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Sensitive Detection of Y448H NS5B Mutant Viruses in Patients Infected with Genotype 1a and 1b HCV

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Introduction

- Genetic diversity of HCV results from rapid virus replication and nucleotide misincorporation by the error-prone NS5B polymerase
- HCV antiviral drug resistance mutations may pre-exist in a viral population in antiviral treatment-naive patients at low frequencies
- The NS5B Y448H mutant confers resistance to various non-nucleoside NS5B inhibitors in vitro and has been observed in patients who received GS-9190[1]
- Standard population sequencing is limited in detecting minor variants (<25%) within a viral population

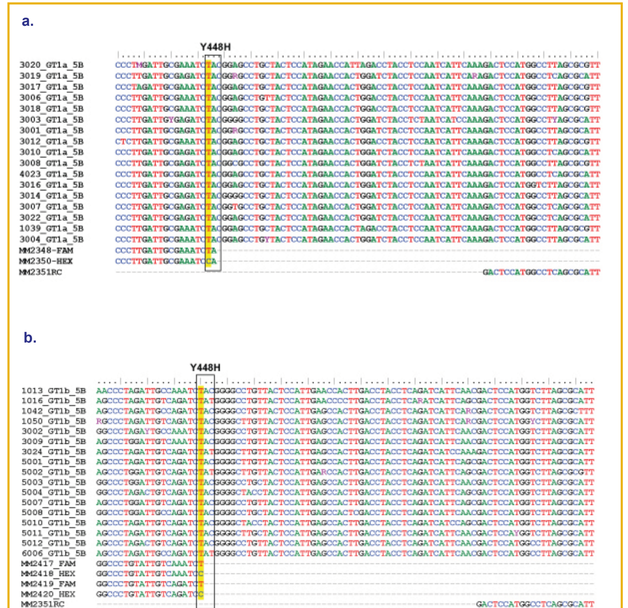
Objectives

- To develop a highly sensitive allele-specific PCR (AS-PCR) assay to detect low levels of NS5B Y448H variants in plasma of HCV genotype 1a and 1b infected patients
- To test for the presence of the NS5B Y448H variants in a panel of antiviral treatment-naïve HCV infected patients by AS-PCR

Methods

- Standard curves were generated for both GT1a and GT1b, using fixed combinations of mutant and wild-type clones.
  - NS5B Y448H mutant variants of 100%, 50%, 10%, 5%, 1%, 0.5%, 0.1% and 0%
  - Performed on a real-time MultiCode Technology (EraGen Biosciences, Madison, WI)
- Huh-Luc cells stably expressing GT1b replicons were treated with 5X-, 10X- or 20X-EC_{50} of GS-9190; RNA was extracted and analyzed for Y448H. Clonal analysis was also performed using standard TopoTA cloning
- NS5B was RT-PCR amplified from 65 antiviral treatment-naïve HCV infected subjects and tested for the presence of Y448H
- Population sequences were obtained from RT-PCR products and single-genome sequencing (SGS) analyses were performed by serial dilution of cDNA

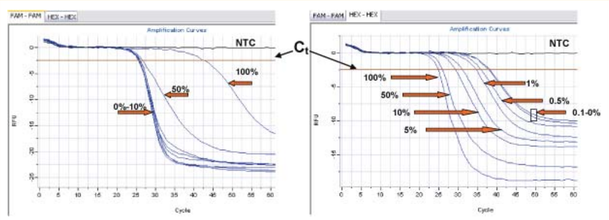
Figure 1. HCV GT1a and GT1b Nucleotide Population Sequence Alignment of the NS5B Y448H Region from Antiviral Treatment-Naïve Patients to Design AS-PCR Primers



