Predicting Treatment Response and the Role of the ISG15/USP18 Ubiquitin-like Signaling Pathway in Hepatitis C Viral Infection

by

Limin Chen

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Department of Molecular Genetics
University of Toronto

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2010

Abstract

Hepatitis C Virus (HCV) infects 170 million people worldwide. The current treatment regimen, which is combination therapy with pegylated interferon (PegIFN) and Ribavirin (Rib), cures only 50% of the patients infected with the most prevalent HCV genotype. Therefore, there is a pressing need to understand the molecular mechanism of interferon resistance and to develop a prognostic tool to predict who will respond to treatment before initiation of therapy. It has been firmly established that the virus-host interaction plays an important role in determining treatment outcomes. My thesis investigated the host factors that are involved in interferon resistance with an aim to provide insights into the molecular mechanism of IFN resistance.

cDNA microarray analysis identified 18 differentially expressed hepatic genes from pretreatment liver tissues of responders (Rs) and non-responders (NRs). Based on the differential expression levels of these 18 genes, a prognostic tool was developed to predict who will respond to therapy, with a positive predicting value (PPV) of 96%. Most of these 18 genes are interferon stimulated genes (ISGs) and they are more highly expressed in NR livers, indicating that preactivation of interferon signaling in the pre-treatment liver tissues contributes to NR. 3 out of the 18 genes are
involved in an ubiquitin-like ISG15/USP18 signaling pathway that plays an important role in interferon response. Over-expression of USP18 and ISG15 in the pretreatment liver tissues of NR promotes HCV production and blunts interferon anti-HCV activity. There exists a distinct cell-type specific ISG activation in the pretreatment liver tissues of Rs and NRs. Up-regulation of the two ISGs that I tested (ISG15 and MxA) was found mainly in hepatocytes in NRs while ISG activation was preferentially observed in macrophages in Rs.

Taking all these data together, pre-activation of interferon signaling and cell-type specific gene activation in the pretreatment liver tissues of patients infected with HCV are associated with treatment non-response. HCV exploits the host interferon system to favour its persistence by enhanced replication /secretion stimulated by a few ISGs (ISG15, USP18) in response to IFN. The developed prognostic tool can be used to stratify patients for treatment and the novel insights of the molecular mechanism of IFN resistance in HCV patients offer potential drug targets for future development.
Acknowledgments

I wish to sincerely thank my supervisor Dr. Aled Edwards for allowing me to stay in his lab to finish this interesting project. His support, guidance, encouragement, motivation, and supervision are greatly appreciated. While providing me with freedom to work independently, Dr. Edwards inspired me to achieve every step of my scientific goals and become a more productive scientist.

I would also like to wholeheartedly thank my research supervisor Dr. Ian McGilvray for assisting me to overcome many obstacles that I have encountered during my PhD research. His input, tutoring, encouragement and support, both emotionally and financially, will never be forgotten.

My supervisory committee members Dr. Jack Greenblatt, Dr. Rob Rottapel, and Dr. Daniela Rotin have also been extremely helpful during this process due to their critical insights and many valuable contributions for my thesis work. In addition, I would like to thank my collaborators, Dr. Jenny Heathcote, Dr. Charles Rice and Dr. Glenn Randall for providing me with all the necessary materials and reagents, including the most precious HCV liver biopsy samples and HCV in vitro culture systems. Without their support this project could not have been completed.

I would also like to thank all the past and present members of Edwards/McGilvray laboratory and management that assisted me in one way or another, with special thanks to Dr. Ivan Borozan, Jing Sun, Larry Meng, Qiong Lin, Yan Chen and Max Ruzanov.

I wish to acknowledge the National CIHR Research Training Program in Hepatitis C (NCRTP-HepC), the Canadian Graduate Scholarship (CGS), and the Canadian Institute for Health Research (CIHR) for their financial support.

To my wife, Jing Sun, and my daughter and my parents for their love, support, understanding and encouragement in making this long, but rewarding journey possible.
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<td>“complete” Early Viral Response</td>
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<td>CHC</td>
<td>chronic hepatitis C</td>
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<tr>
<td>CTL</td>
<td>cytotoxic T-lymphocyte</td>
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<td>dsRNA</td>
<td>double-stranded RNA</td>
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<td>EC50</td>
<td>median effective concentration</td>
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<td>EIF2α</td>
<td>eukaryotic translation initiation factor 2α</td>
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<td>ESCRT-I</td>
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<td>ETR</td>
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<td>interferon regulatory factor 3</td>
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<td>ISGs</td>
<td>IFN-stimulated genes</td>
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<tr>
<td>KNN</td>
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<td>mAb</td>
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<td>Mda5</td>
<td>Melanoma differentiation associated gene 5</td>
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<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
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<td>NK</td>
<td>natural killer</td>
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NPV  negative prediction value
NR  non-responder
OAS1  2’5’-oligoadenylate synthetase 1
pAb  polyclonal antibody
PAMP  pathogen associated molecular patterns
PCA  principal components analysis
PCR  polymerase chain reaction
pegIFN/rib  pegylated interferon/ribavirin
PKR  protein kinase R
PPV  positive prediction value
R  Responder
RdRP  RNA dependent RNA polymerase
RIG-I  retinoic-acid inducible gene I
RNaseL  ribonuclease L
RVR  Rapid Viral Response
siIRR  irrelevant small inhibitory RNA
siRNA  small inhibitory RNA
SNP  single nucleotide polymorphism
SNV  Sindbis virus
SR-BI  scavenger receptor class B type I
ssRNA  single-stranded RNA
STAT-C  specific targeted antiviral therapy for hepatitis C
SVR  sustained virological response
TLR3  Toll-like receptor 3
Ube1L  ubiquitin E1 like
USP  ubiquitin-specific protease
USP18  ubiquitin-specific protease 18
VSV  vesicular stomatitis virus
List of Published Papers

Thesis related:


Thesis non-related:

1. **Limin Chen**, Ivan Borozan, Piotr Milkiewicz, Jing Sun, Xiangbin Meng, Catalina Coltescu, Aled M Edwards, Mario A Ostrowski, Maha Guindi, EJ Heathcote, Ian D McGilvray. Gene expression profiling of early primary biliary cirrhosis: Insights into the


Chapter 1: INTRODUCTION

1.1 HCV: virology, epidemiology and treatment

Hepatitis C virus (HCV) is the only member of the genus *Hepacivirus* in the family *Flaviviridae*.\(^1,2\) Identified as the pathogen that caused the non A-non B hepatitis in 1989, HCV infection is one of the most common causes for liver diseases, currently infecting 170 million people worldwide.\(^3,4\) After exposure to HCV, 60-80% of infected patients develop chronic infection despite the induction of HCV-specific antibodies and a HCV-specific cellular immune response.\(^5,6\) HCV chronic infection frequently results in progressive fibrosis, cirrhosis and an increased risk of hepatocellular carcinoma.\(^7\) As such, HCV is a significant health burden and the leading indicator for liver transplantation in the US and Western Europe.\(^1\)

The HCV genome consists of a positive strand RNA, 9.6Kb in length, encoding a single polyprotein that is processed by both host and viral proteases to generate 3 structural (Core, E1, E2) and 7 non-structural (P7, NS2/3, NS3, NS4A, NS4B, NS5A, and NS5B) proteins (Figure 1-1).\(^8\) There are six major HCV genotypes; genotype 1 is the most prevalent genotype in North American, Asia, and Europe, while genotype 4 is the most common in Egypt.\(^9\)
The HCV life cycle starts with receptor-mediated viral particle endocytosis. Several receptors or co-receptors have been identified, including CD81, the scavenger receptor class B type I (SR-BI), claudin-1, occludin, DC-SIGN and L-SIGN. Once inside the cell, the HCV virion
is de-coated and the positive strand RNA is released into the cytoplasm to function as an mRNA template to direct IRES-mediated translation. This translation generates a single polyprotein (3011aa) that is co- and post-translationally cleaved into 3 structural and 7 non-structural proteins by host signal peptidase and viral proteases. These viral proteins assemble with the newly-replicated viral RNA to form new virions to be released out of cells to infect other naïve cells.

HCV infects only humans and chimpanzees. The clinical course of infection varies, but the majority of humans infected cannot clear the virus and up to 85% will develop chronic infection - defined as HCV RNA persistence in the serum for more than 6 months as detected by PCR. No vaccine is available. The current standard of care antiviral therapy consists of combination therapy with pegylated IFNa and Ribavirin (IFN/RBV). This treatment is often inadequate. Although there is a 60-80% response rate in patients infected with genotype 2 and 3, there is only a 30-50% rate of response for genotype 1. Thus, in North America the majority of patients chronically infected with Hepatitis C (predominantly Genotype 1) will not respond to treatment with combination of PegIFN/Ribavirin. The treatment is also expensive – estimated at more than 25 000$ US per patient - and associated with significant side effects over a prolonged period of treatment (12-24 months).

The fact that the different genotypes respond dramatically differently to combination therapy also indicates that viral genotype plays an important role in determining the outcome of infection, although detailed mechanisms remain poorly understood.
1.2 HCV and innate immune response: induction and evasion mechanisms

1.2.1 Innate immune response to HCV infection: Type 1 IFN production and the Jak/STAT pathway

The first line of host defense to a viral infection is the innate immune response, which promotes the subsequent adaptive immune response. The innate immune response is characterized by the production of type 1 interferon (principally IFNα and β), IFN-stimulated genes (ISGs) and the activation of natural killer (NK) cells. NK cells mainly circulate in the blood, where they account for 5 to 15% of circulating lymphocytes; by contrast, they represent up to 45% of tissue-infiltrating lymphocytes in the liver. The innate immune response is triggered by pathogen associated molecular patterns (PAMPs) such as double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and unmethylated CpG motifs in DNA. IFNβ is produced after activation of RIG-I/Mda5 (retinoic acid inducing gene-I/Melanoma differentiation associated gene 5) or endosomal Toll-like receptor 3 (TLR3) pathways. After IFNβ is expressed and binds to its cognate type 1 IFN receptors, IFNα and interferon stimulated gene (ISG) production is stimulated through the Jak/STAT pathway. Many ISGs are known to have antiviral activity, such as 2’5’-OAS1, PKR, MxA and P56.

2’5’-oligoadenylate synthetase 1 (OAS1) is expressed at low constitutive levels as an inactive monomer in the cytoplasm and is upregulated by type I interferons (IFNs). Following activation by viral double-stranded RNA (dsRNA), the enzyme oligomerizes to form a tetramer that synthesizes 2’5’-oligoadenylates that, in turn, activate the constitutively expressed inactive ribonuclease L (RNaseL), and this then enables RNaseL to cleave cellular (and viral) RNAs.
Protein kinase R (PKR) accumulates in the nucleus and cytoplasm as an inactive monomer, which is activated directly by viral RNAs. Following activation, PKR monomers are phosphorylated and dimerize to form the active enzyme. Activated PKR plays an important role in viral defence by inhibiting translation through phosphorylation of eukaryotic translation initiation factor 2α (EIF2α). The MxA protein accumulates in the cytoplasm on intracellular membranes (such as the endoplasmic reticulum, ER) as oligomers. Following viral infection, MxA monomers are released and bind viral nucleocapsids or other viral components, to trap and then degrade them. The P56 families of proteins have been implicated in IFN’s antiviral actions against HCV, West Nile virus and LCMV. The C-terminal region of P56 mediates its interaction with eIF-3e and causes an impairment of eIF-3 function and resultant inhibition of protein synthesis. In addition to function through impairment of eIF-3 and resultant inhibition of protein synthesis the antiviral effect of P56 was ascribed to its direct interaction with some viral proteins. For example, in HPV virus infection, P56 interacts with the DNA replication origin-binding protein E1 of several strains of HPV to directly inhibit HPV replication.

