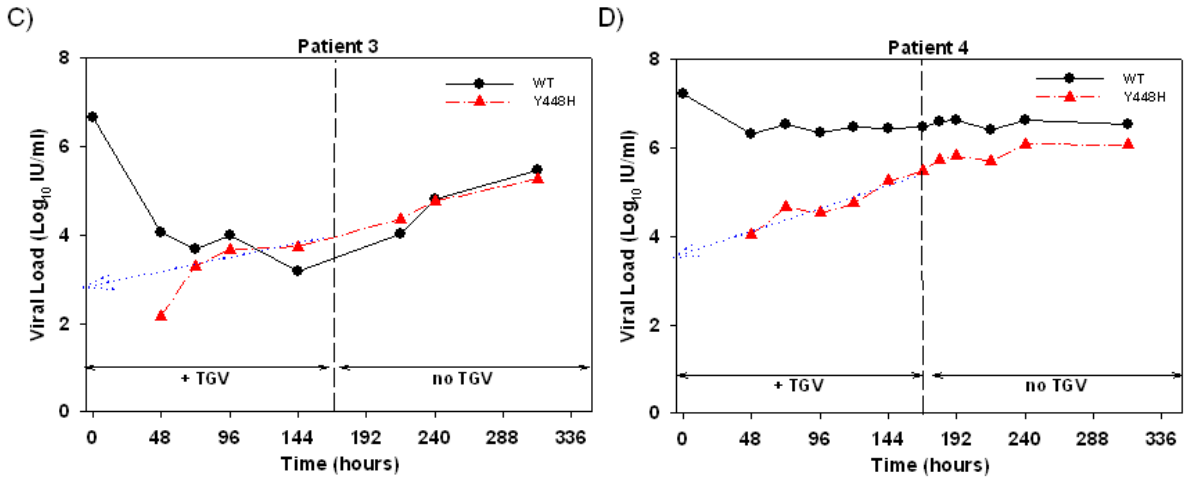
The AS-PCR testing of longitudinal patient samples allowed for the monitoring of Y448H replication during and after the 8-day TGV monotherapy. Patients who received 40 or 120 mg BID TGV over 8 days showed a maximal wild-type viral load decline of 2.5 log_{10} IU/ml. After the 8-day monotherapy, both the wild-type and Y448H viral load gradually increased. The replication kinetics of Y448H mutant after treatment as compared to the wild-type virus suggested that Y448H mutation did not strongly impact the fitness of the virus *in vivo* or that additional mutations developed to compensate for the previously observed replication defect of Y448H in the replicon system [35]. After monotherapy, the persistence of the Y448H mutant may also have been due to the suppression of the wild-type virus that increased the availability of target cells for new infection. At the end of drug treatment in the absence of a HCV inhibitor, a new equilibrium of cells infected with wild type and Y448H viruses was established and both wild-type and mutant viruses compete for the limited, yet available target cells. Because Y448H has a replication capacity comparable to wild-type virus, increased drug dose or a combination therapy may aid in lowering the mutant population so that it would be unable to compete with the wild-type virus for infection of the target cells.

Other NS5B non-nucleoside drug-resistant mutants, such as M414T and M423T have been detected in HCV-infected patients and the replicon system. Pre-existing levels of M414T ranged from 0.11-0.60% in treatment-naïve GT 1 HCV-infected patients and