AS-PCR primers were designed by aligning NS5B population sequences of the antiviral treatment naïve patients, genotypes (GT) GT1a (Fig. a) and GT1b (Fig. b), in order to differentiate between Y448H (CAC) mutant variant and Y448Y (TAC) wild-type (highlighted). One set of GT1a (MM2348-FAM/MM2350-HEX) and two sets of GT1b (MM2417_FAM/MM2418_HEX & MM2419_FAM/MM2420_HEX) forward-labeled primers pairs with a single reverse primer were designed. Sequences in these figures represents a subset of the 65 sequences analyzed in designing the AS-PCR primers

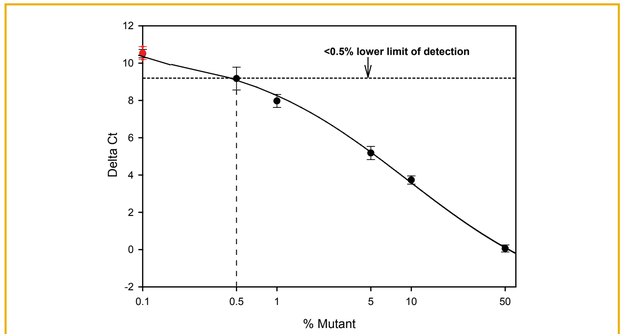
Results

Figure 2. MultiCode RTx PCR Amplification Data of the AS-PCR Y448H Standard Curve from the Wild-type and Mutant Channels



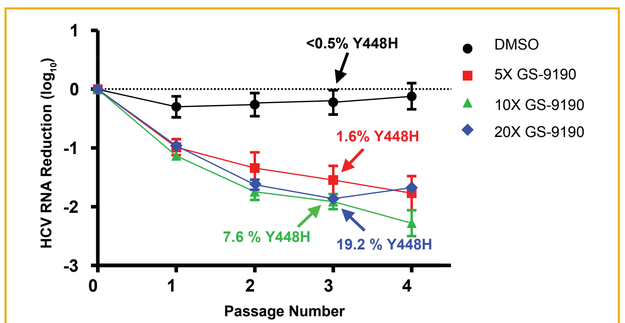
The percent of NS5B Y448H in each sample was determined by plotting the ΔCt, which is the Ct-m of the mutant channel fluorescence (Hex) minus the Ct-wt of the wild-type channel fluorescence (Fam), against the standard curve.
Ct: cycle threshold, NTC: no template control

Figure 3. Standard Curve of the Delta Cycle Threshold (ΔCt) for the NS5B Y448H % Mutant Standards (n=10)



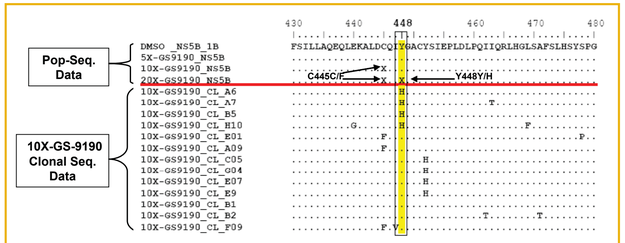
Standard curve of 10 AS-PCR runs derived from the ΔCt calculated from the 50%, 10%, 5%, 1%, 0.5%, 0.1% and 0%(red) NS5B Y448H mutant mixes. ΔCt values of standard curve samples were used to quantify the presence of the 448H mutant in each plasma sample. Calculation of the ΔCt value of the standard curves set the lower limit of detection at 0.5%.

Figure 4. HCV RNA Reduction in Huh-Luc Cells Expressing GT1b Replicons Treated with 5X-, 10X,- and 20X-EC_{50} of GS-9190 and Corresponding AS-PCR Results



RNA was extracted from the Huh-Luc cells expressing GT1b replicons treated with 5X-, 10X-, and 20X-EC50 GS-9190 from passage 3 were analyzed for Y448H mutant by AS-PCR

Figure 5. Clonal Sequence Alignment of NS5B Amino Acids 430-480 Against Population Sequencing Data of Treated Replicon RNA



RNA was extracted from the HuhLuc cells expressing GT1b replicons treated with 5X-, 10X-, and 20X-EC50 GS-9190 and analyzed by population sequencing and Topo-TA cloning. 4/78 clones analyzed contained Y448H (5%). Alignment represents a subset of 13/78 of clones sequenced. The 10X-GS-9190 clonal sequences not represented were wild-type at NS5B Y448.