The innate immune response also involves activation of NK cells, whose main roles are to produce immune-regulatory cytokines (IFNγ, TGF-β, IL3, GM-CSF, and TNFα) and to link the innate and adaptive immune responses by activating dendritic cells (DCs) in TNFα-dependent manner.
Figure 1-2. Type 1 IFN induction and the Jak/STAT pathway

Adapted from O. Haller et al. Virology 344 (2006) 119-130

1.2.2 Adaptive immune response to HCV infection: humoral and cell-mediated immune responses

The adaptive immune response to HCV consists of a B-cell response (HCV-specific antibody) and a T-cell response (CD4+ T helper cells and CD8+ Cytotoxic T lymphocytes).
While anti-HCV antibodies are easily detected in the overwhelming majority of chronic HCV cases,\textsuperscript{30} they are not neutralizing and do not play a major role in determining the outcome of HCV infection. The lack of a role is demonstrated by the fact that a robust humoral response does not provide protection against re-infection in chimpanzees and humans, and the levels of anti-HCV antibodies in patients do not correlate with a more favourable outcome of infection.\textsuperscript{31,32} This said, chimpanzees and humans who have cleared the virus seem to be less likely to develop chronic infections after re-exposure.\textsuperscript{33-35}

However, there is strong evidence that virus-specific CD4+ and CD8+ T cells (cellular immune response) play a major role in viral control and clearance – i.e. in determining the outcome of infection (resolution or persistence).\textsuperscript{36,37} Lymphocyte depletion studies in chimpanzees have demonstrated a crucial role for both CD4+ and CD8+ T cells in mediating protective immunity.\textsuperscript{38,39}

\subsection*{1.2.3 HCV evasion from innate immune response: counteracting IFN pathways and inhibiting NK cell activity}

Even after successful induction of HCV-specific antibodies and T-cell responses, 60-80\% of infected patients develop chronic infections.\textsuperscript{5,6} This fact indicates that HCV has evolved a number of mechanisms to evade both the host innate immune response and the adaptive immune response. HCV evades the host innate immune response by inhibiting IFN production, interfering with IFN signaling transduction (JAK/STAT pathway) and modulating IFN effector molecules.\textsuperscript{40} Quite surprisingly, HCV has also developed a mechanism to inhibit NK cell activity: the E2
structural protein of HCV crosslinks CD81 on NK cells to inhibit cytotoxicity and IFNγ production. These countermeasures appear to be quite efficient, since almost 85% of the HCV-infected patients develop a chronic infection, and up to 60% of those patients do not respond to IFN therapy or experience a relapse when therapy is stopped.

1.2.4 HCV evasion from adaptive immune response: lack of priming or exhaustion of T cell response

A lack of primary induction of T cell response and exhaustion of an initially vigorous response are two important predictors of viral persistence both for HCV and other viruses. Lack of priming may be due to impairment of antigen presentation by DCs and macrophages. For example, Kaposi sarcoma herpes virus (KSHV) encodes a membrane-bound viral E3 ligase (K3 or K5) that promotes MHC class 1 molecule polyubiquitination and degradation in lysosomes. T cell exhaustion might also result from deletion of virus-specific T cells in the presence of continuously high viral load or lack of a functional CD4+ T helper cell response. Most recently, programmed death 1 (PD-1) protein, of the CD28 family of receptors, was shown to be a marker for these exhausted T cells. Binding of PD-1 to one of its ligands, PD-L1 or PD-L2, transmits a negative signal to the T cells expressing PD-1, reducing cytokine production such as interleukin-2, tumor necrosis factor-α and interferon-γ and proliferation. Quite interestingly, high levels of PD-1 expression were found on bulk CD8+ T cells and intrahepatic HCV-specific CD8+ T cells from the livers of patients with chronic HCV infection, with higher expression level of PD-1 on liver infiltrating HCV-specific CD8+ T cells compared with peripheral blood. As in HIV infections, in vitro blockade of PD-1/PD-1L has been shown to improve the functionality of the impaired HCV-specific CD8+ T cells from the peripheral blood.
HCV virus mutational escape might also play a role in evasion. For example, mutations in the hypervariable region of E2, which is the antibody recognizing site, may allow the virus to avoid antibody neutralization. The virus in the liver of HCV infected patients replicates very rapidly with a viral half-life (the time taken for half of the viruses to be cleared) about 3 hours, and about $10^{12}$ virions are produced per day in an infected person. Rapid replication in conjunction with the lack of proof-read ability of the HCV RNA dependent RNA polymerase (RdRP) makes the virus exist as a quasispecies in patients. These diversities of viral genomes favour the virus evading host immune attack.

1.3 Predicting treatment response: current approaches and limitations

As noted earlier, a considerable number of persons chronically infected with HCV are subjected to a highly morbid, highly costly treatment involving the combination of PegIFN and ribavirin. At present there is no reliable way to accurately predict treatment responses prior to initiation of therapy. Much effort has been made to determine which patients will respond to treatment as soon as possible. The current standard is to measure decreases in viral load early during treatment. Patients with a Rapid Viral Response (RVR) - defined as having no detectable virus at 4 weeks of treatment – have an 88% chance of being cured. Unfortunately, only 19% of genotype 1 patients achieve an RVR. Patients with a “complete” Early Viral Response (cEVR) – no detectable virus at 12 weeks of treatment – have a 68% chance of being cured; however, <70% of genotype 1 patients achieve an EVR. The negative predictive value is only about 50% for both. In addition, these measures can only be implemented after treatment has been initiated,
which is sub-optimal: in clinical practice, patients are often unwilling to start treatment due to the side-effects and the low probability of success. An ability to predict treatment response prior to initiating treatment would encourage patients to start and to continue treatment.

Although a few specific targeted antiviral therapies for hepatitis C (STAT-C), such as NS3/4A protease and NS5B polymerase inhibitors, are being developed with the most promising one entering into phase III clinical trial, viral mutations that are resistant to these modalities often occur. As a result, it is quite likely that Pegylated IFN/Ribavirin will remain a mainstay of therapy for the foreseeable future, to be administered alone or in combination with protease/polymerase inhibitors.

1.4 Pre-treatment predictors of response in HCV: genomics-based approaches

In addition to viral and patient characteristics, the genetic diversity of the host contributes to the outcome of infection and treatment of chronic HCV. High-throughput techniques now allow for the rapid and accurate characterization of gene expression in tissues, and for the detection of individual host genetic polymorphisms.

1.4.1 Hepatic gene expression

Gene expression profiling studies that have looked at the effects of HCV infection in the host liver have often aimed to associate changes in the expression of individual genes with clinical outcomes or treatment responses. For example, I used a 19,000 cDNA microarray to study pre-treatment liver biopsy specimens taken from patients with chronic HCV who were subsequently
treated with combination therapy with PegIFNα/RBV. Gene expression levels were compared among 15 non-responder, 16 responder, and 20 normal liver biopsy specimens. I identified a discrete set of 18 genes whose expression differed consistently between responders and non-responders ($p < 0.05$). Many of these 18 genes were IFN sensitive genes (ISGs), and three of them (USP18/UBP43, CEB1, and ISG15) play roles in the same IFN regulatory pathway, suggesting a possible rationale for treatment resistance. These results have now been substantiated by other groups and have been validated prospectively in a larger cohort of chronic HCV patients prior to initiating antiviral treatment. Using four different methods for classification accuracy (KNN, DQDA, DLDA, CART), we demonstrated that the 18 gene signature has a positive prediction value (PPV) of at least 95% for prediction of treatment response, though a negative prediction value (NPV) of only 50% – a predictive capacity similar to the RVR (Rapid Virological Response is defined as having more than 2 log10 viral titer drop at 4 weeks post treatment) and 48 hour drop in viral titers above. We went on to confirm that the USP18/UBP43 protease, a down-regulator of Type I interferon responses identified in our initial microarray experiment, plays an important role in regulating the anti-HCV effect of IFNα.

In another array study by Hayashida et al, they analyzed liver tissue samples obtained prior to the treatment of 69 HCV patients who then received either IFNα monotherapy or IFNα/RBV. Of these 69 samples, 31 were used as a training set to develop an algorithm for predicting interferon efficacy and 38 were used to validate the algorithm. They also applied their methodology to the prediction of the efficacy of IFNα/RBV combination therapy using an additional 56 biopsies. For the IFN group, genes differentially expressed were mainly IFN, lipid metabolism, complement,
and oxidoreductase-related genes. For the IFNα/RBV combination group a different set of genes was identified, including cyclophilin A and multidrug resistance protein with an accuracy of 93% for prediction of SVR/non-responders. The pattern of the genes in this study is different from ours and others, possibly because the majority of the patients (69%) in this study were genotype 2, which have a significantly higher SVR. It would be interesting to reanalyze these data after excluding genotypes 2 patients.

Asselah et al. recently identified a two-gene signature (IFI27 and CXCL9) that accurately predicted treatment response in 79% of patients being treated for chronic HCV. Using a large scale real-time quantitative RT-PCR to analyze the mRNA expression of 58 selected genes in liver biopsies of treatment-naive HCV patients, they found that the two-gene signature had a predictive accuracy of 100%, 70%, and 73% in non-responders, sustained responders and responder-relapsers. The predictive values of these genes also held true when the authors sub-analyzed patients according to both genotype 1 and to the severity of fibrosis. Both IFI27 and CXCL9 belong to the interferon-stimulated gene (ISG) family, and both are up-regulated in the pre-treatment liver tissue of patients who do not respond to treatment.

Feld et al. used a microarray-based approach to shed light on the mechanisms of action of combination PegIFNα/RBV therapy. They extended our previous observation that ISGs are more highly expressed in the pre-treatment liver tissue of non-responders than responders. In this study patients were randomized either to receive or not to receive RBV. All patients received
IFN 24 h prior to liver biopsy; those randomized to the RBV arm were treated with this drug for 72 h prior to the biopsy. The combination of PegIFNα/RBV resulted in greater up-regulation of genes involved in the interferon signaling cascade, and a more pronounced down-regulation of genes involved in IFN-inhibitory pathways, than did monotherapy with IFN. Additionally, pre-treatment ISG expression seemed to be higher in the liver tissue from slow responders than in the liver tissue of rapid responders. During treatment rapid responders had a higher fold induction of ISGs. A major puzzle is why the pre-treatment up-regulation of ISGs in the livers of non-responders is not able to eliminate the virus, whereas the IFN-driven up-regulation of ISGs in responders is associated with viral control. One possibility is that the virus in non-responders has had an opportunity to adapt mutationally to the up-regulation of ISGs.

Taken together, these studies argue that discrete gene subsets have predictive value in the treatment of chronic HCV infection, and offer intriguing insights into the mechanisms underlying treatment response and non-response.

