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Using Stepwise Pharmacogenomics and Proteomics to Predict Hepatitis C Treatment Response in Difficult to Treat Patient Populations

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest

A.O. is currently an employee of Gilead Sciences, but was not an employee at the time this work was completed. All others declare no conflict of interest.

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Abstract

Purpose: In the interferon era of hepatitis C virus (HCV) therapies, genotype/subtype, cirrhosis, prior treatment failure, sex, and race predicted relapse. Our objective is to validate a targeted proteomics platform of 17 peptides to predict sustained virologic response (SVR).

Experimental design: Stored plasma from three, open-label, trials of HIV/HCV-coinfected subjects receiving interferon-containing regimens is identified. LC-MS/MS is used to quantitate the peptides directly from plasma, and *IL28B* genotyping is completed using stored peripheral blood mononuclear cells (PBMC). A logistic regression model is built to analyze the probability of SVR using responders and nonresponders to interferon-based regimens.

Results: The cohort ($N = 35$) is predominantly black (51.4%), male (86%), and with median age 48 years. Most patients achieve SVR (54%). Using multivariable models, it is verified that three human corticosteroid binding globulin (CBG) peptides are predictive of SVR in patients with the unfavorable *IL28B* genotypes (CT/TT). The model performs better than *IL28B* alone, with an area under the curve of 0.870.

Conclusions and clinical relevance: In HIV/HCV-coinfected patients, three human CBG peptides that accurately predict treatment response with interferon-based therapy are identified. This study suggests that a stepwise approach combining a genetic predictor followed by targeted proteomics can improve the accuracy of clinical decision-making.

Keywords

hepatitis C virus; human Immunodeficiency virus; *IL28B*; pharmacogenomics; proteomics

1. Introduction

All-oral combination, direct acting antiviral (DAA) regimens for the treatment of chronic hepatitis C virus (HCV) infection are now the standard of care.^[1] Although current DAA combination regimens offer high sustained virologic response (SVR) response rates of >95% in the majority of cases, several historical baseline predictors of response remain. In patients harboring combinations of these predictors, treatment failure is more likely.^[2–4] Due to the cost of DAA regimens, and the risk of treatment-emergent multidrug resistant mutations, there remains an interest in identifying patients at greatest risk of treatment failure, especially in those harboring baseline predictors of failure. These patients still stand to benefit from personalized treatment tailored to minimize the risk of failure.

In the early DAA era of HCV therapies, HCV genotype and subtype, presence of cirrhosis, prior treatment failures, sex, and race predicted risk of relapse.^[5] In the phase III ION-4 trial of the fixed-dose combination ledipasvir (NS5A inhibitor) and sofosbuvir (NS5B nucleotide polymerase inhibitor) in HIV/HCV-coinfected patients, black race was independently associated with treatment failure.^[3] This racial disparity was not identified in studies of the same regimen in HCV mono-infection. In that study, black patients who relapsed were more likely to have unfavorable CT or TT *IL28B* genotypes.^[6] Furthermore, with this same

regimen, there is the potential to shorten therapy from 12 to 8 weeks in patients who lack negative baseline predictors including prior treatment failure, evidence of cirrhosis, and high HCV RNA (>6 million IU mL⁻¹). A post hoc analysis of the registration trial that assessed 8 versus 12 weeks of ledipasvir/sofosbuvir in HCV-infected subjects without HIV identified female sex, black race, and unfavorable CT or TT *IL28B* genotypes as predictors of failure with shortened therapy.^[7]

We previously demonstrated that HIV/HCV coinfecting patients with the unfavorable *IL28B* genotype (CT/TT) had aberrant baseline induction of innate immune response pathways, resulting in impaired virologic response to pegylated-interferon and ribavirin (PEG-IFN/RBV) therapy, and that treatment response was independent of interferon stimulating gene expression.^[8] Black patients with HCV infection have higher rates of unfavorable CT or TT genotypes, but also aberrant baseline innate immune response regardless of *IL28B* genotype, suggesting additional mechanisms for poorer treatment response.

