Gene Expression Profiling To Predict and Assess the Consequences of Therapy-Induced Virus Eradication in Chronic Hepatitis C Virus Infection

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ABSTRACT

Systems biology has proven to be a powerful tool to identify reliable predictors of treatment response in chronic hepatitis C virus (HCV) infection. In the present study, we studied patients with chronic HCV infection who responded to interferon (IFN)-based therapy, as evidenced by an absence of HCV RNA at the end of treatment, and focused on two issues that have not received much attention. First, we evaluated whether specific genes or gene expression patterns in blood were able to distinguish responder patients with a viral relapse from responder patients who remained virus negative after cessation of treatment. We found that patients with chronic HCV infection who were sustained responders and relapsers after IFN-based therapy showed comparable baseline clinical parameters and immune compositions in blood. However, at baseline, the gene expression profiles of a set of 18 genes predicted treatment outcome with an accuracy of 94%. Second, we examined whether patients with successful therapy-induced clearance of HCV still exhibited gene expression patterns characteristic of HCV or whether normalization of their transcriptome was observed. We observed that the relatively high expression levels of IFN-stimulated genes (ISGs) in patients with chronic HCV infection prior to therapy were reduced after successful IFN-based antiviral therapy (at 24 weeks of follow-up). These ISGs included the CXCL10, OAS1, IFI6, DDX60, TRIM5, and STAT1 genes. In addition, 1,428 differentially expressed non-ISGs were identified in paired pre- and posttreatment samples from sustained responders, which included genes involved in transforming growth factor beta (TGF- β) signaling, apoptosis, autophagy, and nucleic acid and protein metabolism. Interestingly, 1,424 genes with altered expression levels in responder patients after viral eradication were identified, in comparison to normal expression levels in healthy individuals. Additionally, aberrant expression levels of a subset of these genes, including the interleukin-32 (IL-32), IL-16, CCND3, and RASSF1 genes, were also observed at baseline. Our findings indicate that successful antiviral therapy for patients with chronic HCV infection does not lead to normalization of their blood transcriptional signature. The altered transcriptional activity may reflect HCV-induced liver damage in previously infected individuals.

IMPORTANCE

Tools to predict the efficacy of antiviral therapy for patients with HCV infection are important to select the optimal therapeutic strategy. Using a systems biology approach, we identify a set of 18 genes expressed in blood that predicts the recurrence of HCV RNA after cessation of therapy consisting of peginterferon and ribavirin. This set of genes may be applicable as a useful biomarker in clinical decision-making, since the number of genes included in the predictor is small and the correct prediction rate is high (94%). In addition, we observed that the blood transcriptional profile in patients with chronic HCV infection who were successfully treated is not normalized to the status observed in healthy individuals. Even 6 months after therapy-induced elimination of HCV RNA, gene expression profiles in blood are still altered in these patients with chronic HCV infection, strongly suggesting long-term modulation of immune parameters in previously infected patients.

Chronic hepatitis C virus (HCV) infection is a major public health problem leading to liver fibrosis, cirrhosis, and hepatocellular carcinoma (1, 2). An estimated 130 million to 170 million people are chronically infected worldwide. It is expected that the HCV-related mortality rate will continue to increase over the next 2 decades (3). Over the last 5 years, novel direct-acting antivirals (DAA) against HCV have been discovered at a fast pace. The current standard of care, consisting of pegylated alpha interferon (pegIFN- α) and ribavirin in the presence or absence of a first-generation DAA (telaprevir or boceprevir), will change considerably in the next decade, and complete virus eradication by a second-generation DAA (sofosbuvir or simeprevir) treatment in the absence of pegIFN- α may be feasible for most patients. However, it is unlikely that

pegIFN- α and ribavirin will disappear completely from clinical practice, since DAAs are expensive and not affordable for large groups of patients (4).

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TABLE 1 Characteristics of patients and healthy controls

Parameter	Value for group		
	Chronically HCV-infected patients at baseline	Responders at 24-wk follow-up time point	Healthy individuals
No. of individuals	34	11	20
Ratio of no. of males to no. of females	25:9	6:5	10:10
Mean age (yr) (SEM)	49.4 (1.4)		29.0 (1.7)
No. of individuals of Caucasian-Asian-other race	28-1-5		
Mean ALT level (IU/liter) (SEM)	85.4 (10.7)	18.6 (1.5)	
Mean viral load (IU/ml) (SEM)	1.6×10^6 (3.9×10^5)	15 (0)	
No. of individuals with HCV genotype 1-2-3-4-unknown	19-1-12-1-1		
No. of individuals with CC-TC-TT-unknown IL-28B SNP	12-15-4-3		
No. of patients with fibrosis (Metavir score) ^a			
F0	0		
F1	12		
F2	4		
F3	10		
F4	3		
Unknown	5		

^a Metavir scores are as follows: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis with few septa; F3, numerous septa without cirrhosis; F4, cirrhosis.