1.4.2. Gene expression in blood

Given that HCV replicates almost exclusively in the liver, it is likely that the strongest HCV “signal” would be found in liver tissue. However, there is no question that it would be more convenient to develop a predictive test from blood. A number of groups have tried and generally failed to find a gene expression signature in peripheral blood that correlates with treatment outcomes in chronic HCV. One recent study was able to correlate peripheral blood gene
signatures with treatment responses – possibly because they focused on patients co-infected with both HCV and HIV. The study analyzed gene expression profiles in peripheral blood mononuclear cells (PBMCs) to predict treatment response in patients co-infected with HCV and HIV. The authors used a class prediction analysis of gene expression patterns in the PBMCs of 29 patients prior to antiviral treatment in order to predict the response of the patients to combination therapy. Seventy-nine genes correctly classified all 10 patients who did not respond to therapy, 8 of 10 patients with end of treatment response (ETR), and 7 of 9 patients with SVR. The same analysis was performed after therapy was initiated to predict SVR among patients with an EVR. Prediction analysis of the 17 post-treatment samples identified 105 genes that correctly identified all 9 patients with ETR and 7 of 8 patients with SVR. As with the intrahepatic profiles, failure of antiviral therapy was associated with increased expression of ISGs prior to treatment and the inability of these genes to be further stimulated by IFN administration. With the caveat that these results were generated in co-infected patients, overall the findings are consistent with the idea that dysregulation of subsets of IFN-stimulated genes in chronic HCV may be a biomarker of immune dysfunction and non-response to IFN plus RBV. These studies also suggest that non-responders tend to have high ISG expression pre-treatment, which is consistent with our previous findings from gene expression profiling, and are not able to increase ISGs much following initiation of treatment. The basis for this response is unclear.

Gerotto et al. studied the role of IFN-inducible protein kinase (PKR) in PBMCs and liver biopsies of patients with chronic HCV. They demonstrated that non-responders to combination therapy had pre-treatment mRNA levels in PBMCs and in liver that were significantly higher
than the responders. However, no difference in PKR mRNA levels were found in PMBCs of responders compared to the non-responders after in vitro exposure to IFN. Taken together these results indicate an endogenous activation of IFN production in non-responders prior to antiviral therapy.  

Taylor et al. studied gene expression profiles in PMBC samples from a group of patients infected with HCV genotype 1 during the first 28 days of IFN-based combination therapy. Results were analyzed with respect to treatment response (poor viral response – <1.5 log 10 IU/ml decrease of HCV RNA at day 28 – compared to marked viral response – >3.5 log 10 IU/ml decrease) and to race (African-American vs. Caucasian). They demonstrated that patients with a marked viral response had pronounced changes in PBMC gene expression, while patients with a poor viral response did not. ISG expression was strongly altered, suggesting that poor response to IFN-based therapy may be due to blunted induction of interferon responsive gene expression. Whether this lower response is determined by host genetics or due to the environment is unclear; and the results should be approached with caution given that they were generated from peripheral immune cells.

1.4.3. Host genetic polymorphisms

Single nucleotide polymorphisms (SNPs) are the most common form of genetic variation, with more than nine million reported in public databases. A SNP is a single nucleotide variation at a specific location in the genome that is by definition found in more than 1% of the population.
In general, SNPs occur much less frequently in coding regions of the genome than in non-coding regions, with most SNPs being located in non-coding regions.\textsuperscript{74} SNPs in non-coding regions, although they do not alter encoded proteins, can be useful as physical markers for comparative or evolutionary genomics studies. In the coding regions, SNPs can cause alterations in protein structure and hence function, leading to the development of disease or change in response to a drug or environmental toxin.

In several recent studies of patients with chronic HCV, SNP analysis for candidate genes was employed to predict both disease progression\textsuperscript{75} and therapeutic response.\textsuperscript{76,77} Genes that were assessed for disease-associated SNPs included interferon-stimulated genes such as 2–5 oligoadenylate synthetase (2–5 OAS), dsRNA activated protein kinase (PKR) and MxA. These genes are of special interest since the anti-HCV activity of IFN is likely mediated, at least in part, by their induction. Several studies have reported an association with SNPs in the promoter regions of MxA, OAS-1 and PKR.\textsuperscript{76,78,79} While it is difficult to draw firm conclusions from these mostly small studies, it is interesting that both OAS and MxA were found to be associated with treatment response in our impartial gene expression profiling study.

Other host factors that might also determine the response to treatment, such as cytokines, chemokines and their receptors, have also been examined for SNP associations.\textsuperscript{77,80,81} Among the cytokines examined was IFN-γ, which is produced by effector T and natural killer cells and has a critical role in host defense against a variety of intracellular pathogens, including HCV.
IFN-γ efficiently inhibits HCV replication in the replicon system *in vitro* and the intrahepatic level of IFN-γ appears to be associated with viral clearance in the chimpanzee model. In a recent study of two patient cohorts, Huang *et al.* demonstrated an important role between one specific polymorphism of IFN-γ and treatment response. The first cohort included 280 chronic HCV patients who had received INFα-based therapy. The second cohort contained 250 IV drug abusers who either spontaneously cleared the virus or became chronically infected. The authors demonstrated that among the eight analyzed SNPs spanning the entire IFN-γ gene in the two cohorts, only one SNP variant (−764G, located in the proximal I IFN-γ promoter region) was significantly associated with sustained virological response in the first cohort and with spontaneous recovery in the second cohort. Other cytokines such as IL10 have been studied in a similar manner. Yee *et al.* reported that two SNP variants of IL10 (−592A and −819T SNP) were more frequent among the patients with SVR compared to the non-responders to treatment with IFN/RBV. Further gene-specific and genome-wide association studies are needed to link genetic polymorphisms with clinical or treatment outcomes.

Most recently, four groups, using genome-wide association study (GWAS), reported that a single-nucleotide change at roughly 3kb upstream of the promoter region of IL28B gene (which encodes a type III IFNλ3) is associated with both treatment-induced and spontaneous Hepatitis C Virus clearance. Ge *et al.* scanned the genomic DNA sequences from more than 1600 treatment-naïve HCV genotype 1 (G1) infected patients and found that SNP rs12979860 was strongly associated with treatment induced SVR. This SNP was also found to be associated with virus spontaneous clearance.
It is of note that, although SNP rs12979860 is indeed the strongest hit that was found to be associated with treatment-induced or spontaneous HCV clearance from these GWAS, the authors did find that other SNPs were also associated, albeit the associations were statistically weaker. However, from the top 100 SNPs that were associated with SVR, none of them replicated previous reported SNPs (MxA, OAS-1, PKR, IFNγ, and IL-10) that were associated with treatment response. Failure to replicate these previous findings suggests that either those associations were too weak to be picked up by GWAS or those previous SVR associated SNPs were not real. To support this notion, from 637 non-Hispanic Caucasian patients infected with genotype 1, a study by Morgan et al. indicated that none of the 8 previously reported treatment response-associated SNPs was associated with SVR. Taking all these together, further cross-validation using even larger patient populations for GWAS is needed to confirm these associations.

1.5 ISG15 and USP18 in viral disease

The ISGs identified in our microarray study suggested a possible mechanism for treatment nonresponse. Three of the genes that are overexpressed in non-responders - interferon stimulated gene 15 (ISG15), ubiquitin specific protease 18 (USP18/UBP43), and CEB1/Herc5 (a HECT domain ISG15 E3 ligase) - are linked to a ubiquitin-like protein (Ubl)/ubiquitin specific protease (ISG15/USP18) pathway.
1.5.1 ISG15: structure, function, and ISG15 conjugation

Type I IFNs (IFNα, IFNβ, IFNω) are a group of cytokines that have anti-proliferation, anti-viral and immunomodulatory activities.\textsuperscript{76} Initiated by the sensor molecules RIG-I and TLR3, a signal cascade induced following viral or bacterial infection leads to the production of IFNβ, which is secreted and binds to the type I IFN receptors (IFNAR) on the surface of target cells to activate the JAK/STAT signaling pathway. As a result, a few hundred IFN stimulated genes (ISGs) are induced. Although some of these ISGs have direct anti-viral activity\textsuperscript{91-93}, the functions of most of these ISGs remain unknown.

ISG15 is one of the most abundantly expressed genes induced by viral/bacterial infections or type I IFN treatment. As the first identified ubiquitin-like protein, ISG15 shares sequence and structural similarity with ubiquitin (Figure 1-3).
The overall polypeptide fold for ISG15 and its similarity with the ubiquitin fold

A

B


The overall polypeptide fold for ISG15 and its similarity to the ubiquitin fold. A, ribbon diagram of ISG15 showing two separate domains, with color ramped from blue (N terminus, N) to red (C terminus, C) through green (Hinge). The two ubiquitin-like domains (each in β-grasp fold) are oriented differently and connected by a hinge. The last four C-terminal residues of ISG15 are disordered and not resolved, indicating the flexibility of the C-terminal tail. B, overlay of ribbon diagrams for ubiquitin (pink) with the amino- (blue) and C-terminal (green) domains of ISG15 to emphasize the marked similarities in their respective β-grasp folds.
ISG15, like ubiquitin, conjugates to its cellular targets through a series of enzymatic steps. Conjugation involves first an E1 activating enzyme (Ube1L), \(^{94}\) then an E2 conjugating enzyme (UbcH8, UbcH6)\(^{95,96}\), and finally an E3 ligase (EFP, CEB1/Herc5) .\(^{97,98}\) The C-terminal LRLRGG sequence of ISG15 is required for conjugation to the lysine residues of target proteins. ISG15 can be stripped from its target proteins by the USP18 isopeptidase.\(^{99}\)

Unlike ubiquitin, conjugation of ISG15 to its target proteins does not usually cause them to be degraded. Instead, ISG15 conjugation may alter the subcellular localization, structure, stability or activity of targeted proteins.\(^{100}\) A few hundred proteins with diverse functions in the cellular skeleton, stress response, immune response, and chromatin remodeling have been identified as ISG15 conjugation targets. Of particular importance are proteins that play an important role in innate antiviral response, such as PKR, MxA, Stat1, Jak1, and RIG-I.\(^{101,102}\) Although the functional consequences of ISGylation, the process of ISG15 conjugation to its targets, are not known, ISGylation has been implicated in various cellular processes and functions, such as modulating IFN signaling, antiviral activity, pregnancy, and some forms of cancers.\(^{103}\) Several lines of evidence suggest that ISG15 conjugation also plays an important role in the innate antiviral response\(^{104}\): 1) ISG15 is targeted by other viruses: non-structural protein (NS-1B) of Influenza B virus binds to the free form ISG15, preventing ISGylation\(^{94}\); 2) ISG15 inhibits HIV release (but not virus replication)\(^{105}\); 3) over-expression of ISG15 in IFN-α/β receptor knockout mice protected them against Sindbis virus-induced lethality and decreased Sindbis virus replication in multiple organs\(^{106}\); 4) Mice lacking ISG15 deconjugation enzyme (USP18/UBP43), resulting in increased ISGylation, are resistant to LCMV and VSV infection.\(^{104}\)
Two possible mechanisms for an ISG15 antiviral activity have been proposed. First, ISG15 may conjugate to key cellular proteins or viral proteins and inhibit virus replication. For example, in HIV infection, the Gag protein is ubiquitinated in order to recruit the endosomal complex required for transport (ESCRT-I) to the plasma membrane for viral budding. ISG15 has been shown to conjugate with Gag and thus might prevent Gag from being ubiquitinated. As a consequence, HIV release would be inhibited. Conjugation of ISG15 to interferon regulatory factor 3 (IRF3), a key signal-transducing factor in the activation of the antiviral innate immune response, protects IRF3 from ubiquitin-mediated degradation by the 26S proteasome. Second, ISG15 may also act alone, as a cytokine. The free form of ISG15 has been shown to activate natural killer (NK) and cytotoxic T-lymphocytes (CTL) and to stimulate IFN-γ production. This in turn induced dendritic cell maturation and neutrophil recruitment.