Our group previously reported on an unbiased proteomics discovery platform in a well-characterized cohort of chronic HCV infected patients who received PEG-IFN/RBV therapy.^[9] We identified a three-metaprotein signature able to accurately predict treatment response in 90% of patients in the training cohort, 86% in the validation cohort, and 71% in patients carrying the unfavorable *IL28B* polymorphism. Due to the poorer response to PEG-IFN/RBV therapy reported for HIV/HCV-coinfecting patients, we set out to validate the targeted proteomics platform of 17 peptides, as previously described,^[10] in order to develop a model of SVR in this population. Additionally, we simplified the sample preparation by removing the tedious and expensive antibody immunodepletion step used during the discovery study, in order to improve the potential for translation to the clinic.

2. Experimental Section

Study Population:

Three prospective, single-center, open-label trials were performed at the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health from 2001 to 2010. Thirty-five HCV-treatment naive HIV/HCV genotype 1 infected patients were treated with weight-based ribavirin daily in addition to either weekly PEG-IFN alfa-2b at 1.5 μ g kg⁻¹, weekly PEG-IFN alfa-2a, or albinterferon alfa-2b at 900 μ g every 2 weeks. All patients received 48 weeks of therapy and follow-up for 24 weeks after completion of treatment. Only patients who completed a full course of therapy per study protocol were included. Similar to the discovery study design, patients who achieved SVR (undetectable HCV RNA 24 weeks following the completion of therapy) or nonresponse (NR) (failure to achieve HCV RNA decline $2\log_{10}$ at week 12 or detectable viral load at week 24 or any time after week 24 while on therapy) were included. These same treatment outcomes (SVR vs NR) were used in the discovery cohort. All patients gave written informed consent approved by the NIAID Institutional Review Board (IRB) prior to enrollment in the studies. This study was approved by the Duke University IRB.

Genotyping:

IL28B genotyping was conducted in a blinded fashion using Taqman probes for rs12979860 SNP.

Targeted Proteomic Platform:

We have previously reported on an unbiased proteomics discovery platform used in a well-characterized cohort of chronic hepatitis C genotype-1 infected patients.^[9] From this discovery platform data, a set of 17 peptides from ten human proteins was selected and used to develop a targeted MS assay (LC-multiple reaction monitoring [MRM]) to model SVR in this cohort of HIV/HCV-coinfected patients. The tryptic plasma peptides measured and their precursor proteins are reported in Table S1, Supporting Information.

Sample Preparation:

The Duke Proteomics and Metabolomics Shared resource (DPMSR) received 35 human plasma samples from a cohort of HIV/HCV-coinfected patients. All samples were thawed on ice, and a small aliquot was removed from each sample and diluted 35× to perform a Bradford assay to get an initial total protein concentration measurement. Briefly, 100 μg was removed from each according to the Bradford results, and volume normalized with 50 mM ammonium bicarbonate, pH 8, to 100 μL total. TQL stable isotope-labeled peptides were purchased from JPT, solubilized in 20/80 v/v MeCN/100 mM ammonium bicarbonate, and mixed into an equimolar pool. Five microliter of this stable-isotope-labeled internal standard stock pool was spiked into each sample (24 peptides total in the stock at either 100 fmol μL^{-1} or 500 fmol μL^{-1}). Then RapiGest SF surfactant (Waters) was added to give a 0.1% w/v RapiGest concentration, which was pipetted into each sample. Samples were then reduced with 10 mM dithiothreitol (DTT) at 80 °C for 15 min, alkylated with 25 mM iodoacetamide at room temperature in the dark for 30 min, and digested with 2 μg trypsin overnight at 37 °C. After digestion, all samples were acidified to 1% TFA/2% ACN and heated to 60 °C for 2 h to hydrolyze the RapiGest. All samples were then spiked with 500 fmol total of ADH1 YEAST (MassPrep, Waters) as a surrogate standard (5 fmol μg), and samples were centrifuged at 15 000 rpm for 5 min to pellet out any precipitate. A 2 μg aliquot from each sample was injected onto the instrument. A study pool quality control (SPQC) sample was made by combining 5 μL from each sample. This SPQC sample was run at the start, end, and three times intermittently during the sample run queue to provide the LC-MS/MS analytical precision metrics.

Targeted MS:

LC-MS/MS performed in MRM mode to measure 17 peptides to the following proteins: AMBP protein precursor (AMBP), complement C2 precursor (CO2), complement C5 precursor (CO5), complement component C8 gamma chain precursor (CO8G), histidine-rich glycoprotein precursor (HRG), inter-alpha-trypsin inhibitor heavy chain H1 precursor (ITIH1), inter-alpha-trypsin inhibitor heavy chain H2 precursor (ITIH2), lipopolysaccharide-binding protein (LBP), thyroxine-binding globulin precursor (THBG), and corticosteroid-binding globulin (CBG) precursor.

