Short Communication

Validation of interleukin 28B genotyping assay for clinical use

Mark A. Cervinski *, Soya S. Sam, Heather B. Steinmetz, Brendan Wood, Gregory J. Tsongalis

Department of Pathology, The Geisel School of Medicine at Dartmouth, Dartmouth-Hitchcock Medical Center, Lebanon, NH, United States

A R T I C L E   I N F O

Article history:
Received 19 November 2013
Received in revised form 10 January 2014
Accepted 13 January 2014
Available online 23 January 2014

Keywords:
Hepatitis C
Interleukin 28B
Sustained viral response
HCV
IL-28B

A B S T R A C T

Objectives: The favorable CC genotype at rs12979860 upstream of the interleukin (IL)-28B gene is correlated with a greater post-treatment sustained virologic response rate in chronic hepatitis C infected patients. We report on our validation of a clinical genotyping assay for rs12979860 polymorphisms in the IL28B locus.

Design and methods: The rs12979860 genotype was determined using a TaqMan® Real-Time PCR allelic discrimination assay with primers and probes specific for the C and T alleles on the Applied Biosystems 7500 Fast Real-Time PCR System.

Results: The rs12979860 genotype determined by our assay was concordant with the genotypes obtained from a reference laboratory. The allele frequency was similar to that reported in the HapMap project (rs12979860 C = 0.65, T = 0.35) and did not deviate from Hardy–Weinberg equilibrium.

Conclusion: Clinical availability of this assay in conjunction with other factors will allow the prediction of the individual patient’s response to therapy.

© 2014 The Canadian Society of Clinical Chemists. Published by Elsevier Inc. All rights reserved.

Introduction

Chronic hepatitis C (CHC) infection affects approximately 3.2 million people in the U.S. and approximately 150 million people globally [1–10]. CHC infection is the leading cause of cirrhosis, an important risk factor for the development of hepatocellular carcinoma and the leading case for liver transplantation. The prior standard of care treatment for viral hepatitis C (HCV) infection was a 48-week regimen of pegylated interferon alpha and ribavirin (PegIFN/RBV). This regimen achieved a sustained virologic response (SVR) in approximately 50% of HCV genotype 1 infected individuals. Individuals infected with genotypes 2 and 3 have higher rates of SVR following PegIFN/RBV therapy [7,8]. An association between SVR and allelic variants upstream of the interleukin (IL)-28B gene has recently been demonstrated [9]. Of these polymorphic sites, the CC allelic variant at rs12979860, approximately 3 KB upstream of the IL28B gene, shows the greatest association with SVR [9]. Genotype 1 infected patients with favorable CC genotype demonstrate a 2- to 3-fold increase in SVR following PegIFN/RBV therapy when compared to patients with either the heterozygous CT or homozygous TT genotype [11]. While the rs12979860 genotype is a pretreatment predictor of SVR, when considered in a multivariate logistic regression analysis with rapid virologic response (RVR) the IL28B genotype is not a significant predictor of SVR in genotype 1 patients [10].

The rs12979860 genotype demonstrates the same pretreatment predictive ability in HCV genotype 2 and 3 infected patients. In a study combining genotype 2 and 3 patients the SVR rates were 82% for CC patients, 75% for CT patients and 58% for TT genotype patients, respectively [9,12]. For genotype 4 HCV patients the favorable CC genotype demonstrated an 81.8% SVR rate as compared to a 46.5% and 29.4% SVR rate for CT and TT genotypes [13]. This association between the CC genotype and SVR was maintained in a multivariate analysis; however the analysis did not include RVR as a variable.

The rs12979860 genotype appears to also predict treatment success with the new direct acting serine protease antivirals (Telaprevir or Boceprevir) in treatment naïve patients [3–6,9]. However, there does not appear to be an association between IL28B genotype and response to direct acting agents in patients who have failed previous treatment or have since relapsed [12,13]. For the foreseeable future assessment of a patient’s IL28B genotype will remain a useful pretreatment predictor of therapeutic success and for this reason we have chosen to validate a laboratory developed test to fill this need.

There are many technologies available in the molecular pathology laboratory to query the patient’s genotype at rs12979860. We have chosen to validate an assay that utilizes TaqMan® Real Time-PCR (Applied Biosystems) technology for the assessment of our patients’ genotype at rs12979860. In this report we outline our validation study of this laboratory developed test.

Materials and methods

Specimens and controls

Genomic DNA was extracted from 500 randomly selected, de-identified remnant EDTA anticoagulated patient derived whole blood samples with the EZ1 DNA blood kit on the Qiagen EZ1 BioRobot workstation (Qiagen, Valencia, CA). Additionally, 50 samples from physician ordered IL28B testing were extracted in the same manner. All extracted DNA samples were analyzed for purity and yield via absorbance
spectroscopy A260/A280 using the NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Assay controls for the IL28B rs12970860 locus were gBlock™ gene fragments (Integrated DNA Technologies).