Although it was initially suggested that ISGylation plays an important role in the regulation of the JAK-STAT pathway and IFN signaling, IFN signaling is intact in ISG15 knock-out mice. Furthermore, in vivo analyses of ISG15 antiviral activity report contradictory findings. Although the replication of the Sindbis virus-expressing ISG15 was inhibited in IFNAR1 deficient mice and ISG15 null mice have an increased susceptibility to Sindbis, Influenza, and HSV-1 virus infections, there was no difference in the replication of VSV and LCMV in ISG15 null and wild type mice. These data suggest that the antiviral activity of ISG15 might be virus-specific. More recently, ISG15 was shown to be able to negatively regulate IFN signaling by targeting RIG-I. Quite surprisingly, we and others have found that ISG15 promotes viral production in a cell culture model.
1.5.2 **USP18 is a deconjugating enzyme for ISG15 and a negative regulator for IFN signaling**

ISG15 conjugation is reversible and controlled by USP18 (UBP43), an IFN-inducible cysteine protease of the ubiquitin-specific protease (USP) family. USP18 appears to counteract the effects of interferon; lack of USP18 results in enhanced and prolonged STAT1 phosphorylation, DNA binding, and increased induction of hundreds of ISGs. Perhaps as a result of increased IFN signaling and effect, USP18 knock out mice show greater resistance to the cytopathic effects of a number of viruses, including lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), and Sindbis virus (SNV). USP18-deficient cells exhibit high levels of ISG15 modified proteins (ISGylation). Furthermore, they are hypersensitive to type I IFN and undergo apoptosis upon IFN stimulation. Thus, USP18 appears to be a negative regulator of IFN signaling.

Although ISG15 may play a role in the anti-HCV response, the ability of USP18 to regulate the anti-HCV interferon response may be independent of its ability to deconjugate ISG15. Ablation of ISG15 or its E1 activating enzyme Ube1L in mice did not reverse the phenotype of the USP18 knockout, nor affect IFN-induced JAK/STAT signaling, indicating that neither ISG15 nor ISGylation is essential in JAK/STAT signaling. It was recently reported that USP18 negatively regulates JAK–STAT signaling independently of its isopeptidase activity. In that study, USP18 action was specific to type I IFN responses and achieved through a direct interaction between USP18 and the IFNAR2 subunit of the type 1 IFN receptor. Binding of exogenous and endogenous USP18 to IFNAR2 *in vivo* interfered with the JAK-receptor...
interaction and led to inhibition of the downstream phosphorylation cascade and other signaling events. Whether this is a cell- or species-specific mechanism remains to be determined.

Most recently, USP18 was found to modulate the expression levels of the EGF surface receptor at the protein level. Silencing USP18 by specific siRNA resulted in decreased epithelial growth factor receptor (EGFR) expression by 50-80% while overexpression of USP18 stimulated EGFR protein translation in an USP18 protease activity-dependent manner.\textsuperscript{119}

1.6 Model systems to study HCV replication and viral production

Until recently, HCV research has been hampered by the lack of an appropriate model system. The HCV cDNA clone was first sequenced in 1989\textsuperscript{120}, but it was not until 1997, after the discovery that the original HCV cDNA clone lacked a highly conserved 3’-terminal genome fragment, that the first functional full-length cDNA clones were reported through injection of this in vitro synthesized HCV RNA into chimpanzees. At that time, no RNA replication could be achieved in cell culture.\textsuperscript{121}

1.6.1 HCV Replicon system

In 1999, Lohmann et al reported the first cell culture system that allowed HCV RNA replication.\textsuperscript{122} This replicon system was based on a subgenomic genotypye 1b (isolate Con1) that replicates in a human hepatoma cell line (Huh7). As shown in Figure 1-4A, bicistronic replicon RNAs, encoding a selectable marker (Neo\textsuperscript{5}) under control of the HCV IRES in the first cistron and the HCV replicase proteins (NS3-NS5B) under control of a heterologous IRES from
encephalomyocarditis virus in the second cistron, were electroporated into Huh-7-based cell lines. Replication of these RNAs leads to production of a selectable marker, which allows for selection of colonies containing active RNA replication.

The replication efficiency of HCV RNA in the early replicon system was very low, which spurred efforts to optimize the system. The first optimization derived from the discovery of cell lines that were more permissive to HCV replication. Blight et al. isolated more permissive subclones of replicon-transduced Huh7 cells cured by alpha interferon treatment. Of those was Huh7.5 subclone, which is deficient in the retinoic-acid inducible gene I (RIG-I) antiviral response signaling pathway. The replicon system was further improved by selecting for adaptive mutations that dramatically enhanced HCV RNA replication. However, although robust HCV RNA replication in cultured permissive cells was achieved for both genotype 1b and 1a, there was no infectious virus produced from this system.

### 1.6.2 HCV pseudoparticle (HCVpp) system

In the process of searching for a system that reproduced the HCV full lifecycle, retroviral pseudotypes bearing HCV glycoproteins (HCVpp) were generated. HCVpp could be produced by cotransfection of 293T cells with expression vectors encoding (i) HCV E1E2, (ii) the Gag-Pol proteins of either murine leukemia virus or human immunodeficiency virus, and (iii) a retroviral genome encoding a reporter to detect subsequent productive entry (Fig. 1-4B). This HCVpp system is particularly useful to study virus entry.
1.6.3 JFH full-life cycle HCV replication and production

In order to develop a system that allows both HCV RNA replication and viral particle production, it was necessary to understand the mechanisms by which high-level HCV RNA replication was achieved in cell culture (HCV replicon system), yet no infectious virus was produced. It was possible that adaptive mutations result in hypophosphorylation of NS5A, which is essential for HCV RNA replication.\(^{125,132,133}\) NS5A hypophosphorylation maintains the HCV replicase stability (allows HCV RNA replication) while sacrificing the HCV late viral life cycle events, such as assembly and packaging. In support of this idea, most genomes with adaptive mutations do not produce infectious particles in cell culture despite efficient replication.\(^{127-129}\) All these studies led to the hypothesis that a HCV clone capable of replicating in cell culture without adaptive mutations might yield infectious virus. Such an HCV isolate (JHF-1), isolated from a Japanese patient with a rare case of acute fulminant hepatitis, was indeed identified.\(^{134-136}\) When full length JFH-1 RNA is transfected into Huh7 cells, infectious virus particles (HCVcc) are produced (Figure 1-4C).\(^{137}\) Higher titers could be obtained in Huh7.5 cells and derived sublines.\(^{123,124}\) HCVcc is infectious in chimpanzees and uPA-SCID mice transplanted with human hepatocytes.\(^{138,139}\)
Figure 1-4. Systems for the study of HCV replication, entry, and infectivity

(A) HCV replicon systems, shown here in one of their simplest iterations, allow for productive viral RNA replication in cell culture. Bicistronic replicon RNAs, encoding a selectable marker (Neo) under control of the HCV IRES in the first cistron and the HCV replicase proteins (NS3-NS5B) under control of a heterologous IRES from encephalomyocarditis virus in the second cistron, are delivered to Huh-7-based cell lines by electroporation. Replication of these RNAs leads to production of the selectable marker and allows for selection of colonies containing active RNA replication. Transduction of resistance to the drug G418 is shown in this figure, but replicons expressing a number of reporter genes have been developed, as have methods to efficiently measure HCV proteins and RNA from these systems. (B) The HCV pseudoparticle system (HCVpp) provides a method to investigate glycoprotein-mediated events in the HCV life cycle. In this system, recombinant retroviruses that contain HCV functional glycoproteins on their surface are generated in 293T cells. These particles can be used to infect permissive cell lines, such as Huh-7.5. The retrovirus genomes have been engineered to express a reporter gene, such as luciferase, allowing for a quantitative measure of cell entry. (C) The HCVcc infectious virus system uses either JFH-1 HCV genomic RNA or chimeras of this genome with heterologous sequences (such as J6). These RNAs are electroporated into permissive cell lines and yield infectious HCV virions that can be used to infect naïve cells or animal models. Productive infection can be monitored by detection of the expression of NS5A, by a number of reporter genes, or by direct measure of viral RNA.

Adapted from Tellinghuisen, et al., J Virology 2007; 81(17):8853-8867
1.6.4 The chimeric SCID-Alb/uPA mouse model

HCV research had also been hindered by a lack of a small animal model system before the development of the SCID-Alb/uPA mouse.\textsuperscript{140} In this animal, progressive depletion of the mouse hepatocytes occurs in severe combined immune deficiency (SCID) mice carrying a plasminogen activator transgene (Alb-uPA). Human hepatocytes can then be transplanted into these mice to engraft and regenerate the liver. The resulting chimeric human-mouse liver can then be infected with HCV or HBV. Although this animal model cannot be used to study the immune response following HCV infection- a critical deficiency- this system is the only small animal model that supports a full viral life cycle and virion production. It has been very useful in studying virus-host interactions, as well as in antiviral screening. It is also valuable for distinguishing direct virus-mediated transcriptional changes from immune-mediated effects, which is impossible to achieve by studying patient liver biopsies.

In summary, the fact that only half of the patients infected with HCV respond to the current best regime of treatment with pegylated interferon and ribavirin highlights several major issues in the HCV research field that need to be solved:

**First**, it is important to understand why some patients respond to treatment while others do not. One focus of my PhD research was to understand the molecular mechanism of interferon resistance in those patients who do not respond to therapy. This will help to develop new drug targets for better treatment of HCV infected patients.

**Second**, it is important to develop a prognostic test to predict which patients would respond to treatment. The other focus of my PhD research was to identify a set of biomarkers (gene
expression patterns in HCV-infected pretreatment livers) that might be used to predict treatment response prior to initiation of therapy.