Combination therapy of pegIFN- α and ribavirin results in complete viral eradication in ~50% of patients with chronic HCV infection. However, a substantial number of patients show no significant response to therapy (nonresponse) or develop viral relapse after the cessation of IFN-based therapy (5). Viral and host factors that influence the therapeutic response to IFN-based regimens have been described, including HCV genotype, gender, baseline viral load, early virological response, and fibrosis grade (6, 7). More recently, host immunity-related predictive markers that discriminate between responders and nonresponders have been described, which include serum interferon gamma-induced protein 10 (IP-10) levels at baseline, *IL-28B* gene polymorphisms, as well as baseline and therapy-induced expression levels of IFN-stimulated genes (ISGs) and non-ISGs in the livers of patients (8–15).

Our group previously reported that therapy-induced virus eradication does not lead to normalization of the intrahepatic immune response to a resting state, supported by the retention of high numbers of regulatory T cells and sustained immunopathology in livers of responder patients (16) as well as altered immune regulation of T cell responses in blood (17). These findings suggest that therapy-induced elimination of HCV from the liver does not fully normalize the immune status of patients with a sustained viral response (SVR). Since the interactions between virus and host are highly complex and may determine disease outcome and therapeutic efficacy, systems biology approaches may help gain more insight into the underlying mechanisms. Although HCV infects primarily hepatocytes in the liver, this organ is not easily accessible, and blood may reflect certain changes induced by the disease. In the present study, we compared blood transcriptional responses before and after treatment, aiming to better characterize patients with chronic HCV infection who responded to IFN-based therapy. We focused on two issues that have not received much attention in previous studies. First, we evaluated whether specific genes or gene patterns in blood were able to distinguish responder patients with a viral relapse from patients who completely eliminated the virus after cessation of treatment. Second, we deter-

mined whether patients with successful therapy-induced clearance of HCV still exhibited transcriptional patterns in blood characteristic of HCV-positive patients or whether normalization of their transcriptome was observed by using high-throughput expression profiling.

MATERIALS AND METHODS

Study population and antiviral treatment. All HCV-infected patients were treated for 24 or 48 weeks with pegIFN- α 2a (Pegasys; Roche) or pegIFN- α 2b (Pegintron; Merck) and ribavirin (Copegus [Roche] or Rebetol [Merck]) at Erasmus MC. Thirty-four treatment-naïve chronically HCV-infected patients were retrospectively included in this study on the basis of undetectable serum HCV RNA at the end of treatment (see Table 1 for patient details). All patients were negative for hepatitis B virus (HBV) or HIV. Additionally, 20 healthy controls were included in this study. The institutional review board of Erasmus MC approved the protocols. Informed consent was obtained from all individuals. Clinical and virological parameters were determined by routine diagnostics used by Erasmus MC. HCV RNA was tested by using the Roche Ampliprep/Cobas TaqMan HCV/HPS assay (Roche Molecular Systems Inc., Branchburg, NJ, USA), with a lower limit of detection of 15 IU/ml. In addition, the interleukin-28B (IL-28B) single nucleotide polymorphism (SNP) rs12979860 was determined for all patients by using competitive allele-specific PCR (KASP; LGC Genomics, Hoddesdon, United Kingdom).

Definitions of response. The end-of-treatment response was defined as undetectable HCV RNA at week 48 of therapy for HCV genotype 1 and at week 24 for HCV genotypes 2 and 3. SVR was defined as undetectable serum HCV RNA levels (<15 IU/ml) 24 weeks after the end of therapy. A viral relapse was defined as undetectable HCV RNA at the end of therapy but a reappearance of serum HCV RNA during the follow-up period.

Assessment of frequency of leukocyte subpopulations in whole blood. To determine the frequency of distinct leukocyte subpopulations, whole blood (collected into sodium heparin tubes) was lysed with ammonium chloride; stained with antibodies against CD3-fluorescein isothiocyanate (FITC), CD4-phycoerythrin (PE)-Cy7, CD8-Pacific Blue, CD19-allophycocyanin (APC)-H7, and CD56-APC (eBioscience, Vienna, Austria, or BD, Breda, the Netherlands); and evaluated by flow cytometry (Canto-II; BD). The data were analyzed by using BD FACSDiva software. The frequency of granulocytes, lymphocytes, and monocytes was deter-

mined on the basis of their forward-scatter (FSC)–side-scatter (SSC) profile.

Sample processing and microarray. Peripheral blood samples were collected in Tempus Blood RNA tubes (ABI, Foster City, CA, USA) at baseline from all patients and healthy controls and at 24 weeks of follow-up from patients who achieved SVR and stored at -80°C . Total RNA was isolated from whole blood by using the Tempus Spin RNA isolation kit (Applied Biosystems, Bleiswijk, the Netherlands), and globin mRNA was removed from total RNA preparations by using the GlobinClear kit (Life Technologies). The integrity of isolated RNA was assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The RNA integrity number (RIN) of samples was always >8 . The concentrations of RNA were measured with a NanoDrop ND-111 UV-visible (UV-VIS) spectrophotometer. RNA (100 ng) was labeled by using the MessageAmp Premier RNA amplification kit (Applied Biosystems) and hybridized onto GeneChip Human Genome U133 Plus 2.0 arrays (Affymetrix), according to the manufacturers' recommendations. Hybridization signals were detected by using the GeneArray 3000 scanner (Affymetrix). Image analysis was performed by using Gene Chip operating software (Affymetrix). Microarray Suite version 5.0 software (Affymetrix) was used to generate .dat and .cel files for each experiment.