MRM method generation and sample quantitation were performed with Skyline.^[11] Peptide selection for targeted MS experiments was based on previously reported proteomics discovery experiments,^[9] using targeted MS conditions previously described in detail.^[12] Briefly, approximately 2 μ g of protein was analyzed on a NanoAcquity UPLC (Waters). Samples were ionized directly using a nanoelectrospray source into a Xevo TQ-MS mass spectrometer (Waters) in positive electrospray mode. Skyline document including the analysis of the samples by LC-MS/MS has been made publicly available for download at: <https://panoramaweb.org/labkey/targetedms/Duke%20Proteomics%20Core/SRM%20Quantitation%20Results/downloadDocument.view?runId=7422>.

Association Analysis and Predictive Modeling:

Descriptive analyses used median and inter-quartile range for continuous variables and proportions for categorical variables. Univariable analyses were completed using the *t*-test. The *IL28B* good responder (CC) genotype was considered a perfect predictor because all patients ($N=7$) with this genotype achieved SVR. Logistic regression models were built with the remaining 28 subjects with poor responder genotypes (CT/TT) to analyze the probability of a SVR (vs NR). Models for SVR were selected using a least absolute shrinkage and selection operator (LASSO) and optimized using grid search with cross-validation. Potential predictors, selected a priori, included all 17 validated candidate proteins, five control yeast proteins, study, gender, age and race. Additionally, protein level predictors formed by averaging related peptides were used in an alternative model. These analyses were performed in R using the *cv.glmnet* function included in the *glmnet* package and Pearson correlation between peptides was calculated with the *cor* function in the *stats* package.

3. Results

3.1. Study Population

The cohort was predominantly black (51.4%), male (86%), and with median age of 48 years. Most patients had controlled HIV (60%), and median CD4 count was 483 cells mm^{-3} (Table 1). Just over half of patients achieved SVR (54%) and 20% had the favorable *IL28B* genotype (CC). Approximately 25% of patients had severe fibrosis on histopathology from liver biopsy (METAVIR stage 3–4).

3.2. Quantitative LC-MS/MS of Putative Biomarker Peptides

The multiplexed assay for 17 peptides to ten serum proteins was completed without immunodepletion (removal of abundant plasma proteins using antibodies), and the LC-MS/MS analysis time was approximately 30 min. Figure 1A shows an example of the LC-MS/MS chromatographic separation of the peptides from a typical serum sample in this cohort, along with the five peptides to the surrogate standard protein ADH1 YEAST. After calculating the ratio of the native peptides to their internal standards, the five replicates of the SPQC measured across the study were used to measure precision of the assay, and exclude irreproducible peptides. Three peptides showed greater than 25% coefficient-of-variation (CV) across the analysis (Table S1, Supporting Information), but among the

remaining 14 peptides, the average percent CV of the SPQC was 9.5%, while the average biological variance is 31%.

These 14 peptides were included in an unsupervised hierarchical cluster across all 35 patient samples (Figure 1B). This cluster shows that peptides to the same protein tend to cluster together, demonstrating coexpression and generally good performance of multiple peptides to each protein in the multiplexed assay. This clustering may not always be demonstrated, as different peptides to a single protein may have varying real expression due to protein posttranslational modifications, SNP-encoded amino acid substitutions, different MS response functions or protein cleavage. The cluster is colored and samples labeled by response to therapy, but most proteins are not strongly predictive (see next section on predictive modeling), thus sustained responders (SVR) and nonresponders (NR) do not segregate using this un-supervised cluster.

Pearson correlation was completed for all peptides to assess for associations of proteins or peptides (Figure 2). Positive correlations appeared in two primary groups clusters: (1) all four peptides of the CBG-HUMAN protein and (2) all peptides for the CO5, CO8G, HRG, ITIH1, and ITIH2-HUMAN proteins. Meanwhile, negative correlations were noted for THBG and LPB-HUMAN proteins and CO5, CO8G, HRG, ITIH1, and ITIH2-HUMAN proteins.