**Third,** it is important to develop more specific and effective therapeutics.

**Last,** the key to eradicating the disease is to develop a vaccine.
References


15. Theresa L. Whiteside and Ronald B. Human Natural Killer Cells in Health and Disease Herberman Pittsburgh Cancer Institute and Department of Pathology, University of Pittsburgh


60. Fried MW, Hadziyannis SJ, Shiffman M, Messinger D, Zeuzem S. Rapid virological response is a more important predictor of sustained virological response (SVR) than genotype in patients with chronic hepatitis C virus infection. 43rd Meeting of the European Association for the Study of Liver Disease (Milan, 2008)


98. Wong JJ, Pung YF, Sze NS, Chin KC. HERC5 is an IFN-induced HECT-type E3 protein ligase that mediates type I IFN-induced ISGylation of protein targets. Proc Natl Acad Sci U S A. 2006 ;103(28):10735-40


Chapter 2  Search for a response signature in liver tissues of patients chronically infected with HCV

Combination therapy of Pegylated IFN and Ribavirin for the treatment of patients chronically infected with HCV is only effective in 50% of the patients, and there is no reliable method to predict responsiveness in these patients before initiation of therapy. In this chapter, I describe how I identified differentially expressed genes (a response signature) from pre-treatment liver biopsies of patients chronically infected with HCV using cDNA microarrays. In a retrospective study, I divided patients into two groups: those who respond to pegylated IFN/Ribavirin treatment (Responder, R) and those who do not (non-responders, NR). All these patients had their liver biopsies done before initiation of treatment and the samples were stored in the RNA stabilization solution (RNAlater, Qiagen) in a -20°C freezer until used for RNA extraction. 18 genes whose expression levels are constantly and statistically different between R and NR were identified from 19,000 clones on the microarray chip. Based on expression levels of these 18 genes, or even 8 genes, I could correctly classify 30 out of 31 patients studied.

This piece of work entitled “Hepatic Gene Expression Discriminates Responders and Nonresponders in Treatment of Chronic Hepatitis C Viral Infection” was published in Gastroenterology 2005;128:1437-1444

My role in this paper: experimental design, clinical sample collection, performing the experiments, summarizing/analyzing data and writing up the paper
Hepatic Gene Expression Discriminates Responders and Nonresponders in Treatment of Chronic Hepatitis C Viral Infection

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Published in Gastroenterology 2005;128:1437-1444

ABSTRACT

Background & Aims: Pegylated interferon (IFN)-α plus ribavirin is the most effective treatment of chronic hepatitis C but has unpleasant side effects and high costs. A large proportion of patients do not respond to therapy for reasons that are unclear. We used gene expression profiling to investigate the molecular basis for treatment failure. Methods: Expression profiling was performed on percutaneous needle liver biopsy specimens taken before therapy. Gene expression levels were compared among 15 nonresponder, 16 responder, and 20 normal liver biopsy specimens. Differential gene expression was confirmed using real-time polymerase chain reaction. Results: We identified 18 genes whose expression differed significantly between all responders and all nonresponders (P < .005). Many of these 18 genes are IFN sensitive and 3 (ISG15/USP18/CEB1) are linked in a novel IFN-regulatory pathway, suggesting a possible rationale for treatment resistance. Using a number of independent classifier analyses, an 8-gene subset accurately predicted treatment response for 30 of 31 patients. The classifier analyses were applicable to patients with genotype 1 infection and were not correlated with viral load, disease activity, or fibrosis. Conclusions: Hepatic gene expression profiling identified consistent differences in patients who subsequently fail treatment with pegylated IFN-α plus ribavirin: up-regulation of a specific set of IFN-responsive genes predicts nonresponse to exogenous therapy. These data may be of use in predicting clinical responses to treatment.
Abbreviations used in this paper: IFN, interferon; KNN, nearest neighbor analysis; LDA, linear discriminant analysis; NR, nonresponder; PCA, principal components analysis; PCR, polymerase chain reaction; R, responder

Introduction

More than 170 million people worldwide are infected chronically with hepatitis C virus (HCV). Currently there is no vaccine or small molecule therapy for this disease, which can lead to liver failure and cancer. The most effective treatment is pegylated interferon (IFN)-α plus ribavirin, which has morbid side effects, variable cure rates, and high costs.1

Although the interaction of the virus with hepatic microenvironments creates a cellular state that is nonresponsive to treatment,3,4,5 the underlying molecular mechanisms are unknown and it is not possible to predict treatment outcomes before initiating therapy. Viral and host factors both play a role; for example, infection with HCV genotypes 1 or 4 is associated with at best a 60% response rate, and increasing degrees of hepatic fibrosis can decrease response rates.1 Mutations in viral (NS5A, NS5B) and host (MxA, OAS, PKR) proteins can enhance (NS5A, NS5B) or partially inhibit (MxA) the response to IFN-based treatment.6-10 Increased hepatic MxA protein expression is associated with poorer treatment responses.11 While these results are intriguing, the heterogeneity of viral and host phenotypes makes it unlikely that any single factor will accurately predict the cellular response to treatment.

The ultimate response to treatment can only be gauged after treatment with pegylated IFN-α plus ribavirin has been initiated. Patients undergo at least a 12-week course of combination therapy and then are assessed for an antiviral response. An early viral response (2-log decrease in
baseline HCV RNA titers) suggests the eventual outcome, although only with 60%–90% accuracy.\textsuperscript{1} However, this 3-month regimen is associated with maximum morbid side effects and is expensive.\textsuperscript{1,12} We hypothesized that pretreatment nonresponder (NR) and responder (R) liver tissue would show consistent differences in gene expression levels and that these differences could be used to predict treatment outcomes.

**Materials and Methods**

**Patients and Biopsies**

**Chronic HCV**

Thirty-one patients with chronic HCV (23 genotype 1, 4 genotype 2, 3 genotype 3, and 1 genotype 6) were treated at University Health Network from October 2001 to May 2004. All treatment-naive patients considering treatment with IFN/ribavirin underwent percutaneous liver biopsy (via a 15-gauge needle) and had baseline viral loads determined. Treatment consisted of pegylated IFN-\(\alpha\)2a/2b 180 \(\mu\)g weekly by subcutaneous injection and oral ribavirin 800–1200 mg daily (depending on genotype and weight) for 24 (genotype 2/3) or 48 (genotype 1/6) weeks. Quantitative HCV RNA was determined at completion of therapy and 6 months after. Patients were designated as NRs if HCV RNA was detectable at the end of therapy, as relapsers if HCV RNA was undetectable at the end of treatment but was detectable at the 6-month follow-up, and as having a sustained viral response if HCV RNA was undetectable at both the end of therapy and the 6-month follow-up. Compliance was excellent (30 of 31 patients completed therapy). For the purposes of this study, patients were designated as Rs if the initial post-treatment HCV RNA titer was negative.
Normal liver tissue

Biopsies were performed on normal (HCV-negative) liver tissue as the first step of 20 right hepatectomy operations performed on living transplant donors.

Ethics

All patients gave informed consent for the research protocol, which was approved by the hospital research ethics board.

RNA Extraction and Amplification

A portion of each liver biopsy specimen (0.5–1.0 cm if percutaneous 15-g core) was immersed in RNAlater (Qiagen, Mississauga, Ontario, Canada). Total RNA was extracted, and 2 μg of total RNA from each biopsy specimen or from Universal Human Reference RNA (Stratagene, La Jolla, CA) was amplified using the MessageAmp aRNA kit (Ambion, Austin, TX). Gene expression profiles from amplified RNA were highly correlated to those developed from nonamplified RNA (correlation coefficient ≥0.85, data not shown).

Complementary DNA Microarrays

Human single spot microarrays comprising 19,000 human clones were used (UHN Microarray Center; http://www.microarrays.ca/support/glists.html). For each array, 5 μg of liver amplified antisense RNA was compared with 5 μg of reference amplified antisense RNA. After reverse transcription, liver complementary DNA was labeled with Cy5 and reference RNA with Cy3. Hybridization was performed overnight at 37°C (DIGEasy; Roche Diagnostics, GmbH, Mannheim, Germany). Arrays were read with a GenePix 4000A laser scanner and quantified.
with GenePix Pro software (Axon Instruments, Union City, CA). Microarray data were normalized using an R-based, intensity-dependent LOWESS scatter plot smoother (http://142.150.56.35/~LiverArrayProject/home.html).14-16

Real-Time Polymerase Chain Reaction

Two-step real-time polymerase chain reaction (PCR) was performed after reverse transcription of 5 μg of amplified antisense RNA with 5 μg pd(N)6-random hexamer primer (Amersham, Oakville, Ontario, Canada). The resulting complementary DNA was used as a template for real-time PCR quantification with the QuantiTect SYBR PCR Kit (Qiagen), and real-time PCR (normalized to β-actin) was performed using the DNA Engine Opticon 2 cycler (MJ Research, Reno, NV). For primers, see Table 2-1.
Table 2-1. Real-Time PCR Primers for 18 differentially-expressed genes in the pretreatment livers of Rs and NRs

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>PCR product length (base pairs)</th>
<th>Gene name</th>
</tr>
</thead>
<tbody>
<tr>
<td>229295</td>
<td>CAGACCTGACAATCCACCT</td>
<td>AGCTCATACTGCCCCTCCAGA</td>
<td>164</td>
<td>Ubiquitin-specific protease 18</td>
</tr>
<tr>
<td>37942</td>
<td>GATTGCCTGGAGGAATCAAAC</td>
<td>TTGGATTTTCCCTTTTTTGTC</td>
<td>160</td>
<td>Cyclin E binding protein 1</td>
</tr>
<tr>
<td>149319</td>
<td>CGCAGATCACCCAAGAGATT</td>
<td>GCCCTTGTATTCCTCACCACCA</td>
<td>185</td>
<td>IFN-α-inducible protein 1</td>
</tr>
<tr>
<td>136508</td>
<td>TCAGCGAGGCCAGTAATCTT</td>
<td>GCAGGACATTCCAAGATGT</td>
<td>154</td>
<td>2′,5′-oligo adenylate synthetase 2</td>
</tr>
<tr>
<td>324912</td>
<td>CTCGCTGATGAGCTGCTCT</td>
<td>ATACTTGTGGGTGGCGTAGC</td>
<td>148</td>
<td>IFN-α–inducible protein (clone IFI-6-16)</td>
</tr>
<tr>
<td>324284</td>
<td>GTCAACACCAAGCCACAGTG</td>
<td>GGGCGAATGTTCACAAAGTT</td>
<td>110</td>
<td>2′,5′-oligoadenylate synthetase 3, 100 kilodaltons</td>
</tr>
<tr>
<td>5474956</td>
<td>GCTGTAGCCGTCTCTGCTG</td>
<td>AAAAGGCCAATCCCATGT</td>
<td>135</td>
<td>Ribosomal protein, large P2</td>
</tr>
<tr>
<td>325364</td>
<td>GCAGCCAAGTTTACCAGAAG</td>
<td>GCCCTATCTGGTGATGCAGT</td>
<td>109</td>
<td>IFN-induced protein with tetrapricopeptide repeats 1</td>
</tr>
<tr>
<td>120600</td>
<td>CTTTGTGGGAGCTCTTTG</td>
<td>CAGCTGCTGCTTTCTCCT</td>
<td>131</td>
<td>Viperin</td>
</tr>
<tr>
<td>176650</td>
<td>CCGTGTGCAGCCTATCAAG</td>
<td>TTTACATTGCGGATGATGGA</td>
<td>129</td>
<td>RPS28</td>
</tr>
<tr>
<td>5745506</td>
<td>CTGCAAGAGCTTTCATCCR</td>
<td>GTCTCTGGCTCTGCTCA</td>
<td>134</td>
<td>Phosphoinositide-3-kinase adaptor protein 1</td>
</tr>
<tr>
<td>325130</td>
<td>GTGCATTGCAAGGTCAGA</td>
<td>CTGGTGATAGGCCCATCAGGT</td>
<td>140</td>
<td>Myxovirus (influenza virus) resistance 1</td>
</tr>
<tr>
<td>52905</td>
<td>CCAACCATTGGAGGGTCAC</td>
<td>ACCCTTCTCCAGCATTTCT</td>
<td>130</td>
<td>Dual specificity</td>
</tr>
<tr>
<td>Clone ID</td>
<td>Forward primer</td>
<td>Reverse primer</td>
<td>PCR product length (base pairs)</td>
<td>Gene name</td>
</tr>
<tr>
<td>---------</td>
<td>----------------</td>
<td>----------------</td>
<td>-------------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>127270</td>
<td>AGCCCCCTGTCTTGAGATCT</td>
<td>CGAGAAGGTTGAGGTGGAGA</td>
<td>133</td>
<td>Activating transcription factor 5</td>
</tr>
<tr>
<td>487534</td>
<td>GGTGCCATGGATGCTTTT</td>
<td>AGAGAGGCATCCTCCAGACA</td>
<td>124</td>
<td>Leucine aminopeptidase 2</td>
</tr>
<tr>
<td>207669</td>
<td>GCAGGAAGACAGTGAGGAGC</td>
<td>GAGCCAGCACTTCTGGGTAG</td>
<td>125</td>
<td>D11lgp1e-like</td>
</tr>
<tr>
<td>3930678</td>
<td>AGCGGAAGGAGGAGAAAAAG</td>
<td>GTACTCTTGAGGCAAGTGAGG</td>
<td>121</td>
<td>Eukaryotic translation elongation factor 1 γ</td>
</tr>
<tr>
<td>231624</td>
<td>GTCCTGATGGGCTTCGT</td>
<td>TTTGTTGTGGGTTCTTCCA</td>
<td>132</td>
<td>Syntaxin binding protein 5 (tomosyn)</td>
</tr>
</tbody>
</table>