Genotyping

Allelic discrimination reactions for the rs12979860 C and T alleles were performed using either commercially available or custom TaqMan® Real Time-PCR primers or probes from Applied Biosystems on the Applied Biosystems 7500 Fast Real-Time PCR system. Commercially available primers and probes were used in a limited fashion to determine the allelic ratios of a large population of remnant patient samples as a training set. Custom primer and probes were then synthesized for validation of the clinical assay (Table 1). The PCR cycling conditions were as follows: initial denaturation at 95 °C for 20 s; followed by 40 cycles of denaturation at 95 °C for 3 s, and annealing/extension at 60 °C for 30 s. Fluorescence signal intensity due to degradation of TaqMan probe was quantified during the annealing/denaturation phase of each PCR cycle via the software supplied with the Applied Biosystems 7500 Fast Real-Time PCR system. The identity of the IL28B allelic variants was assessed by comparing the relative endpoint fluorescence created by the degradation of each fluorescently labeled TaqMan probe (FAM and VIC). The resulting fluorescent intensities of all patient and control samples were plotted against each other resulting in distinct clusters representing the three possible genotypes. In order to validate the allelic discrimination assay conditions outlined above 50 patient samples from physician ordered testing were analyzed via the in-house IL28B rs12979860 method and results were compared to the results obtained from an external reference laboratory.

Results

To validate the allelic discrimination assay, 500 randomly selected de-identified remnant EDTA anticoagulated patient derived whole blood samples were assayed as defined in the Materials and methods. 493 of the 500 randomly selected samples generated valid results. Due to low yield of extracted DNA we could not further analyze the reason for failure of the seven remaining samples. Following this initial study the laboratory elected to discontinue the use of these commercially available primers and probes in favor of custom synthesized primers.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers and probe sequences for the IL28B assay.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward Primer</td>
<td>GCCTGTCGTGTACTGAACCA</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>GCCGGCAGTGGCAATTCAC</td>
</tr>
<tr>
<td>Probe 1 (C)-VIC</td>
<td>TGGTTCGCGCCTTC</td>
</tr>
<tr>
<td>Probe 2 (T)-FAM</td>
<td>CTGGTTCAGCCCTTC</td>
</tr>
</tbody>
</table>

Fig. 1. Allelic discrimination plot of rs12969860 quality control materials. QC materials containing the extracted DNA for rs12979860 CC (red), CT (green) and TT (blue) were analyzed at decreasing total input DNA concentrations of: A–C, 10, 1, and 0.1 ng DNA homozygous CC at rs12979860; D–G, 10, 1, 0.1 and 0.01 ng DNA heterozygous CT at rs12979860; and H–K, 10, 1, 0.1 and 0.01 ng DNA homozygous TT at rs12979860, respectively. The black square at the lower left represents the no-template control.
and probes so that we would have knowledge of the sequence of the primers and probes.

The custom primers and probes synthesized by Life Technologies were used to undertake the validation of the clinical assay (Table 1). Using these primers and probes the analysis of control materials of known IL28B genotype for the rs12979860 variant via the allelic discrimination assay revealed distinct clusters on the allelic discrimination plots. These clusters represented genotypes that were homozygous rs12979860 C, heterozygous rs12979860 C/T or homozygous rs12979860 T (Fig. 1). This analysis demonstrates that the allelic discrimination assay accurately determined the genotype of known materials with respect to the rs12979860 allelic variants. Dilution of the above control materials revealed that the assay was able to accurately assign genotype in samples containing as little as 10 ng of genomic DNA (Fig. 1). All patient samples analyzed were thus analyzed using 10 ng of genomic DNA.

An additional 50 patient samples collected following physician requested IL28B genotyping were analyzed with custom primers and probes. The results of our allelic discrimination assay were compared to the results obtained from our contracted reference laboratory. Comparison of the results from the in-house assay to the reference laboratory results demonstrated no discordant genotypes.

To assess the reproducibility of this assay 15 of the physician requested IL28B genotyping samples (5 with rs12979860 genotype CC, 5 with genotype CT and 5 with genotype TT) were analyzed by two separate operators on a total of six separate analytical runs on non-consecutive days. All fifteen samples had concordant genotype results on all six runs demonstrating the reproducibility of this allelic discrimination assay.

We found that of the 543 samples generating usable results 43% had the favorable CC genotype, 43% had the CT and 13% had the unfavorable TT rs12979860 genotype, similar to allele ratios reported in the dbSNP database (http://www.ncbi.nlm.nih.gov/snp). The overall allele frequency of this large validation set was rs12979860 C = 0.65, T = 0.35, and did not deviate from Hardy–Weinberg equilibrium. A sub-analysis of the 50 physician ordered samples analyzed with the custom primers and probes from HCV infected patients demonstrated a slightly different distribution of genotypes with 34% carrying the favorable CC genotype, 52% the heterozygous CT genotype and 14% the unfavorable TT rs12979860 genotype. This difference is likely a sampling artifact and does not represent a difference in assay performance. The overall allelic frequency of this sub-group was rs12979860 C = 0.6 and T = 0.4, and again did not deviate from Hardy–Weinberg equilibrium.

Discussion

Here we describe a laboratory-developed test that uses Real-Time TaqMan PCR technology to determine the rs12979860 genotype of patients undergoing treatment for HCV infection. These results demonstrate that we are able to accurately and reliably assign the patient’s IL28B genotype. The availability of this assay is important as the patient’s rs12979860 genotype serves as a pretreatment predictor of therapeutic success with PegIFN/RBV and the direct acting serine protease inhibitor antivirals [8,13–15]. A patient’s rs12979860 genotype is only one of many factors that will determine the success or failure of a therapeutic regimen. However, the only laboratory measure that is predictive of treatment success prior to the initiation of therapy is the IL28B genotype. As this allelic discrimination assay is readily applicable to many molecular pathology laboratories the local availability of this test can help predict treatment success with both the previous PegIFN/RBV therapeutic regimen and the newer direct acting antivirals. This assay in combination with one of the FDA approved HCV viral load assays and a laboratory developed test for HCV genotyping will allow the molecular pathology laboratory to offer onsite comprehensive testing for patients undergoing treatment for chronic HCV infection.

References