Short Communication: Serum-Based Assay Accurately Detects Single Nucleotide Polymorphisms of IL28B and SOCS3 in HIV/Hepatitis C Virus-Coinfected Subjects

Ashton Shaffer,¹ Jon J. Hubbard,² Kerry Townsend,² Shyam Kottlil,² Michael A. Polis,²
Henry Masur,² and Anita Kohli^{1,3}

Abstract

Single nucleotide polymorphisms (SNPs) have become important in predicting treatment response to interferon containing anti-hepatitis C virus (HCV) therapy in HCV and HIV/HCV-infected patients. A reliable method for extracting host DNA from serum for genotyping assays would present a practical alternative for clinicians and investigators seeking to perform SNP analyses in HCV-infected patients, particularly in resource-limited settings. Human genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) and serum of 51 HIV/HCV coinfecting patients using the QIAamp DNA Blood Mini Kit and QIAamp Min Elute Virus Spin Kit, respectively. Genotyping assays for the IL28B SNP (rs12979860) and SOCS3 SNP (rs4969170) were performed using the commercially available ABI Taqman allelic discrimination kit and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using 50 cycles. Results of the genotyping assays using DNA from both PBMCs and cell-free serum were determined separately and then analyzed for concurrence. Genotype analyses performed using DNA isolated from PBMCs or cell-free serum showed a 100% agreement between the IL28B genotyping results from the serum and PBMC isolates and 98% agreement for SOCS3 SNP. This novel serum-based assay to isolate DNA fragments from the serum of HIV/HCV-coinfecting subjects can accurately determine a subject's genotype for IL28B (rs12979860) and SOCS3 (rs4969170). This assay could be immediately valuable for detecting clinically relevant SNPs from serum in cases in which PBMCs are not available.

Introduction

RECENT ADVANCES HAVE EXPLORED the value of using single nucleotide polymorphisms (SNP) of the human genome as an invaluable resource for prediction susceptibility and outcome of therapy.¹ It is widely expected that such efforts will be more commonly employed and applied to most diseases. However, there exist significant restrictions in collecting DNA samples from patients, and thus, performing SNP detection using noncellular DNA would greatly enhance our ability to individualize treatment [Genetics in medicine ACMG statement]. In this regard, SNPs have become important in predicting treatment response in both hepatitis C virus (HCV) monoinfected² and HIV/HCV coinfecting patients.³ Recent studies demonstrate that a polymorphism of

the IL28B gene (SNP rs12979860) is a reliable, clinically useful predictor of cure or sustained virologic response (SVR) among patients treated with peg-interferon and ribavirin.⁴ In addition, pretreatment expression levels and polymorphisms of another gene, SOCS3, an inhibitor of the interferon (IFN)- α -induced JAK/STAT pathway associated with obesity and insulin resistance, have also been associated with interferon-based treatment outcomes.^{2,5–8} While SVR is associated with reduced morbidity and improved survival,⁵ predicting which patients will respond to therapy is challenging.

Due to lack of availability of DNA samples collected in clinical trials, a reliable method for extracting host DNA from serum for genotyping assays would present a practical alternative for clinicians and investigators seeking to perform

¹Critical Care Medicine Department, Clinical Research Center, National Institutes of Health, Bethesda, Maryland.

²Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

³Clinical Research Directorate/Clinical Monitoring Research Program, Leidos Biomedical Research, Inc. (formerly SAIC-Frederick, Inc.), Frederick National Laboratory for Cancer Research, Frederick, Maryland.

SNP analyses, particularly in resource-limited settings. In this regard, there is at least one recent report that has used serum samples to perform IL28B SNP typing with dried blood spots, serum, and epithelial cells.⁹ However, this technique has not been extended to other SNPs. In this study, we compare the reliability and reproducibility of genotyping analysis for IL28B SNP rs12979860 (hereafter “IL28B SNP”) and SOCS3 SNP rs4969170 (hereafter “SOCS3 SNP”) from both human peripheral blood mononuclear cells (PBMCs) and serum.

Materials and Methods

Patients coinfecting with HIV and HCV on stable anti-retroviral therapy were enrolled in three National Institute of Allergy and Infectious Disease (NIAID) IRB approved trials and treated with pegylated IFN- α 2a (180 μ g weekly), pegylated IFN- α 2b (1.5 μ g/kg weekly), or albuterferon- α 2b (900 μ g every other week), all in combination with RBV (1–1.2 g/day) for 48 weeks. Eligibility criteria have been described previously.^{10–12} All patients signed an informed consent for participation that was approved by the NIAID Institutional Review Board. Serum and whole blood samples were collected at regular protocol-defined intervals, processed, and stored at –80°C. The time of storage of samples ranged from 4 to 9 years (mean 6.9 years). All subjects signed informed consent that included genotyping in the original treatment protocol.