### Statistics, Clustering, and Classifier Analyses

Comparisons between continuous variables were performed using the 2-sample Welch $t$ statistic with the multtest package, which includes an estimation of adjusted $P$ values by permutation. Unsupervised hierarchical clustering and unsupervised principal components analyses (PCA) were performed using the R mva package. Nearest neighbor classifier analyses (KNN) were performed using the R class package, and linear discriminant analyses (LDA) were performed with the R MASS package. Details can be found at http://142.150.56.35/LiverArrayProject/home.html.
Results

A Gene Expression Profile That Discriminates Rs and NRs

The patients in this study were well matched for most clinical variables with the exception of viral genotype and sex (Table 2-2). There were no significant differences between R and NR patients when compared for age, baseline viral load, disease activity, hepatic fibrosis, compliance to therapy, or dose reduction. Patients with genotype 1 infection had the highest failure rate with therapy and accounted for all NR patients in our cohort.
Table 2-2. Patient Characteristics: All 31 Patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>NR</th>
<th>R</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>15</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>46.4 ± 14</td>
<td>48.3 ± 10</td>
<td>.6896</td>
</tr>
<tr>
<td>Sex (no. male)</td>
<td>7/15</td>
<td>13/16</td>
<td>.0443</td>
</tr>
<tr>
<td>Genotype 1 infection</td>
<td>15/15</td>
<td>8/16</td>
<td>.0015</td>
</tr>
<tr>
<td>Viral load (IU/mL)</td>
<td>(2.4 \times 10^6 \pm 3.7 \times 10^6)</td>
<td>(3.8 \times 10^6 \pm 4.3 \times 10^6)</td>
<td>.3529</td>
</tr>
<tr>
<td>Activity</td>
<td>1.63 ± 0.44</td>
<td>1.81 ± 0.51</td>
<td>.3049</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>2.50 ± 0.84</td>
<td>2.65 ± 0.94</td>
<td>.6305</td>
</tr>
<tr>
<td>Completed course of treatment</td>
<td>14/15</td>
<td>16/16</td>
<td>.72</td>
</tr>
<tr>
<td>Pegylated IFN-α plus ribavirin dose &gt;80%</td>
<td>14/15</td>
<td>12/16</td>
<td>.69</td>
</tr>
<tr>
<td>Alcohol (10 drinks/week)</td>
<td>2/12</td>
<td>2/13</td>
<td>.66</td>
</tr>
<tr>
<td>Smoking (1 pack/day)</td>
<td>5/9</td>
<td>4/8</td>
<td>.74</td>
</tr>
<tr>
<td>Race (no. black)</td>
<td>3/15</td>
<td>0/16</td>
<td>.083</td>
</tr>
</tbody>
</table>

NOTE. All patient characteristics were recorded in a prospectively maintained database. In general, data are presented as mean ± SD. Where data are presented in fractions, the denominator represents the number of patients for whom full data were available. Statistics are either Welch t test or \(\chi^2\) analysis. The number of patients who received at least 80% of the dose of pegylated IFN-α plus ribavirin for at least 80% of the time was recorded over the entire course of therapy.

\(^a\) \(P < .05\)
To define which genes discriminate between HCV infection of Rs and NRs, we compared gene expression levels from 15 NR, 16 R, and 20 normal liver biopsy specimens. We determined that the levels of 18 genes differed between R and NR groups with $P < .005$ (Table 2-3) and verified these differences using real-time PCR (Figure 2-1). Within these 18 genes, most of the difference between NR and sustained virologic response samples was a relative up-regulation in NR tissue; R gene expression profiles actually cocluster with normal liver (Figure 2-2). Hierarchical cluster analysis clearly segregated all NR samples in one family, with all but 2 R samples and all normal liver samples segregated in another cluster (Figure 2-2). Thus, there is a consistent difference in the NR response to HCV reflected in the expression of 18 genes.
Table 2-3. Eighteen Genes That Differ Between NR and R Hepatic Gene Expression Profiles

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Name</th>
<th>Symbol</th>
<th>NR/R</th>
<th>P (NR vs R)</th>
<th>NR/normal</th>
<th>P (NR vs normal)</th>
<th>R/normal</th>
<th>P (R vs normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>149319</td>
<td>IFN-α-inducible protein (clone IFI-15K)</td>
<td>G1P2/ISG15/IFI15</td>
<td>4.37</td>
<td>.0001</td>
<td>9.69</td>
<td>.0001</td>
<td>2.22</td>
<td>.0001</td>
</tr>
<tr>
<td>136508</td>
<td>2′, 5′-oligoadenylate synthetase 2</td>
<td>OAS2</td>
<td>3.80</td>
<td>.0001</td>
<td>6.58</td>
<td>.0001</td>
<td>1.73</td>
<td>.0009</td>
</tr>
<tr>
<td>324912</td>
<td>IFN-α-inducible protein (clone IFI-6-16)</td>
<td>G1P3/IFI616</td>
<td>2.83</td>
<td>.0001</td>
<td>4.72</td>
<td>.0001</td>
<td>1.67</td>
<td>.0002</td>
</tr>
<tr>
<td>324284</td>
<td>2′, 5′-oligoadenylate synthetase 3</td>
<td>OAS3</td>
<td>2.54</td>
<td>.0001</td>
<td>3.42</td>
<td>.0001</td>
<td>1.35</td>
<td>.005</td>
</tr>
<tr>
<td>5474956</td>
<td>Ribosomal protein, large P2</td>
<td>RPLP2</td>
<td>2.53</td>
<td>.0001</td>
<td>3.70</td>
<td>.0001</td>
<td>1.46</td>
<td>.0002</td>
</tr>
<tr>
<td>37942</td>
<td>Cyclin E binding protein 1</td>
<td>CEB1</td>
<td>2.15</td>
<td>.0001</td>
<td>2.55</td>
<td>.0001</td>
<td>1.19</td>
<td>.0777</td>
</tr>
<tr>
<td>325364</td>
<td>IFN-induced protein with tetratricopeptide repeats</td>
<td>IFIT1</td>
<td>2.14</td>
<td>.0001</td>
<td>2.83</td>
<td>.0001</td>
<td>1.32</td>
<td>.0127</td>
</tr>
<tr>
<td>120600</td>
<td>Viperin</td>
<td>VIPERIN/cig5</td>
<td>1.82</td>
<td>.0002</td>
<td>1.78</td>
<td>.0001</td>
<td>0.98</td>
<td>.8031</td>
</tr>
<tr>
<td>176650</td>
<td>40S ribosomal protein S28</td>
<td>RPS28</td>
<td>1.75</td>
<td>.0004</td>
<td>2.38</td>
<td>.0001</td>
<td>1.35</td>
<td>.0002</td>
</tr>
<tr>
<td>5745506</td>
<td>Phosphoinositide-3-kinase adaptor protein 1</td>
<td>PB3KAP1</td>
<td>1.60</td>
<td>.005</td>
<td>1.66</td>
<td>.0022</td>
<td>1.04</td>
<td>.8283</td>
</tr>
<tr>
<td>325130</td>
<td>Myxovirus (influenza virus) resistance 1, IFN-inducible protein 78</td>
<td>MX1</td>
<td>1.58</td>
<td>.0013</td>
<td>1.98</td>
<td>.0001</td>
<td>1.25</td>
<td>.0394</td>
</tr>
<tr>
<td>52905</td>
<td>Dual specificity phosphatase 1</td>
<td>DUSP1</td>
<td>1.56</td>
<td>.0003</td>
<td>0.59</td>
<td>.002</td>
<td>0.38</td>
<td>.0001</td>
</tr>
<tr>
<td>127270</td>
<td>Activating transcription factor 5</td>
<td>ATF5</td>
<td>1.56</td>
<td>.0046</td>
<td>0.96</td>
<td>.6984</td>
<td>0.62</td>
<td>.0024</td>
</tr>
<tr>
<td>487534</td>
<td>Leucine aminopeptidase 3</td>
<td>LAP3</td>
<td>1.56</td>
<td>.0003</td>
<td>2.10</td>
<td>.0001</td>
<td>1.35</td>
<td>.0067</td>
</tr>
<tr>
<td>229295</td>
<td>Ubiquitin-specific protease 18</td>
<td>USP18/UBP43</td>
<td>1.52</td>
<td>.0001</td>
<td>1.72</td>
<td>.0001</td>
<td>1.13</td>
<td>.0791</td>
</tr>
<tr>
<td>207669</td>
<td>D11lgp1e-like</td>
<td>LGP1</td>
<td>1.51</td>
<td>.0014</td>
<td>1.38</td>
<td>.0094</td>
<td>0.92</td>
<td>.1351</td>
</tr>
<tr>
<td>Clone ID</td>
<td>Name</td>
<td>Symbol</td>
<td>NR/R</td>
<td>P (NR vs R)</td>
<td>NR/normal</td>
<td>P (NR vs normal)</td>
<td>R/normal</td>
<td>P (R vs normal)</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------</td>
<td>--------</td>
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<td>-------------------</td>
<td>----------</td>
<td>----------------</td>
</tr>
<tr>
<td>3930678b</td>
<td>Eukaryotic translation elongation factor 1 γ ETEF1</td>
<td>0.65</td>
<td>.0032</td>
<td>0.75</td>
<td>.0009</td>
<td>1.15</td>
<td>.7341</td>
<td></td>
</tr>
<tr>
<td>231624b</td>
<td>Syntaxin binding protein 5 (tomosyn) STXBP5</td>
<td>0.65</td>
<td>.0034</td>
<td>0.96</td>
<td>.7156</td>
<td>1.47</td>
<td>.0126</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Gene expression ratios were compared among NR, R, and normal liver gene expression values. Statistics are calculated using the Welch t test.