Figure 6. AS-PCR and Population Sequencing Results from the 65 Antiviral Treatment Naïve Patients Tested for HCV NS5B Y448H

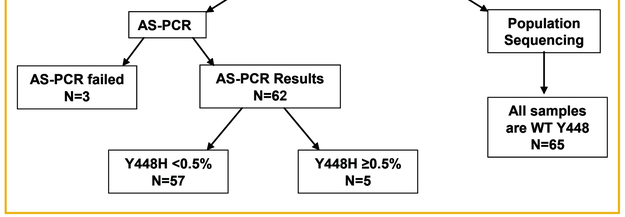
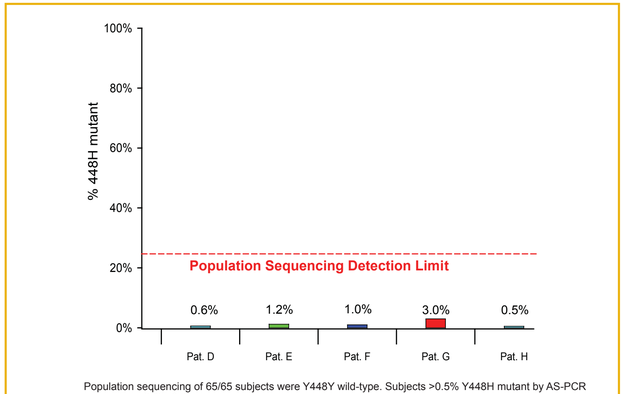


Figure 7. Low Levels of Y448H in Treatment-Naive Subjects with Detectable Mutant by AS-PCR



Population sequencing of 65/65 subjects were Y448Y wild-type. Subjects >0.5% Y448H mutant by AS-PCR ranged from 0.5%-3.0%

Table 1. Y448H Results of Subjects by Population Sequencing, AS-PCR and/or Single Genome Sequencing Methods Prior to/after 7 Days of GS-9190 Monotherapy

Subject	Time Point	GT	Pop-Seq @448	SGS Analysis @ 448H clone / total clones (%)	AS-PCR % Y448H
A	bl	1a	WT	H: 0/31	<0.5%
	d8	1a	WT	H: 1/16(6.3%)	2.0%
B	bl	1a	WT	H: 0/39	<0.5%
	d8	1a	WT	H: 5/51(9.8%)	3.3%
C	d8	1b	H/R/C/Y	H: 6/13 (46.2%) N: 1/13	>50%

Results Summary

- An AS-PCR assay has been developed capable of detecting the NS5B Y448H mutant in genotype 1a or 1b HCV replicons or infected patients when present at levels as low as 0.5%
- HCV replicons treated with GS-9190 showed by AS-PCR 1.6% to 19.2% Y448H by passage 3
- AS-PCR showed a good correlation with single genome sequencing and clonal analysis
- Naturally occurring low levels of Y448H variants were detected in 5/62 (8%) treatment-naive patients infected with HCV GT1 by AS-PCR

Conclusions

- **An AS-PCR assay has been developed capable of detecting low levels of the NS5B Y448H mutant down to 0.5% in genotype 1a and 1b HCV**
- **This assay can be used to monitor the selection and decay of the Y448H mutant in HCV infected patients during and off treatment with GS-9190 and other "Site 3" NS5B inhibitors**
- **Naturally occurring low levels of Y448H variants suggests the need for combination therapy with multiple anti-HCV agents with distinct resistance profiles**

References & Acknowledgements

1. "Antiviral Response and Resistance Analysis of Treatment-Naive HCV Infected Subjects Receiving Single and Multiple Doses of GS-9190,". Poster 833, AASLD 2010, October 29 – November 2, 2010, Boston, MA

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