Array data processing and analysis. The standard quality metrics of Affymetrix human arrays were used to exclude failed experiments from further analyses, including noise values (raw Q; measurement for the pixel-to-pixel variation of probe cells on the chip), background values, a signal ratio of ≤ 3 of the 3'/5' probe sets for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and actin, and internal labeling and hybridization spike-in controls. The microarray data from all subjects were normalized by using GC robust multiarray analysis (GC-RMA) (18), which sequentially performs background correction, nonspecific binding adjustment, quantile normalization, and expression summarization by median polish. The final intensity of individual probe sets in each sample was then displayed as $\log_2$ -transformed values.

Supervised analysis. Differentially expressed genes were identified by using significance analysis of microarrays (SAM). For each gene, the SAM algorithm computed the difference in expression levels between classes relative to the variation expected in the mean difference. When paired HCV cases were analyzed, the algorithm of SAM for two-class paired samples was used. To correct multiple comparisons, the false discovery rate (FDR) was controlled by performing random permutation 100 times for sample classes. SAM first computes for each gene a modified F-statistic (or t-statistic for two-class data) in which a "fudge factor for standard deviation" is included in the denominator to stabilize the gene-specific standard deviation estimates. These F-statistic values are sorted from lowest to highest. Next, the class labels, e.g., response and relapse as well as baseline and follow-up, were permuted, and recomputation of ordered F-statistics was performed for each permutation. The expected ordered statistics are estimated as the averages of the ordered statistics over the set of permutations. A cut point is then defined as $F(i^*)(\Delta)$, where $i^*(\Delta)$ is the first index, i , in which the actual ordered F-statistic value is higher than the expected ordered F-statistic value by a Δ threshold value and is a function of this Δ . Genes with an F-statistic value higher than this cut point are considered to be "significant" (19–21). Differentially expressed genes were selected by a q value (FDR-adjusted P value) of $<1\%$ in comparisons between healthy cases and HCV cases or by a q value of $<10\%$ or a factor of >1.5 -fold in comparisons between paired HCV cases (paired cases at baseline and at the 24-week follow-up time point; $n = 8$). From the differentially expressed genes that were identified, two gene sets were determined. (i) Therapy-reversible genes are genes that are significantly differentially expressed in responder patients at baseline but not at 24 weeks of follow-up, using the expression levels in healthy blood as a reference, and significantly differentially expressed between paired HCV cases. (ii) Genes with sustained aberrant expression are genes that are significantly differentially expressed in responder patients both at baseline and at 24 weeks of

follow-up, using the expression levels in healthy blood as a reference, and not significantly differentially expressed between paired HCV cases.

Class prediction. Class prediction analysis was performed by using 3 different methods: prediction analysis for microarray, nearest centroid, and support vector machines (SVMs). The genes selected by supervised analysis were tested for their ability to act as baseline classifiers for predicting the therapeutic response of HCV patients (sustained response and relapse). The leave-one-out cross validation was performed to compute the misclassification rate, which omits one sample each time and predicts the class label with the remaining samples for the omitted sample. For each sample omitted, the entire analysis is repeated from the start, including the determination of univariately significant genes for the reduced training sample. From this gene list, a multivariate predictor is constructed and applied to the sample that was omitted. Whether that prediction was correct or not is recorded. The 3 methods generated comparable predictors with respect to the number of genes included and the correct classification rate; the predictive classifiers from support vector machines were reported and used for further analyses. The SVM algorithm used a weighted linear combination of gene expressions as a discriminator between the two classes. The expression of all input genes is used to train the SVM algorithm. The SVM algorithm removes genes that have a low absolute value of weight in the linear combination, and a new SVM classifier is developed by using the remaining genes. After identifying a new linear discriminant, the genes that have the lowest absolute weights in the new discriminant are removed, and a new SVM classifier is developed. This process continues iteratively until the best prediction is reached.

Pathway enrichment analysis. The selected genes were functionally annotated by using canonical pathways and toxicological functions from Ingenuity Knowledge Base (Ingenuity Systems Inc., Redwood City, CA). All genes presented on the Affymetrix Human U133 Plus 2.0 array were used as the reference set for the human genome to compute the enrichment score for each pathway, which is a ratio of gene occurrences between differentially expressed genes and the reference set. Fisher's exact test and FDR determined by the Benjamini-Hochberg method were used to determine the statistical significance of pathways.

Statistical analysis. Continuous variables are represented as means $\pm$ standard errors of the means (SEM), unless indicated otherwise. Baseline analyses were performed by using descriptive statistics with IBM SPSS Statistics (v21; IBM, Armonk, NY, USA). Student's t tests (means) were used to assess the significance of differences in distributions. For comparisons with small patient groups, for example, the relapser group, the non-parametric Mann-Whitney test was used. A two-tailed P value of 0.05 was considered statistically significant in all statistical analyses. Graphical representations were generated with GraphPad Prism (version 5.01; GraphPad, San Diego, CA).

Microarray data accession number. Microarray data were deposited in the GEO under accession no. [GSE59312](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE59312).