DNA isolations

Human genomic DNA was isolated from stored PBMCs using the QIAamp DNA Blood Mini Kit according to the manufacturer’s specifications (Qiagen Biosciences, Germantown, MD) with an average input of approximately 1.0×10^6 viable cells as determined using Guava ViaCount (Guava Technologies, Hayward, CA). Serum samples were thawed to room temperature and pipetted up and down multiple times and 200- μ l aliquots were prepared for future use. Human genomic DNA was extracted from 200 μ l of stored serum using the QIAamp Min Elute Virus Spin Kit, using the option for carrier mRNA (Qiagen Biosciences, Germantown, MD) per the manufacturer’s specifications for extraction of viral RNA.

Genotype determination

DNA yield from PBMCs was measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) with an average of 115.5 ± 104.2 ng/ μ l DNA isolated and with an average 260/280 ratio of 1.89 ± 0.17 obtained. Quantification of DNA extracted from serum was not possible due to the presence of carrier RNA introduced during the extraction process. Genotyping assays were performed in a blinded fashion on coded DNA specimens using the commercially available ABI Taqman allelic discrimination kit (Applied Biosystems, Albany, NY) in 20 μ l reactions consisting of approximately 5 μ l of DNA input, and nuclease-free water, genotype Master Mix, and either the IL28B SNP (Applied Biosystems, Albany, NY product #4331349; custom primer sequences forward: GCCTGTCGTGTACTGAA CCA, reverse: GCGCGGAGTGCAATTCAAC, reporter primers: TGGTTCCGCGCCTTC and CTGGTTTCAACGCC TTC) or SOC3 SNP (product #4351379, rs4969170) allele-specific Taqman probes with specific volumes as per kit instructions. Reactions were run on the ABI7500 Real-Time

PCR system (Applied Biosystems, Albany, NY) under standard conditions and for 50 cycles. Results of the genotyping assays for IL28 and SOCS3 SNPs using DNA from both PBMCs and cell-free serum were determined separately and then analyzed for concurrence.

Results

Fifty-one HIV/HCV coinfecting patients with available PBMCs and serum were included. Table 1 shows a summary of the patients’ demographic data. The study subjects were predominantly male (88%), African American (63%), HCV genotype 1 (80%), and had relatively higher levels of CD4 T cells. Overall, there was a 37% SVR rate among this cohort. Results using plasma are quite similar and serum was chosen due to sample availability (data not shown).

A comparison of the results of the genotype analyses performed using DNA isolated from PBMCs or cell-free serum showed a 100% agreement between the IL28B genotyping results from the serum and PBMC isolates and 98% agreement for SOCS3 SNP (Table 2). One subject could not be genotyped for SOCS3 SNP in one serum sample on multiple attempts and therefore was considered undetermined.

Discussion

In this study we describe a novel method to isolate DNA fragments from the serum of HIV/HCV-coinfecting subjects

TABLE 1. PATIENT DEMOGRAPHICS

Variables	
Age, mean years $\pm$ SD	48.1 $\pm$ 7.1
Male gender, <i>n</i> (% male)	45 (88.2)
Race	
African American <i>n</i> (%)	32 (62.7)
White <i>n</i> (%)	18 (35.3)
Other <i>n</i> (%)	1 (2.0)
Baseline HIV VL	
Undetectable (<50 copies/ml) <i>n</i> (%)	34 (66.7)
Mean $\pm$ SD	10537 $\pm$ 36390
Baseline CD4 cell count, mean cells/ μ l $\pm$ SD	590 $\pm$ 86
Baseline HCV VL, <i>n</i> >800,000 IU/ml (% >800K)	38 (74.5)
HCV genotype	
Genotype 1 <i>n</i> (%)	41 (80.4)
Genotype 2 <i>n</i> (%)	6 (11.8)
Genotype 4 <i>n</i> (%)	1 (2.0)
Genotype 5 <i>n</i> (%)	1 (2.0)
HCV treatment response	
Sustained virologic response (SVR) <i>n</i> (%)	19 (37.3)
Nonresponse (NR)	9 (17.6)
Relapse (RL)	14 (27.5)
Viral breakthrough (VB)	6 (11.8)
Treatment discontinued	3 (5.9)
Liver Biopsy	<i>N</i> =43
HAI fibrosis	<i>n</i> (%)
0–2 (early stage fibrosis) (0–2)	28 (65.1)
3–4 (advanced fibrosis) (3–4)	15 (34.9)
Steatosis	<i>n</i> (%)
Absent (0–trace)	35 (81.4)
Present (1–3)	8 (18.6)

VL, viral load; HCV, hepatitis C virus.