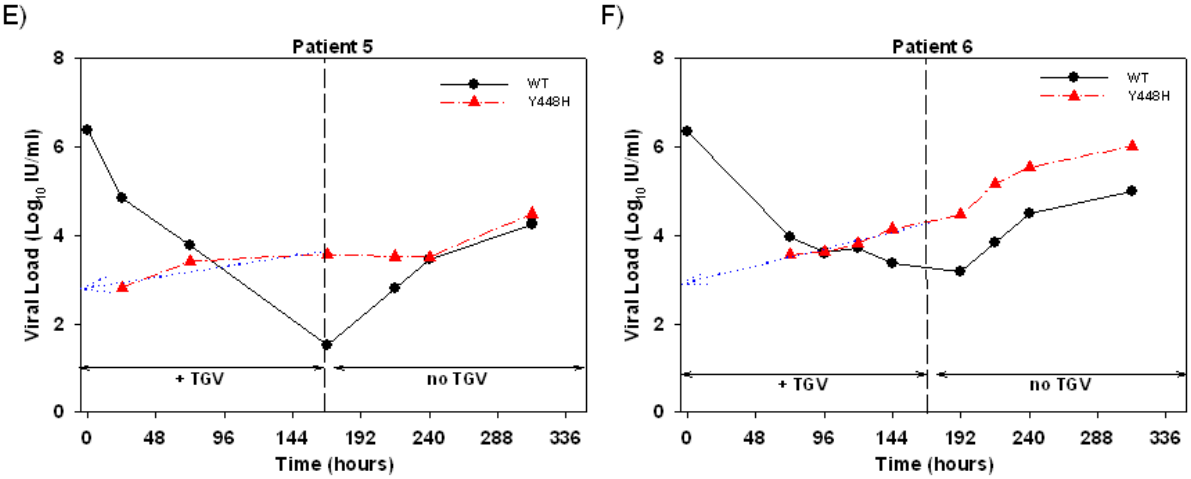
•Genotype 1a, 120 mg BID
•Pre-treatment Y448H levels: 0.019%; -3.72 $\log_{10}$

•Genotype 1a, 40 mg BID
•Pre-treatment Y448H levels: 0.020%; -3.70 $\log_{10}$



•Genotype 1a, 120 mg BID
•Pre-treatment Y448H levels: 0.023%; -3.65 $\log_{10}$

•Genotype 1a, 120 mg BID
•Pre-treatment Y448H levels: 0.027%; -3.57 $\log_{10}$



•Genotype 1a, 40 mg BID
•Pre-treatment Y448H levels: 0.029%; -3.54 $\log_{10}$

•Genotype 1a, 120 mg BID
•Pre-treatment Y448H levels: 0.038%; -3.42 $\log_{10}$

To be continued...