RESULTS

Chronically HCV-infected patients with different treatment outcomes show comparable baseline clinical parameters and immune compositions in blood. In this study, we examined clinical material from 34 treatment-naïve patients chronically infected with HCV who were treated with pegIFN- α and ribavirin (Table 1) and were negative for serum HCV RNA by the end of therapy. Out of these patients, 7 individuals showed a reappearance of serum HCV RNA during the follow-up period of 24 weeks and were classified as viral relapsers. The other 27 patients remained virus negative and were classified as sustained viral responders. As shown in Fig. 1A and B, the viral loads and alanine aminotransferase (ALT) levels at baseline were similar between sustained responders and responders who relapsed after the end of treatment. Moreover, these levels were comparable in patients infected with HCV genotype 1 and those infected with HCV geno-

FIG 1 Chronically HCV-infected patients with different therapeutic responses display similar viral loads and ALT levels at baseline and similar frequencies of peripheral blood leukocyte subpopulations. (A) Mean HCV loads ($\pm$ SEM) at baseline showed no significant differences between responders ($n = 27$) and relapsers ($n = 7$) after IFN-based therapy and were independent of the HCV genotype based on nonparametric Mann-Whitney tests and Student's t tests, respectively. (B) Mean ALT levels ($\pm$ SEM) at baseline showed no significant differences between patient groups based on nonparametric Mann-Whitney tests and Student's t tests, respectively, and were again found to be independent of the HCV genotype. (C). At baseline, responders and relapsers showed comparable frequencies of granulocytes, total lymphocytes, and monocytes (top); CD3⁺, CD4⁺, and CD8⁺ T lymphocytes (middle); and CD19⁺ B lymphocytes and NK cells (bottom) in peripheral blood. Values are presented as percentages of the total leukocytes. No significant differences between patient groups stratified by therapeutic response to IFN-based therapy were observed based on nonparametric Mann-Whitney tests.

type 3. To determine whether the frequencies of distinct leukocyte subpopulations differed between patient groups, the relative contribution of these cells was determined by flow cytometry in peripheral blood. As shown in Fig. 1C, prior to

IFN-based therapy, no significant differences in the percentages of granulocytes, lymphocytes, and monocytes were observed. Further subdivision of lymphocytes into CD3⁺ T cells, CD19⁺ B cells, and CD56⁺ CD3[−] NK cells also showed similar

TABLE 2 Baseline gene predictors used to distinguish patients with chronic HCV infection who are successfully treated from patients who relapse after ending IFN-based therapy^a

Gene	Gene symbol	Functional association(s)
Spindlin 1	SPIN1	Cell cycle; reproductive process in a multicellular organism
TAF13 RNA polymerase II, TBP-associated factor, 18 kDa	227278_at	Basal transcription factors
HAUS augmin-like complex, subunit 2	HAUS2	Mitotic cell cycle
E4F transcription factor 1	E4F1	DNA replication
DEAD (Asp-Glu-Ala-Asp) box polypeptide 49	DDX49	ATP-dependent helicase activity
AE binding protein 2	AEBP2	Chromatin organization, modification
Ring finger protein 26	RNF26	Protein-DNA and protein-protein interactions
SEC63 homolog (<i>Saccharomyces cerevisiae</i>)	SEC63	Protein folding, localization, and transport
	235901_at	Proteolysis; cell-cell signaling
Phospholipase C, beta 2	PLCB2	Inositol phosphate metabolism, calcium signaling pathway, chemokine signaling pathway, T cell receptor
Glutaryl-CoA dehydrogenase	GCDH	Fatty acid, lysine degradation, tryptophan metabolism
Glucosidase, beta (bile acid) 2	GBA2	Lipid and glycolipid metabolism
Diacylglycerol O-acyltransferase 1	DGAT1	Glycerolipid and retinol metabolism
Leucyl/cystinyl aminopeptidase	LNPEP	Renin-angiotensin system
Glycoprotein Ib (platelet), beta polypeptide	206655_s_at	ECM-receptor interaction; hematopoietic cell lineage
Immunoglobulin heavy constant mu	IGHM	B cell development, activation
Leukotriene B4 receptor	LTB4R	Neuroactive ligand-receptor interaction
KIAA1949	KIAA1949	
Nitrilase 1	NIT1	
Yippee-like 2 (<i>Drosophila melanogaster</i>)	YPEL2	
Family with sequence similarity 65, member A	FAM65A	

^a TBP, TATA box binding protein; CoA, coenzyme A; ECM, extracellular matrix.

frequencies at baseline between patient subgroups stratified by their long-term treatment response.

Baseline gene markers predict therapeutic response of patients with chronic HCV infection. Having demonstrated that general clinical characteristics and leukocyte profiles are not associated with long-term therapeutic outcomes, we studied gene expression patterns in whole blood at baseline to identify genes that might predict viral relapse of patients with chronic HCV infection following successful IFN-based therapy. The expression profiles of patients with chronic HCV infection prior to therapy were first studied in comparisons with healthy individuals. Statistical analysis for microarrays, with a cutoff of a q value (FDR-adjusted P value) of $<1\%$ by significance analysis of microarrays (SAM), showed that at baseline, the numbers of significantly differentially expressed genes in responders and relapsers versus healthy individuals were 1,239 and 1,272, respectively. Since the frequencies of the major leukocyte subpopulations were similar between experimental groups (Fig. 1C), differential expression profiles were unlikely to be the result of variations in cell composition. Starting with these differentially expressed genes, an optimized predictive signature was generated by using supervised learning algorithm-support vector machine classification with cross validation, consisting of 21 probe sets, corresponding to 18 genes (Table 2). Except for the immunoglobulin heavy constant mu (IGHM) gene, none of the genes included in the signature are immune genes, while the majority of genes encode metabolic enzymes. The baseline expression level of this 21-probe signature in patients compared to healthy controls is shown in Fig. 2. Importantly, we observed that patients who developed viral relapse showed distinctive patterns at baseline compared to patients who responded and remained negative for HCV RNA. The 21-probe signature classified 34 chronically HCV-infected patients with an end-of-treatment response as having a relapse or not during