TABLE 2. GENOTYPING OF IL28B AND SOCS3 SINGLE NUCLEOTIDE POLYMORPHISMS FROM PERIPHERAL BLOOD MONONUCLEAR CELLS AND CELL-FREE SERUM

Pt #	IL28B PBMC genotype	IL28B serum genotype	SOCS3 PBMC genotype	SOCS3 serum genotype
1	TT	TT	AA	AA
2	TT	TT	AA	AA
3	TT	TT	AG	AG
4	TT	TT	AA	AA
5	CC	CC	AG	AG
6	TT	TT	AA	AA
7	CC	CC	AA	AA
8	CC	CC	AG	AG
9	TT	TT	AG	AG
10	CC	CC	GG	GG
11	CT	CT	GG	GG
12	CT	CT	GG	GG
13	CT	CT	AA	AA
14	CT	CT	AA	AA
15	CT	CT	AG	AG
16	CT	CT	AG	AG
17	TT	TT	AA	AA
18	CC	CC	AG	AG
19	TT	TT	GG	GG
20	CC	CC	AA	AA
21	CC	CC	GG	GG
22	TT	TT	AA	AA
23	TT	TT	GG	GG
24	CC	CC	AG	AG
25	TT	TT	GG	GG
26	CC	CC	AA	AA
27	CT	CT	AG	AG
28	CT	CT	GG	GG
29	CT	CT	AG	AG
30	CC	CC	AG	AG
31	CC	CC	AG	AG
32	CC	CC	AG	AG
33	CT	CT	AA	AA
34	CT	CT	AA	AA
35	CT	CT	GG	GG
36	TT	TT	AA	AA
37	TT	TT	AG	AG
38	TT	TT	AG	AG
39	CT	CT	AG	AG
40	CT	CT	GG	GG
41	TT	TT	AG	AG
42	CT	CT	AA	AA
43	CC	CC	AG	AG
44	CT	CT	GG	GG
45	CC	CC	AA	AA
46	CC	CC	AG	AG
47	CT	CT	AG	AG
48	CC	CC	AG	Undetermined
49	CC	CC	AA	AA
50	TT	TT	AG	AG
51	CT	CT	AA	AA
% Concordance		100%		98%

PBMC, peripheral blood mononuclear cell.

in order to accurately determine each subject's genotype for each of two SNPs: IL28B (rs12979860) and SOCS3 (rs4969170). When compared to the standard assay using DNA isolated from PBMCs, we were able to obtain 100% and 98% accuracy for IL28B and SOCS3, respectively, using

the serum-based assay. This assay could be immediately valuable for detecting clinically relevant SNPs from serum in cases in which PBMCs are not available.

Since the description of polymorphisms in the IL28B SNP as an important predictor of HCV treatment outcomes,^{4,13} genotyping for this SNP has become a routine clinical assay used by physicians in the evaluation of HCV-infected patients prior to treatment. Additionally, in a recent multicenter study that treated 500 HCV genotype 3-infected subjects, baseline steatosis was an independent predictor of SVR¹⁴; however, a correlation among the SOCS3 SNP in particular, hepatic steatosis, and treatment outcomes was not performed because PBMCs were not collected. Our assay provides a practical approach to determine the SOCS3 and IL28B genotypes in patients with stored serum to determine its role in clinical liver outcomes.

Moving forward, we believe this technique will have wider applications in various diseases. As described previously, the collection of cellular DNA material in larger multicenter trials is complex and limited. Hence, this technique offers a unique opportunity to perform targeted SNP analysis using serum stored over many years. As treatment for HCV is rapidly evolving into all oral directly acting agents, our assay, which provides accurate determination of the IL28B SNP and SOCS3 SNP genotypes from DNA extracted from serum, will allow clinicians and researchers to test all patients undergoing novel treatment for HCV more easily. In addition, this serum-based assay technique has the potential to be adapted to other novel SNPs, to identify and confirm novel host genetic mediators of disease susceptibility and treatment outcome. Although our assay can be useful in determining SNPs from stored serum samples, it raises the important issue about genetic testing of stored samples. In many cases, samples stored over time may not have linked informed consents from study subjects that permit genetic testing. In such cases, a special ethics committee review of proposal weighing in on the advantages of such testing needs to be conducted prior to use.

Conclusions

In conclusion, we describe the results of a serum-based genotyping assay for the determination of two SNPs that are predictive of HCV treatment response and are associated with clinical outcomes. Although we tested only two SNPs, this method could likely be adapted to detect additional SNPs, including those that are commercially available and custom designed, and may have wider applications in medical research in which SNP determination is relevant. This serum-based assay provides clinicians and researchers with a simpler alternative to tedious PBMC collection and processing and may be of particular use in resource-limited settings.