3.3. Association Peptide Measurements with Clinical Variables

The 17 human peptides were assessed for association with treatment response and *IL28B* genotype. Only peptides of the liver protein CBG were associated with treatment response (Table S1, Supporting Information; Figure 3), while multiple peptides were associated with *IL28B* genotype including peptides from CO5, HRG, ITIH1, and CBG (p and FDR <0.05 , Table S1, Supporting Information). Association with treatment response and *IL28B* genotype was also assessed by aggregating the individual peptide predictors into protein-level predictors. Using protein-level predictors, only CBG protein was associated with treatment response ($p = 0.005$, FDR = 0.046) and only HRG protein was associated with *IL28B* genotype ($p = 0.001$, FDR = 0.014) (Table S1, Supporting Information).

Upon investigation of the effect of *IL28B* genotype on treatment response, it was noted that all favorable genotype (CC) patients achieved SVR, making this genotype a perfect predictor in this small pilot study. Thus, the model was built removing these patients and including only those patients with the less favorable or untypable *IL28B* genotypes ($N = 28$). The SVR in this subgroup was 43% (12/28). Four variables remained in the model: age (adjusted OR 0.95), CBG-HUMAN-HLVALSPK (aOR 0.01), CBG-HUMAN-SETEIHQGFQHLHQLFAK (aOR 0.82), and CBG-HUMAN-MNTVIAALSR (aOR 0.99). There was a correlation noted between the CBG peptides and race, but only one met statistical significance (MNTVIAALSR, $p = 0.036$), which was lost after correction for multiple testing. Although race was an independent predictor of treatment response, this predictive power was lost when CBG peptides were included in the model. Neither level of fibrosis nor HCV RNA level was associated with the CBG metaprotein.

In this small cohort, the specificity of the *IL28B* genotype was 1.00 (perfect predictor for the favorable CC genotype); sensitivity was 0.368 with an overall accuracy of 65.7%. The test characteristics of the proteomics model in patients with the unfavorable *IL28B* genotypes (CT/TT) was better than *IL28B* alone: sensitivity 0.750 and specificity 0.938, with an accuracy of 86%. The proteomics model had an area under the receiver operator characteristic curve (AUROC) for SVR of 0.870 (Figure 4).

4. Discussion

We used a targeted proteomics platform to quantitate 17 peptides from ten proteins in a predominantly black cohort of HIV/HCV-coinfected patients, the majority of whom had unfavorable *IL28B* genotype. Using multivariable models, we verified three human CBG peptides to be highly predictive of treatment response to PEG-IFN/RBV. The test characteristics for the CBG model included a positive likelihood ratio (LR) of 12.0, a negative LR of 0.27, and an AUROC of 0.870, consistent with a very good test for use in medical decision making. This study supports the need to explore proteomic approaches to identifying patients most at risk of relapse after HCV therapy, and to better inform mechanisms behind relapse.

CBG is a major high-affinity plasma transport protein for glucocorticoids and progestins and is expressed primarily in the liver. Serum cortisol and CBG levels are inversely related. The primary role of CBG in proinflammatory states like chronic HCV infection is to deliver cortisol, a modulator of immune response, to sites of injury and inflammation. A prior study reported that CBG was associated with *IL28B* genotype, and that this may be independent of HCV treatment response, suggesting that CBG may be a marker of host immune response to HCV.^[10] Thus further investigations of this targeted proteomic platform in patient cohorts receiving DAA combination regimens may provide further insight into the mechanisms of relapse, especially among patients who remain difficult to treat or in whom there is consideration of shortening therapy.

Although interferon-free regimens are now the standard of care, these data remain relevant for several reasons. This proteomics platform identified three peptides that mitigated the impact of race in the model. The impact of race on SVR remains a clinically important question in the new DAA era for HCV therapy. In addition to the ION-4 study in HIV/HCV patients, a recent report of SVR in the Veterans Affairs population identified black race as a risk factor for lower SVR with ledipasvir/sofosbuvir.^[13] The CBG protein may provide further insight into potential biologic mechanisms that underpin the association of race and SVR. Furthermore, genotype 3 infection has high relapse rates even with DAA regimens, and has emerged as a more difficult to treat infection, especially in the setting of cirrhosis.^[14] The reasons for higher relapse in genotype 3 infection are unclear. Current data suggest that extension of therapy and/or the addition of ribavirin to regimens can decrease relapse, and this remains a recommendation in the national HCV guidance for some regimens.^[1] However, longer duration increases the cost of therapy, and ribavirin is associated with hemolytic anemia and is poorly tolerated. Thus, development of an accurate proteomic biosignature could help to optimize regimens for those who are less likely to achieve SVR,

and avoid unnecessary cost and side effects in those more likely to respond to shorter or ribavirin free regimens.