the follow-up phase. A correct prediction rate of 94% was achieved, with a sensitivity of 0.71 and a specificity of 1 (see Table S5 in the supplemental material). No optimization was observed when the classification was limited to patients infected with HCV genotypes 1 and 3. The hierarchical clustering analysis of baseline expression levels of these genes in responders and relapsers was performed on both microarray measurements and reverse transcription-PCR (RT-PCR) measurements, as shown in Fig. S1A and S1B in the supplemental material, respectively. The hierarchical clustering of both microarray and RT-PCR data was able to separate responders and relapsers, consistent with the prediction results developed from machine learning.

Diminished ISG mRNA expression in virological responders after successful IFN-based therapy. Previously, it was shown that the expression levels of ISG mRNA in hepatocytes or in peripheral blood are associated with the outcome of IFN-based therapy for chronic HCV infection (11, 22, 23). However, the predictive signature (21-probe set) did not include any ISG, indicating that baseline expression of ISG cannot predict viral relapse after successful IFN-based therapy. To gain more insight into longitudinal ISG mRNA expression following IFN-based therapy in sustained responders, we compared the ISG mRNA expression profiles of paired samples from sustained responders at baseline and at 24 weeks of follow-up. The ISG set was defined previously by Chaussabel et al. using peripheral blood of systemic lupus erythematosus (SLE) patients (24). As shown in Fig. 3A, compared to expression at baseline, decreased expression levels of ISG were clearly observed in the responders at 24 weeks of follow-up when the patients were HCV RNA negative. Analysis of gene expression patterns in a larger cohort showed high ISG expression levels prior to therapy compared to healthy individuals (Fig. 3B). After successful

FIG 2 Chronically HCV-infected patients who are responders or relapsers after IFN-based therapy are classified by a set of 18 genes (21 probe sets). A heat map shows the expression levels of 21 probe sets. Genes are clustered based on complete linkage using Spearman correlation coefficients as the distance measure. The black lines separate responders ($n = 27$) from relapsers ($n = 7$). The panel on the right-hand side presents a heat map depicting the same genes in healthy individuals ($n = 20$). Fibrosis scores, IL-28B SNP genotypes, and HCV genotypes are indicated for individual patients; black boxes indicate that the score or genotype has not been determined. The intensity values are normalized to the median value for a specific gene across all patients. Red represents relatively high expression levels, and blue represents relatively low levels.

IFN-based antiviral therapy, high expression levels of the ISG set were no longer observed, and at this time point, they more resembled the levels found in healthy individuals. For instance, decreased expression levels at 24 weeks of follow-up were observed for CXCL10 (2.1-fold), OAS1 (1.6-fold), IFI6 (1.7-fold), DDX60 (1.3-fold), TRIM5 (1.3-fold), and STAT1 (1.5-fold), compared to the expression levels at baseline.

Successful IFN-based therapy modulates and reverses expression levels of non-ISGs. As described above, the ISG set expression profiles in paired samples of sustained responders at baseline and at 24 weeks of follow-up differed as a consequence of successful IFN-based therapy. In addition to the 18 ISGs, 1,428 differentially expressed non-ISGs were identified in paired pre- and posttreatment samples from sustained responders using supervised SAM and a cutoff of a q value of $<10\%$, as described in Materials and Methods. A group of metabolic, immune, and signaling pathways was overrepresented by these genes determined by pathway enrichment analysis, such as metabolism pathways of alanine and aspartate, Jak-STAT signaling, and the endocytosis pathway (see Table S1 in the supplemental material). Out of these 1,428 genes, a subset of 45 genes was defined as therapy reversible in sustained responders (Fig. 3C); 16 of these genes were upregulated, and 29 were downregulated. The altered expression levels of these genes were observed in responder patients only at baseline and not at 24 weeks of follow-up based on a q value of $<10\%$ from supervised SAM statistics. The 45 therapy-reversible genes encode