Acknowledgments

S.K., A.K., and J.H. conceived the study. S.K., J.H., and A.K. designed the experiments. A.S. and J.H. performed the experiments. A.K., H.M., M.P., K.T., J.H., and A.S. performed data analysis. A.K., J.H., S.K., M.P., K.T., H.M., and A.S. wrote the paper.

This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract no. HHSN261200800001E.

The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. This research was supported in part by the Intramural Research Program of the NIH [National Institute of Allergy and Infectious Diseases and NIH Clinical Center].

Author Disclosure Statement

No competing financial interests exist.

References

- Schork NJ, Fallin D, and Lanchbury JS: Single nucleotide polymorphisms and the future of genetic epidemiology. *Clin Genet* 2000;58:250–264.
- Persico M, Capasso M, Russo R, Persico E, Croce L, Tiribelli C, *et al.*: Elevated expression and polymorphisms of SOCS3 influence patient response to antiviral therapy in chronic hepatitis C. *Gut* 2008;57:507–515.
- Neukam K, Camacho A, Caruz A, Rallon N, Torres-Cornejo A, Rockstroh JK, *et al.*: Prediction of response to pegylated interferon plus ribavirin in HIV/hepatitis C virus (HCV)-coinfecting patients using HCV genotype, IL28B variations, and HCV-RNA load. *J Hepatol* 2012;56:788–794.
- Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, *et al.*: Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 2009;461:399–401.
- Walsh MJ, Jonsson JR, Richardson MM, Lipka GM, Purdie DM, Clouston AD, *et al.*: Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1. *Gut* 2006;55:529–535.
- Lee SS, Sherman M, Ramji A, Greenbloom S, Elkashab M, Pluta H, *et al.*: Randomised clinical trial: The efficacy of treatment, guided by a shorter duration of response, using peginterferon alfa-2a plus ribavirin for hepatitis C virus other than genotypes 2 or 3. *Aliment Pharmacol Ther* 2012;35:37–47.
- Machado MV and Cortez-Pinto H: Insulin resistance and steatosis in chronic hepatitis C. *Ann Hepatol* 2009;8(Suppl 1):S67–75.
- Kawaguchi T, Yoshida T, Harada M, Hisamoto T, Nagao Y, Ide T, *et al.*: Hepatitis C virus down-regulates insulin receptor substrates 1 and 2 through up-regulation of suppressor of cytokine signaling 3. *Am J Pathol* 2004;165:1499–1508.
- Halfon P, Ouzan D, Khiri H, Penaranda G, Castellani P, Oules V, *et al.*: Detection of IL28B SNP DNA from buccal epithelial cells, small amounts of serum, and dried blood spots. *PLoS One* 2012;7:e33000.
- Neumann A, Polis M, Rozenberg L, Jackson J, Reitano K, McLaughlin M, *et al.*: Differential antiviral effect of PEG-interferon-alpha-2b on HIV and HCV in the treatment of HIV/HCV co-infected patients. *AIDS* 2007;21:1855–1865.
- Murphy AA, Herrmann E, Osinusi AO, Wu L, Sachau W, Lempicki RA, *et al.*: Twice-weekly pegylated interferon-alpha-2a and ribavirin results in superior viral kinetics in HIV/hepatitis C virus co-infected patients compared to standard therapy. *AIDS* 2011;25:1179–1187.
- Osinusi A, Bon D, Nelson A, Lee YJ, Poonia SJ, Shivakumar B, *et al.*: Comparative efficacy, pharmacokinetic, pharmacodynamic activity, and interferon stimulated gene expression of different interferon formulations in HIV/HCV genotype-1 infected patients. *J Med Virol* 2014;86:177–185.
- Naggie S, Osinusi A, Katsounas A, Lempicki R, Herrmann E, Thompson AJ, *et al.*: Dysregulation of innate immunity in hepatitis C virus genotype 1 IL28B-unfavorable genotype patients: Impaired viral kinetics and therapeutic response. *Hepatology* 2012;56:444–454.
- Shah SR, Patel K, Marcellin P, Foster GR, Manns M, Kottlil S, *et al.*: Steatosis is an independent predictor of relapse following rapid virologic response in patients with HCV genotype 3. *Clin Gastroenterol Hepatol* 2011;9:688–693.

Address correspondence to:

Anita Kohli

CCMD/CC/NIH

10 Center Drive, Building 10/Room 11C442

Bethesda, Maryland 20814

E-mail: kohlia@niaid.nih.gov