This study has several weaknesses. The cost of high throughput proteomic sequencing and the limited funding for this project precluded more extensive enrollment and limited the sample size for analysis. The limitation of the treatment outcomes to only SVR and nonresponder treatment responses limit the generalizability of the results. Thus, our findings require validation in a larger cohort with inclusion of all treatment responses. While this study was based on PEG-IFN-based regimens, which are no longer the standard of care, racial disparity in treatment response persists even in current DAA-based practice and remains poorly understood. Moreover, this study serves as a proof of concept for the process of translational biomarker discovery, refining genomic predictors with proteomics, a process applicable to other pharmaceutical agents and diseases.

5. Conclusions

In a cohort of predominantly black patients coinfecting with HIV/HCV, we identified three human corticosteroid binding globulin peptides that can accurately predict treatment response with PEG-IFN based therapy. This study suggests that a stepwise approach combining a strong genetic risk predictor (*IL28B*) followed by a targeted peptide panel can significantly improve the accuracy of clinical decision making in subpopulations with poorer treatment prognosis. This platform warrants validation in current standard of care DAA regimens and in other difficult to treat populations such as genotype 3 infection, DAA nonresponders, and decompensated liver disease. A combined genomic-proteomic signature could potentially help guide treatment decision-making in these difficult to treat populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Significance Statement

Although direct acting antiviral (DAA) regimens for the treatment of chronic hepatitis C virus (HCV) infection are now standard of care, baseline predictors of response reported in the interferon era remain relevant. Due to the cost of these regimens, and the risk of treatment-emergent resistant mutations, there remains a need to identify patients at greatest risk of treatment failure. Additionally, current data suggest that extension of therapy and/or the addition of ribavirin to regimens can decrease relapse. Development of an accurate proteomic biosignature could help to optimize regimens for those who are less likely to achieve sustained virologic response (SVR) and avoid unnecessary cost and harmful side effects in those more likely to respond to shorter or ribavirin free regimens. In the current study, we identified three human corticosteroid binding globulin (CBG) peptides predictive of SVR analyzing responders and nonresponders to interferon-based regimens. The test characteristics of the model in patients with the unfavorable *IL28B* genotypes (CT/TT) were better than *IL28B* alone with an area under the curve for SVR of 0.870. This study suggests that a stepwise approach combining a strong genetic risk predictor followed by a targeted peptide panel can improve the accuracy of clinical decision-making and should be validated in DAA therapies.

Figure 1.
A) Example chromatogram from the analysis of a single sample in the cohort, displaying the multiplexed LC-MS/MS analysis of the 17 putative biomarker peptides. B) Unsupervised hierarchical clustering of the peptides across the cohort of 35 samples. Three peptides with poor quantitative precision were excluded from this analysis (see Table 1).

Figure 2.
Pearson correlation of 17 putative biomarker peptides.

Figure 3.

Boxplots showing the relative expression levels for the peptides HLVALSPK (A) and MNTVIAALSR (B) to CBG as a function of response to therapy. Box-and-whiskers show quartile ranges.

Figure 4.
AUROC for SVR for the proteomics model.

Table 1.

Patient characteristics.

Characteristic	Result (N = 35)
Age, median [IQR]	48 (42, 52)
Race, N [%]	
Black	18 (51.4)
White	12 (34.3)
Latino	4 (11.4)
Female gender, N [%]	5 (14.3)
METAVIR, stage 3–4 ^{a)} , N [%]	7 (24.1)
CD4 percentage, median [IQR]	30 (22, 35)
CD4 absolute (cells mm ⁻³), median [IQR]	483 (332, 716)
Log HIV RNA, median [IQR]	1.69 (1.69, 3.1)
Log HCV RNA, median [IQR]	6.3 (5.65, 6.41)
HCV treatment response, N [%]	
Rapid virologic response (RVR), N [%]	10 (28.6)
SVR, N [%]	19 (54.3)
<i>IL28B</i> Genotype, N [%] ^{b)}	
Favorable (CC)	7 (20)
Unfavorable (CT/TT)	22 (62.9)

^{a)} Six patients did not have available histopathology.;

^{b)} Six samples were not typable for *IL28B* SNP.; IQR, interquartile range.