proteins with a wide range of activities that are involved in immunity, autophagy, and enzymatic activities. These 45 genes and the other non-ISGs are presented in Table 3 and Table S2 in the supplemental material, respectively. The expression patterns of these 45 genes in paired samples from 8 responder patients at baseline and at 24 weeks of follow-up compared to healthy controls clearly showed alterations in gene expression levels as a consequence of successful treatment (Fig. 3D). The hierarchical clustering of 45 genes in paired samples of individual responders is shown in Fig. S2A in the supplemental material. At the group level, changes were observed for genes encoding proteins whose functions include regulation of cell proliferation and DNA synthesis, i.e., SMAD5 (1.85-fold decreased by therapy; $q < 0.01\%$); apoptosis, i.e., NLRP1 (1.3-fold increased by therapy; $q < 0.01\%$); nucleic acid and protein metabolism, i.e., TRIM5 (1.29-fold decreased by therapy; $q < 0.01\%$); and protein transport and localization, i.e., ATG16L2 (1.5-fold increased by therapy; $q < 0.01\%$) (Fig. 3E). The gene expression patterns and their association with long-term disease outcome identified by microarray were confirmed by RT-PCR measurements (see Fig. S2B in the supplemental material). The modulation of gene expression in all responding individuals before and after successful treatment exhibited an effect similar to the one described for paired responders (Fig. 3F). When these genes were assessed in healthy individuals, we observed a clear pattern of differential expression for most of the genes between responders at baseline and healthy controls, while this pattern was

FIG 3 Successful IFN-based treatment of patients with chronic HCV infection partly restores deregulated gene expression in responders. (A) The expressions of 18 ISGs were downregulated in 8 responders after successful IFN-based therapy at 24 weeks of follow-up compared to levels in the same patients prior to therapy. (B) Expression levels of 18 ISGs in healthy individuals ($n = 20$), responders at baseline ($n = 27$), and responders at 24 weeks of follow-up. Relatively high expression levels were observed in responders at baseline compared to healthy individuals but not in responders at 24 weeks of follow-up. The paired samples are indicated by numbers above the heat map. (C) Out of 1,446 genes differentially expressed between pre- and posttreatment responders, 45 were defined as therapy reversible in HCV responders. (D) Modulation of gene expression levels of 45 therapy-reversible genes is shown in a heat map for paired samples of individual responders ($n = 8$) to IFN-based therapy at baseline and at 24 weeks of follow-up. (E) Individual gene expression levels of ATG16L2 (1.5-fold increased by therapy; $q < 0.01\%$), TRIM5 (1.29-fold decreased by therapy; $q < 0.01\%$), NLRP1 (1.3-fold increased by therapy; $q < 0.01\%$), and SMAD5 (1.85-fold decreased by therapy; $q < 0.01\%$) are shown for paired samples of individual responders ($n = 8$) to IFN-based therapy at baseline and at 24 weeks of follow-up. (F) The expression pattern of the same 45 genes in healthy individuals ($n = 20$), responders at baseline ($n = 27$), and responders at follow-up week 24 ($n = 9$) is shown in a heat map. Prior to IFN-based therapy, these genes showed a >1.5 -fold alteration of expression levels in responders compared to healthy individuals. The deregulated expression of these genes was reversed by successful antiviral therapy, as determined at follow-up week 24. In all heat maps, genes are clustered based on complete linkage using Spearman correlation coefficients as the distance measure. The paired samples are indicated by numbers above the heat map.

less pronounced between responders at 24 weeks of follow-up and healthy controls (Fig. 3F).

Sustained aberration of expression of certain genes after successful antiviral therapy. To examine the prolonged effect of suc-

cessful IFN-based therapy on the transcriptome of patients with chronic HCV infection, gene expression profiles of responders at 24 weeks of follow-up were further compared to profiles from healthy individuals as a reference. We identified 1,424 genes dif-

TABLE 3 Genes with expression levels reversible by IFN-based therapy in patients with chronic HCV infection^a