*a* Up-regulated in NR.

*b* Down-regulated in NR.

*c* IFN-sensitive gene.
Figure 2-1. Real-time PCR verification of genes predicted to be altered by microarray.

Real-time PCR verification was performed as described in Materials and Methods. In all cases, 4 genotype 1 R samples were compared with 4 genotype 1 NR samples and 3 normal liver samples (white, normal; gray, R; black, NR). Values along the y-axis represent the ratio, in arbitrary units, of a given gene versus β-actin. Data are expressed as mean ± SEM. *P < .05 NR versus R; Welch t test. OAS-3, P = .054; CEB-1, P = .105; RPS28, P = .053; STXBP5, P = .12.
Figure 2-2. Hierarchical cluster analysis using the 18 genes present in all 31 samples. Unsupervised hierarchical cluster analysis was performed as described in Materials and Methods, restricting the analysis to the 18 genes in Table 2-3. Red denotes an increase and green a decrease when compared with the reference RNA pool. An asterisk denotes the patients who experienced a relapse following treatment with pegylated IFN-α plus ribavirin. Note that normal liver tissue coclusters with patients who respond to treatment, while all NR samples form part of a discrete cluster.
A Gene Subset Will Accurately Differentiate NRs and Rs

Unsupervised cluster analyses do not assign predictive end points (in this case, response to treatment). To determine whether the genes that differed between R and NR tissue could be used to predict treatment response, we used 2 supervised classifier analyses (KNN and LDA) and corroborated these results with a further unsupervised cluster analysis (PCA). Because different gene combinations will have different predictive abilities, we randomly drew 50,000 combinations of 6, 8, 10, 12, and 14 genes from Table 2-3 and assessed their individual ability to correctly classify the 31 NR and R samples. We determined a subset of 8 genes with the most consistent ability to correctly classify NR and R samples, comprising GIP2/IFI15/ISG15, ATF5, IFIT1, MX1, USP18/UBP43, DUSP1, CEB1, and RPS28.

Using this predictive gene subset, unsupervised hierarchical cluster analysis identified 2 clusters: one comprised of all NR samples but one, and the other of all R samples but one (Figure 2-3A). Both KNN and LDA accurately identified 30 of 31 samples, while PCA clearly separated R and NR samples into 2 distinct groups (Figure 2-3B). For comparison, if all 18 genes are used, the KNN prediction rate decreases to 28 of 31.
Figure 2-3. Cluster and classifier analysis using the 8-gene predictor set: all patients. (A) Hierarchical cluster analysis of all samples, restricting the analysis to the 8 genes in the predictor set. (B) KNN, LDA, and PCA of all samples using the 8-gene predictor set. Unsupervised (hierarchical cluster, PCA) and supervised (KNN, LDA) analyses were performed as described in Materials and Methods. As in Figure 2-2, an asterisk denotes treatment relapsers.
Because patients with genotype 1 infection are the least likely to respond to treatment, and because classifier analyses are influenced by the numbers and characteristics of the samples in a teaching set, we examined whether the predictive gene subset was valid within the 23 patients with genotype 1 infection in our cohort. As shown in Table 2-4, there were no significant clinical differences in these patients. The predictive gene subset correctly classified 21 of 23 samples using KNN and LDA, while PCA and unsupervised hierarchical clustering again clearly created 2 distinct clusters (Figure 2-4). Together, our results argue that a gene subset can predict NR and R status independent of genotype.
Table 2-4. Patient Characteristics: Patients With Genotype 1 Infection Only

<table>
<thead>
<tr>
<th>Variable</th>
<th>NR</th>
<th>R</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>15</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>50.2 ± 5.1</td>
<td>43.9 ± 9.0</td>
<td>.1032</td>
</tr>
<tr>
<td>Sex (no. male)</td>
<td>7/15</td>
<td>6/8</td>
<td>.1917</td>
</tr>
<tr>
<td>Viral load (IU/mL)</td>
<td>$2.40 \times 10^6 \pm 3.7 \times 10^6$</td>
<td>$4.87 \times 10^6 \pm 5.1 \times 10^6$</td>
<td>.2597</td>
</tr>
<tr>
<td>Activity</td>
<td>1.63 ± 0.44</td>
<td>1.75 ± 0.46</td>
<td>.5681</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>2.50 ± 0.84</td>
<td>2.56 ± 0.98</td>
<td>.881</td>
</tr>
<tr>
<td>Completed course of treatment</td>
<td>13/14</td>
<td>7/7</td>
<td>.85</td>
</tr>
<tr>
<td>Pegylated IFN-α plus ribavirin dose &gt;80%</td>
<td>14/15</td>
<td>7/8</td>
<td>.72</td>
</tr>
<tr>
<td>Alcohol (10 drinks/wk)</td>
<td>2/12</td>
<td>2/5</td>
<td>.41</td>
</tr>
<tr>
<td>Smoking (1 pack/day)</td>
<td>5/9</td>
<td>3/4</td>
<td>.76</td>
</tr>
</tbody>
</table>

NOTE. Patient characteristics restricted to those patients infected with genotype 1 HCV. Again, data are presented as mean ±SD. Where data are presented in fractions, the denominator represents the number of patients for whom full data were available. Statistics are either Welch t test or $\chi^2$ analysis.
Figure 2-4. Cluster and classifier analysis using the 8-gene classifier set: patients with genotype 1 infection only. A hierarchical cluster analysis of the 23 genotype 1 samples, restricting the analysis to the 8 genes in the predictor set. (B) KNN, LDA, and PCA of genotype 1 samples using the 8-gene predictor set. Unsupervised (hierarchical cluster, PCA) and supervised (KNN, LDA) analyses were performed as described in Materials and Methods. As in Figure 2-2, an asterisk denotes treatment relapsers.
Discussion

Our study compared hepatic gene expression profiles from liver biopsy specimens taken from 31 patients before treatment with pegylated IFN-α plus ribavirin. We identified 18 genes, confirmed by real-time PCR, with expression levels that differed consistently between NR and R liver tissue and were not correlated to any obvious clinical parameter. The raw data set can be accessed at http://142.150.56.35/LiverArrayProject/home.html. Levels for these 18 genes in R liver were closer to uninfected tissue than to NR liver, with a general up-regulation of gene expression in NR liver. Interestingly, many of these genes are IFN responsive, suggesting that the NR patients have adopted a different, yet characteristic, equilibrium in their host-virus immune response.

Although this study examined a relatively small set of patients (31), 3 arguments suggest that the results are broadly applicable. First, although the discriminatory genes were identified based solely on mathematical grounds, several have been previously linked either to HCV infection or to the response to viral infection. For example, polymorphisms of OAS have been weakly linked to self-limited HCV infection and polymorphisms of Mx1 have been weakly linked to response status. Hepatic messenger RNA levels for OAS, Mx1, and GIP2 are increased in chronic HCV, but none alone have been linked to treatment outcome. Many of the others are IFN-sensitive genes with antiviral activity and are consistent with an alteration in IFN responsiveness being linked to treatment nonresponse. The genes that are not directly IFN responsive may play roles in cellular pathways important for IFN responses (PI3AP1, DUSP1) and are involved in inflammatory cell activation and maturation. Second, the predictive subset of 8 genes performed well across all 4 statistical analyses (hierarchical clustering, KNN, LDA, and PCA). Third, the composition of the classifier set was unrelated to confounding clinical factors, such as
viral load, degree of fibrosis, and age. In multivariate analyses, USP18 expression was significantly affected by degree of fibrosis (data not shown), but none of the other 17 genes were linked to any of the clinical factors.

Two genes in the classifier gene set, ISG15/IFI15 and USP18/UBP43, are noteworthy for belonging to a novel IFN-regulatory pathway. In our study, NR and R patients were distinguished by up-regulated expression of ISG15 and USP18 in their pretreatment liver biopsy tissue. ISG15 is a ubiquitin-like protein that is believed to be important to innate immune functions. The USP18/UBP43 protease specifically removes ISG15 from ISG15-modified proteins; loss of USP18 in mice leads to IFN hypersensitivity. The USP18-ISG15 pathway is important in innate immunity against viral infection; a recent, elegant study showed that USP18 knockout mice are resistant and wild-type mice are susceptible to fatal intracerebral infection by lymphocytic choriomeningitis virus or vesicular stomatitis virus, concurrent with decreased viral replication and increased protein ISGylation in the knockout mice. Although the investigators suggest that the pathway may be relevant to human disease, ours is the first demonstration of the potential relevance of the USP18-ISG15 pathway in human viral infection. In our study, USP18 up-regulation was one of the factors predicting a lack of response to treatment with IFN, consistent with a role for USP18 in modifying the antiviral IFN response.

In conclusion, our study shows that NR and R patients differ fundamentally in their innate IFN response to HCV infection. These differences suggest novel aspects of HCV pathogenesis and form the basis for a predictive subset of genes that can predict treatment responses before initiation of pegylated IFN-α plus ribavirin therapy.
Acknowledgement:

The authors thank the physicians and surgeons of the Toronto Multi-Organ Toronto Transplant Program for their support and interest, particularly Drs David Grant, Mark Cattral, Paul Greig, and Gary Levy, and thank Drs Elizabeth Edwards and Kaiguo Mo for assistance with the real-time polymerase chain reaction studies. A.M.E. is the Banbury Chair of Medical Research. The authors also thank the CIHR National Research Training Program–HCV for its interest in this study.
References


Chapter 3: Confirmation of the HCV response signature-prospective validation and cell type-specific expression of ISG proteins identified by immunohistochemical staining

The HCV response signature was identified in a retrospective study. In order to know whether this signature could be used to predict treatment response prospectively, 78 patients chronically infected with HCV were recruited. Liver biopsies were obtained prior to the initiation of treatment and the expression levels of host genes were determined on a cDNA microarray chip containing 19,000 genes/ESTs.

Protein expression of 2 ISGs (ISG15 and MxA) in paraffin-embedded liver biopsy tissue was examined by immunohistochemistry(IHC) study and the protein expression patterns were correlated with response status.

This piece of work entitled “Cell-type specific gene expression signature in liver underlies response to interferon therapy in chronic hepatitis C infection” was published in Gastroenterology 2010;138(3):1123-1133.e3. Epub 2009 Nov 6.

My role in this publication: study concept and design, acquisition of data, analysis and interpretation of data, drafting and revising of the manuscript.

Cell-type specific gene expression signature in liver underlies response to interferon therapy in chronic hepatitis C infection

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From the Banting and Best Department of Medical Research\textsuperscript{1}, Department of Molecular Genetics\textsuperscript{2}, Departments of Pathology\textsuperscript{3}, Medicine\textsuperscript{4}, Medical Biophysics\textsuperscript{5}, and Surgery\textsuperscript{6}, University of Toronto, Toronto, Ontario

None of the authors have financial disclosures.

No conflict of interests exist.