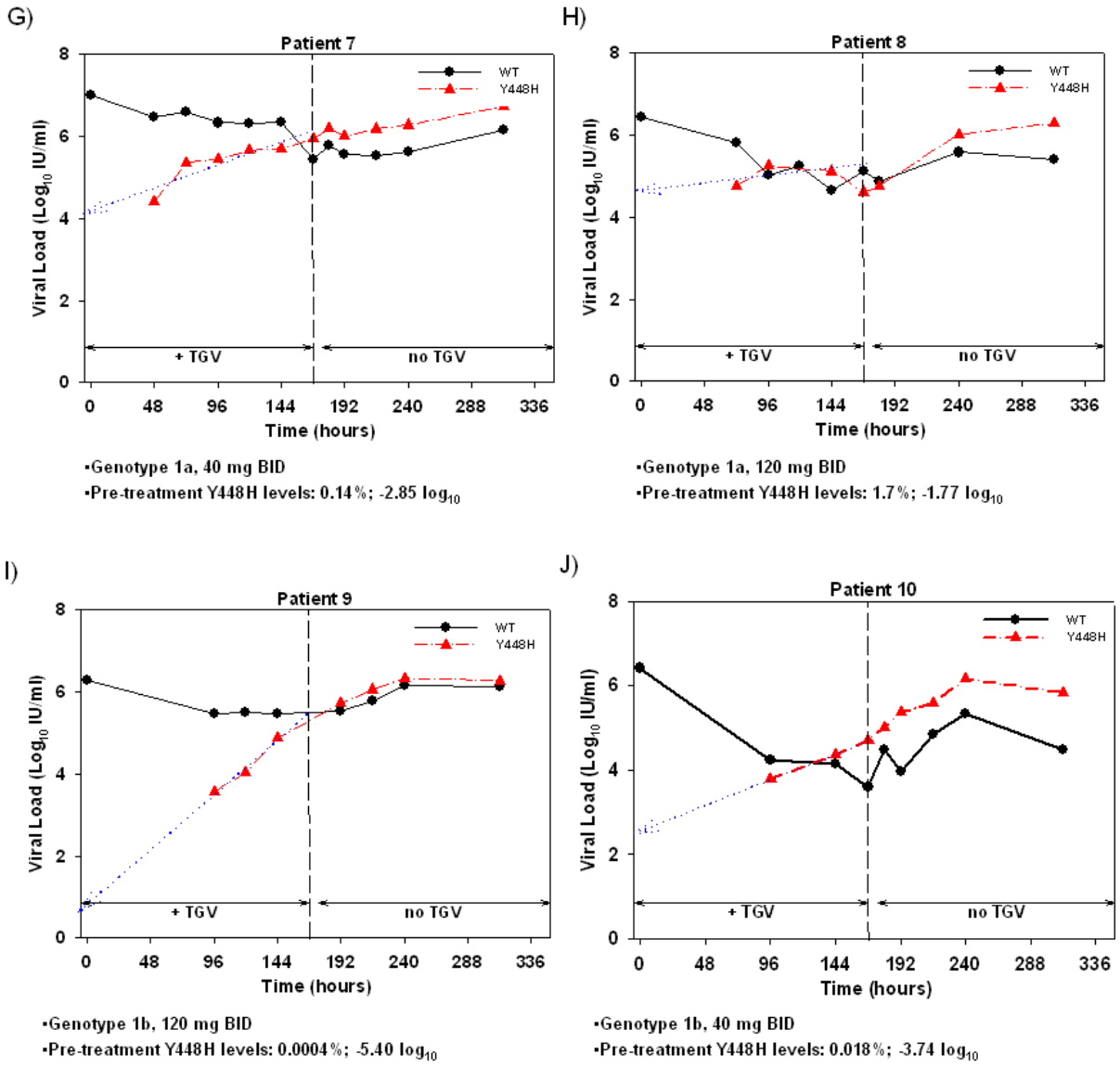


Figure 4: Replication Kinetics of Y448H during Tegobuvir Treatment and Back-Calculation of Pre-Existing Y448H Levels in HCV-infected Genotype 1 Patients. Patients received either 40 or 120 mg BID of Tegobuvir (GS-9190) over 8 days. AS-PCR was performed on longitudinal samples collected during and after monotherapy. This allowed for the monitoring of Y448H replication kinetics during treatment to back-calculate pre-existing Y448H levels by linear regression analysis.

was 0.22% in treatment-naïve GT 1b-con1 replicon cells by AS-PCR [36]. In two phase 1b clinical studies of Filibuvir (PF-00868554), a NS5B non-nucleoside inhibitor, with GT 1 HCV-infected patients, the mean maximal HCV RNA reduction was -2.30 $\log_{10}$ IU/ml for patients treated with 700 mg BID. Filibuvir (PF-00868554) selected for the drug-resistant mutant M423T, which showed a 761-fold reduced susceptibility towards the drug [3,37].

The Y448H AS-PCR assay we describe has a 0.5% lower limit of detection. Since replication kinetics showed a linear dependence of Y448H viral load with time. Pre-existing Y448H levels, which may be lower than 0.5%, were estimated by back-calculation with a linear regression analysis. It was observed that the Y448H population still increased during the TGV-treatment, indicating that the 40 or 120 mg doses were not sufficient to fully suppress the wild-type and mutant populations. Therefore, we made the assumption that the Y448H replication rate between the baseline and the first time point with Y448H detection during monotherapy remains constant and correlated with the fitted linear regression line. This assumption may have resulted in the overestimate or underestimate of pre-existing Y448H levels due to the inclusion or exclusion of certain time points for the linear regression analysis or potential changes in mutant replication rate after initiation of drug therapy. For some patients, such as Patients 3, 7 and 8, the estimated pre-existing Y448H level might be an overestimate because the fitted linear regression line did not include the first Y448H detection time point at 24 hours. To determine more precisely the absolute pre-existing mutant levels in a baseline sample, more sensitive methods with the capacity to measure frequencies on the order of 0.01% would need to be developed.

In summary, the increased sensitivity of the AS-PCR method detected the Y448H NS5B mutant at earlier time points in patients during monotherapy and in replicon cells treated with TGV. This allowed for the back-calculation and estimation of the median pre-existing Y448H levels as -3.61 $\log_{10}$ (0.025%) in HCV-infected GT 1 patients and -4.06 $\log_{10}$ (0.01%) in GT 1 replicon cells by linear regression analysis. These results enhance the understanding of pre-existing mutant subpopulations and reinforce the need for HCV combination therapies that can suppress the pre-existing mutants to achieve durable antiviral responses.

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