Probe	Gene	Gene symbol	Ratios of gene expression level	
			Responders at 24 wk of follow-up vs responders at baseline	Responders at baseline vs healthy controls
232125_at			0.59	2.56
1557236_at	Apolipoprotein L, 6	APOL6	0.67	2.42
227740_at	UHM kinase 1	UHMK1	0.68	2.14
205187_at	SMAD family member 5	SMAD5	0.54	2.09
205091_x_at	RecQ protein-like (DNA helicase Q1-like)	RECQL	0.80	2.03
212417_at	Secretory carrier membrane protein 1	SCAMP1	0.73	1.96
219844_at	Chromosome 10 open reading frame 118	C10orf118	0.65	1.94
232940_s_at	Myeloid/lymphoid or mixed-lineage leukemia 3	MLL3	0.79	1.90
235003_at	UHM kinase 1	UHMK1	0.66	1.84
210705_s_at	Tripartite motif-containing 5	TRIM5	0.78	1.75
213158_at			0.85	1.68
234988_at	Valosin-containing protein (p97)/p47 complex-interacting protein 1	VCPIP1	0.85	1.60
1552978_a_at	Secretory carrier membrane protein 1	SCAMP1	0.74	1.60
221510_s_at	Glutaminase	GLS	0.82	1.56
1552644_a_at	Polyhomeotic homolog 3 (<i>Drosophila</i>)	PHC3	0.86	1.55
217813_s_at	Spindlin 1	SPIN1	0.75	1.54
202775_s_at	Splicing factor, suppressor of white-apricot homolog (<i>Drosophila</i>)	SFSWAP	1.09	0.67
213300_at	ATG2 autophagy-related 2 homolog A (<i>S. cerevisiae</i>)	ATG2A	1.15	0.67
201221_s_at	Small nuclear ribonucleoprotein, 70 kDa (U1)	SNRNP70	1.14	0.66
202032_s_at	Mannosidase, alpha, class 2A, member 2	MAN2A2	1.30	0.66
218274_s_at	Ankyrin repeat- and zinc finger domain-containing 1	ANKZF1	1.16	0.65
218376_s_at	Microtubule-associated monooxygenase-, calponin-, and LIM domain-containing 1	MICAL1	1.19	0.65
232946_s_at	NAD synthetase 1	NADSYN1	1.12	0.65
201244_s_at	v-raf-1 murine leukemia viral oncogene homolog 1	RAF1	1.20	0.64
203249_at	Enhancer of zeste homolog 1 (<i>Drosophila</i>)	EZH1	1.19	0.63
209110_s_at	Ral guanine nucleotide dissociation stimulator-like 2	RGL2	1.32	0.62
212708_at	Male-specific lethal 1 homolog (<i>Drosophila</i>)	MSL1	1.30	0.61
230142_s_at	Cold-inducible RNA binding protein	CIRBP	1.23	0.60
218920_at	Family with sequence similarity 193, member B	FAM193B	1.25	0.59
232145_at	Chromosome 2 open reading frame 68	C2orf68	1.39	0.59
210113_s_at	NLR family, pyrin domain-containing 1	NLRP1	1.32	0.58
202875_s_at	Pre-B cell leukemia homeobox 2	PBX2	1.28	0.56
226340_x_at	WAS protein homolog 1, 2, 3, 7 pseudogene	WASH1,2P,3P,7P	1.19	0.55
214780_s_at	Myosin IXB	MYO9B	1.15	0.55
232543_x_at	Rho GTPase-activating protein 9	ARHGAP9	1.27	0.55
227668_at	Chromosome 17 open reading frame 56	C17orf56	1.30	0.54
225995_x_at	WASP homolog 1,2 pseudogene	WASH1,2P	1.14	0.54
225191_at	Cold-inducible RNA binding protein	CIRBP	1.32	0.53
1569257_at	Formin-like 1	FMNL1	1.59	0.51
219577_s_at	ABC1, member 7	ABCA7	1.25	0.51
202392_s_at	Phosphatidylserine decarboxylase	PISD	1.39	0.51
210428_s_at	Hepatocyte growth factor-regulated tyrosine kinase substrate	HGS	1.16	0.49
225883_at	ATG16 autophagy-related 16-like 2 (<i>S. cerevisiae</i>)	ATG16L2	1.54	0.48
206722_s_at	Lysophosphatidic acid receptor 2	LPAR2	1.23	0.48
232077_s_at	Yippee-like 3 (<i>Drosophila</i>)	YPEL3	1.69	0.43

^a UHM, U2AF homology motif; ABC1, ATP binding cassette, subfamily A.

ferentially expressed between sustained responders assessed during the follow-up period and healthy individuals using supervised SAM, as described in Materials and Methods. These differentially expressed genes were further categorized with respect to their expression levels in responders at baseline. Sustained aberration was assigned to the genes ($n = 584$) that also showed aberrant expression in responders at baseline compared to healthy individuals (Fig. 3C; see also Table S3 in the supplemental material). Genes that exhibited aberrant expression levels compared to those in

healthy individuals, at baseline and at 24 weeks of follow-up, included the CCND3 (1.59-fold and 1.5-fold decreased at baseline and at follow-up, respectively; $q < 0.01\%$), TNFRSF25 (2.0-fold and 1.6-fold decreased at baseline and at follow-up, respectively; $q < 0.01\%$), and RASSF1 (1.69-fold and 1.54-fold decreased at baseline and at follow-up, respectively; $q < 0.01\%$) genes, which were associated with cell cycle control and cell growth (Fig. 4). Moreover, the expression levels of immune genes such as the ICAM2 (1.4-fold and 1.6-fold decreased at baseline and at follow-up, respectively; $q <$

FIG 4 Sustained aberration of gene expression observed in responder patients after successful IFN-based therapy. The mRNA expression levels of genes in healthy individuals ($n = 20$), responders at baseline ($n = 27$), and responders at 24 weeks of follow-up ($n = 11$) are shown. Compared to healthy individuals, significantly reduced mRNA expression levels of RASSF1, TNFRSF25, and CCND3 mRNAs (top) as well as ICAM2, IL-16, and IL-32 mRNAs (bottom) were observed for chronically HCV-infected patients at baseline as well as after successful antiviral therapy.

0.01%), IL-16 (1.5-fold and 1.5-fold decreased at baseline and at follow-up, respectively; $q < 0.01\%$), and IL-32 (2.3-fold and 2.4-fold decreased at baseline and at follow-up, respectively; $q < 0.01\%$) genes were also not normalized after successful IFN-based therapy in responders at 24 weeks of follow-up.

DISCUSSION

The present study shows distinctive blood transcriptional signatures prior to administration of antiviral therapy in chronically HCV-infected patients exhibiting a sustained virological response and in patients who relapse after the end of treatment. In addition, we demonstrate that the blood transcriptional signature observed 24 weeks after the end of successful therapy does not normalize to the expression profile observed in healthy individuals.

We found that chronically HCV-infected patients who are sustained responders or relapsers after IFN-based therapy show comparable baseline clinical parameters and immune compositions in blood. However, evaluation of gene expression profiling at baseline demonstrated that these chronically HCV-infected patients with different treatment outcomes are classified by a set of 18 genes, which enables us to predict whether patients will relapse after the end of IFN-based therapy or will be sustained responders. Considering the costs of novel DAA-based therapies and the severe adverse effects of IFN-based therapies, predictive markers are instrumental to determine the most optimal therapeutic strategy for chronic HCV infection. A number of studies have examined the expression of hepatic genes to discriminate responders and nonresponders (8, 9, 12). For example, Chen et al. identified intrahepatic expression of 18 genes, which were able to predict the end-of-treatment response and nonresponse to IFN-based therapy for patients chronically infected with HCV genotype 1 (8).

Onomoto and colleagues profiled mRNA expression of IFN-related genes using liver biopsy specimens from patients with chronic HCV infection and found that 31 IFN-related genes exhibited distinct intrahepatic expression patterns between nonresponders and healthy individuals. A predictive model based on these 31 IFN-related genes correctly predicted the treatment responses in nonresponders infected with HCV with an accuracy of 86.1% (12). We found no overlapping genes between those studies and our own. To our knowledge, the blood gene signature identified in the present study is the first predictor of long-term treatment outcome for responder patients with chronic HCV infection. The predictive accuracy of 94% with our 18-gene signature was higher than those of existing hepatic gene signatures and is applicable to patients infected with either HCV genotype 1 or HCV genotype 3. To validate the importance of this 18-gene signature, an independent cohort is desired, and therefore future studies by other research groups will be important.

Besides elimination of HCV RNA from the liver and circulation, successful therapy of chronically HCV-infected patients with IFN-based therapy results in the improvement or normalization of ALT levels, albumin levels, platelet counts, and APRI (aspartate aminotransferase to platelet ratio index) values (25, 26). However, to our knowledge, no studies were conducted to determine whether the transcriptome is normalized as a consequence of elimination of virus by successful IFN-based regimens. The present study compared transcriptional profiles of responders during follow-up with profiles obtained from healthy individuals and compared paired profiles from patients prior to and after IFN-based therapy. From these two comparisons, we identified 1,424 and 1,446 differentially expressed genes, respectively. Among

these differentially expressed genes, 45 were found have expression levels that were reversible by IFN-based therapy with respect to their baseline expression levels in responder patients. These genes encode important proteins that function in highly diverse aspects of antiviral immunity and include SMAD5 (involved in transforming growth factor β [TGF- β] signaling), TRIM5 (a positive regulator of innate immune responses), and NLRP1 (a member of the family of innate immune receptors).

Compared to healthy individuals, we and others found that patients with chronic HCV infection have high expression levels of ISGs at baseline (27). Importantly, therapy-induced viral eradication led to decreased expression levels of ISGs, likely due to a removal of the pathogenic trigger. However, despite the absence of the virus, after 24 weeks of follow-up in responder patients, blood transcriptional analysis showed that 584 genes (including the HLA-B, ICAM2, KLF13, MAP2K2, SLC, TOLLIP, and TRIM11/41 genes) were differentially expressed and not reversible by successful therapy. Moreover, the expression levels of IL-16 and IL-32 are also nonreversible, with the latter being involved in the induction of tumor necrosis factor (TNF) production by macrophages (provided in the RefSeq database, 2014) and exhibiting a positive association with HCV-related inflammation and fibrosis (28). Another gene found to be differentially expressed was the RASSF1 gene, which is associated with cell cycle regulation and cell proliferation and is a well-studied tumor suppressor gene for lung cancer and hepatocellular carcinoma (29–32). The biological relevance of our findings still needs to be determined and requires detailed *in vitro* or *in vivo* models. However, these studies are important to conduct, since the genes identified may include possible candidates involved in progression of liver pathology due to previous HCV infection or, conversely, in damage repair. Furthermore, the long-term consequences of previous HCV infections are not well defined, and persistent changes in the transcriptome after viral eradication may be relevant for HCV reinfections or for ongoing immunopathology. Reminiscent of these findings, we previously reported that high numbers of regulatory T cells are retained in the liver of chronically HCV-infected patients months after successful therapy-induced viral clearance (16). Furthermore, we also observed sustained alteration of HCV-specific T cell responses and their regulation in blood of patients who responded to IFN-based therapy (17).

In future studies, it is important to examine whether the persistently altered gene expression in sustained viral responders observed in this study is unique for the activity of an IFN-based regimen. The status of normalization of the blood transcriptome should also be evaluated in patients treated with IFN-free regimens consisting of novel direct-acting antivirals. In addition to the serum levels of HCV RNA and ALT, blood gene markers indicating long-term genomic normalization should be considered additional parameters for defining a sustained antiviral response.

The findings in our present study indicate (i) the existence of a distinctive blood transcriptional signature prior to administration of antiviral therapy in chronically HCV-infected patients that identifies patients who relapse after the end of treatment and patients with a sustained viral response, (ii) that altered expression levels of a considerable number of genes were not reversed by successful IFN-based therapy in responder patients, (iii) that successful virus eradication does not result in complete transcriptional normalization in responder patients, and (iv) that further long-term follow-up studies in independent cohorts are required to determine if sustained aberration of these genes in responder

patients affects disease progression or immunity following infection with pathogens or reinfection with HCV.

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