**Keywords**: HCV response signature, gene expression profiling, prospective validation, multivariate analysis, immunohistochemistry

**Running title**: HCV treatment response signature validation

**Grant support**: This work was funded by grants from the Canadian Institute of Health Research (No. 62488 to I.D.M). L.C. and I.B are supported by the National Canadian Research Training Program in Hepatitis C (NCRTP-HepC). L.C is also supported by Canada Graduate Scholarship (CGS) from the Canadian Institute of Health Research.
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**Abbreviations:**

CHC: chronic hepatitis C
HCV: hepatitis C virus
IFN: interferon
mAb: monoclonal antibody
NR: non-responder
pAb: polyclonal antibody
pegIFN/rib: pegylated interferon/ribavirin
R: responder
Rib: ribavirin
SVR: sustained virological response
ABSTRACT:

Background & Aims: Chronic hepatitis C virus (CHC) infection is treated with interferon/ribavirin, but only a subset of patients respond. Treatment nonresponders have marked pre-treatment upregulation of a subset of interferon stimulated genes (ISGs) in their livers, including ISG15. We here study how the nonresponder gene expression phenotype is influenced by clinical factors, and uncover the cellular basis of the phenotype through ISG15 protein expression.

Methods: 78 CHC patients undergoing treatment were classified by clinical (gender, viral genotype, viral load, treatment outcome) and histological (inflammation, fibrosis) factors and subjected to gene expression profiling on their pre-treatment liver biopsies. An ANOVA model was used to study the influence of individual factors on gene expression. ISG15 immunohistochemistry was performed on a subset of 31 liver biopsies.

Results: 123 genes were differentially expressed in the 78 CHC livers when compared to 20 normals (p <0.001, fold change ≥ 1.5 fold). Of genes influenced by a single factor, genotype (1 vs 2/3) influenced more genes (17) than any other variable; when treatment outcome was included in the analysis, this became the predominant influence (24 genes), and the effect of genotype was diminished. Treatment response was linked to cell-specific activation patterns: ISG15 protein upregulation was more pronounced in hepatocytes in treatment nonresponders, but in Kupffer cells in responders.

Conclusions: Genotype is a surrogate marker for the nonresponder phenotype. This phenotype manifests as differential gene expression and is driven by activation of different cell types: hepatocytes in treatment nonresponders, and macrophages in treatment responders.
INTRODUCTION

Almost 3% of the world’s population is infected with hepatitis C virus (HCV). It is the most common newly diagnosed cause of liver disease and the most common reason for a liver transplant. Treatment of chronic hepatitis C (CHC) is difficult: the current standard of care is pegylated-interferon IFNα (PegIFN) combined with ribavirin. This regimen has only a 50% response rate overall, with high costs and high morbidity.

Why patients respond to IFN-based treatment differently likely reflects differences in the viral/host response. On the viral side, genotype is the single most important factor predicting treatment response. Patients infected with HCV genotypes 2 and 3 respond considerably better than those infected with genotypes 1 and 4, with response rates of approximately 80%, compared to 45% for genotype 1. Viral load at the initiation of therapy is less predictive, though SVR is achieved more often in patients with low titers (<800 000 IU/ml). Host factors are also involved. Hepatic fibrosis is important, with fewer responders in patients with advanced hepatic fibrosis. Race may also play a role: African-American patients tend to have lower response rates to therapy than Caucasian patients, while Asian patients have higher response rates. There are also genetic bases for treatment response: polymorphisms in inflammatory genes, such as IFNγ and IL-10, have been shown to be associated with rates of response.

We have shown that differences in hepatic gene expression levels determined from liver biopsies obtained prior to treatment initiation are associated with treatment outcome. Genes strongly
correlated with response status are enriched in interferon stimulated genes (ISGs); their levels of expression are higher in nonresponders (the “high ISG” group) than in responders (the “low ISG” group). Among the ISGs correlated with response, ISG15 (a ubiquitin-like protein) is consistently upregulated in the pre-treatment liver tissue of patients who then do not respond to PegIFN/Rib.

Among all the factors associated with treatment response viral genotype and the host gene-expression profiles may correlate best with response. However, it has not been established whether the individual host and viral factors are independent markers of treatment response, or if some are simply surrogates for others. In this study we extend our initial observations by considering a larger CHC population (78 patients). We describe results that strongly argue that cell-specific gene expression patterns define two states of CHC infection that influence treatment response.
MATERIALS AND METHODS

Patients and liver biopsies:

Chronic HCV: 78 patients with CHC (56 genotype 1, 22 genotype non-1) were treated at University Health Network from October 2001 through May 2004. All patients underwent pretreatment percutaneous liver biopsy and had baseline viral loads determined. Treatment was either PegIFN2a 180µg or PegIFN2b 1.5µg/kg s/c weekly, and oral ribavirin 800-1200mg daily for 24 (genotype 2/3) or 48 (genotype 1, 4 and 6) weeks. Quantitative HCV-RNA was determined at completion of therapy and 6 months after (lowest limit of detection, LLD 50 IU/ml, Roche). A patient was designated a nonresponder (NR) if HCV-RNA was detectable at the end of therapy, as a relapser if HCV-RNA was undetectable at the end of treatment but was detectable at the 6 month follow-up, and as having a sustained viral response (SVR) if both end-of-treatment and 6 month follow-up HCV-RNA were undetectable. For the purposes of this study, responders (R) were considered to be all patients who had no detectable HCV RNA at the end of treatment.

Normal liver tissue: Normal liver tissue was obtained as the first step of a living donor right hepatectomy in 20 HCV-, HBV-negative patients.

Ethics: All patients gave informed consent for the research protocol, which was approved by the hospital Research Ethics Board.

RNA extraction, amplification, and microarray analysis:
A portion of each liver biopsy (0.5-1.0cm if percutaneous core) was immersed in RNAlater (Qiagen), RNA was extracted and amplified, and gene expression levels were determined using human single spot 19000 clone cDNA microarrays as in our previous studies (UHN Microarray Center). Microarray data was normalized using an R-based, intensity-dependent lowess scatter plot smoother as before.

**Statistical analysis:**

Statistical significance of differences between measured gene expressions levels was evaluated using a two-sample Welch \( t \)-statistic implemented in the \( R \) package *multtest*, which includes an estimation of adjusted \( p \)-values by permutation. Comparisons of clinical, demographic and histological categorical variables were done using either a Fisher's exact test or a Chi-squared test. Unsupervised hierarchical clustering was performed using the \( R \) package *stats*. Where appropriate, Student's \( t \) test was used to compare 2 categorical values and one-way ANOVA was used to compare more than 2 categorical values. For western blot studies the presented work is representative of at least three independent experiments.

**ANOVA model for microarray experiments:**

Our ANOVA model contains four clinical factors (gender, viral genotype, viral load, treatment response) and two histological factors (disease activity, fibrosis). The full model was fitted on a gene-by-gene basis to log2-transformed expression ratios of CHC and normal liver. Our model assumes all effects to be fixed. The main objective of the analysis was to identify genes
with significant changes between the levels of each factor after adjusting for all other effects specified in the model. The significance of each computed effect is thus relative only to other factors present in the model. Because our data are unbalanced we used a type III sum of squares approach where the sums of squares is based on comparing the full model to models with each factor removed one at a time. In this way the calculated sum of squares for unbalanced datasets is independent of the order of factors used. The significance of each effect is then computed with the F-test. For the sake of clarity we identified genes that most clearly were influenced by a single factor: genes for which there were significant interactions between factors are not presented.

**Immunohistochemical studies:**

All percutaneous liver biopsies were fixed in 10% neutral buffered formalin, paraffin-embedded, sectioned at 4μm and stained with hematoxylin/eosin. **MxA immunostaining** - antigen was heat-retrieved with Tris-EDTA pH9.0, stained with anti-MxA mAb (M143, Dr. Otto Haller, University of Freiburg, Germany) at 1:300 for 1hr and finished with MACH4 UniversalAP Polymer Kit (Biocare Medical, Concord, CA). **ISG15 immunostaining** - antigen was heat-retrieved with citrate buffer at pH6.0, stained with rabbit anti-human ISG15 pAb (developed in our laboratory) at 1:300 for 1 hr and finished as above. **CD68 immunostaining**- antigen was retrieved by pepsin digestin and incubated with anti-CD68 mAb (PG-M1, Dako ) at 1:100 for 1hr, and finished with streptavidin-biotin-HRP (ID Labs Inc). **Smooth muscle actin (SMA) immunostaining**: anti-SMA mAb (Sigma, clone 1A4) was used at 1:3000 for 1 hr and finished with streptavidin-biotin-HRP as above. Endogenous peroxidase and biotin activities were
blocked with 3% aqueous H₂O₂ and Lab Vision avidin-biotin blocking kit. **ISG15 quenching test:** First, 10ng of purified human ISG15 in each of 6 lanes was separated on SDS-PAGE gel and transferred onto nitrocellulose membrane. This was cut into 6 lanes and incubated 2hrs with anti-ISG15 pAb (1:300) and increasing concentrations of purified ISG15 (0, 2, 20, 200, 2000, 4000ng/ml); the film was developed with OptiBlaze WESTfemtoLUCENT (G-Biosciences, MO, USA). Second, MxA or ISG15 antibody was neutralized with 9 volumes of recombinant ISG15 (2μg/ml) overnight (4°C) and then used for IHC at 1:300.

Cell staining for ISG15 or MxA was assessed throughout the entire length of each core of each biopsy (≥30 high power fields per biopsy). An immunohistochemical score was assigned based on the proportion of immunoreactive cells in a given cell type. Staining in individual cell types (hepatocyte, macrophage, lymphocyte, bile duct epithelium) was scored from 0 (no staining in any cell), 1 (occasional cell), 2 (multiple cells), to 3 (virtually all cells). Various semiquantitative scoring systems for evaluation of immunohistochemical staining have been developed – ours was chosen for its ease and reproducibility. The evaluation was done in a blinded manner by two independent liver pathologists; the evaluation was repeated, with the samples re-arranged, again in a blinded manner, several days after the first evaluation.
Results:

Patient demographics:

Demographic and clinical variable data on the 78 CHC patients are summarized in Table 3-1. The majority (72%) were infected with HCV genotype 1. Patients were denoted “low fibrosis” if their Ishak scores were 0-2, and “high fibrosis” if their scores were 3-4. Similarly, patients were classified “low activity” if their METAVIR A scores were 0-2 and “high activity” for scores of 3-4. For this study, high viral load was defined as >8x10^6 IU/ml. Comparing genotype 1 and non-1 patients there were no statistical differences in clinical (age, sex, viral load) or histological variables (disease activity, degree of fibrosis). In total, there were 23 non-responders and 55 responders (42 SVR, 13 relapsers).
Table 3-1. Patient demographics

<table>
<thead>
<tr>
<th></th>
<th>Genotype 1 (56)</th>
<th>All other Genotypes (22)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>50.87 +/- 9.87</td>
<td>51.64 +/- 10.22</td>
<td>0.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>33 (59%)</td>
<td>12 (55%)</td>
<td>1</td>
</tr>
<tr>
<td>High</td>
<td>23 (41%)</td>
<td>10 (45%)</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>33 (59%)</td>
<td>12 (55%)</td>
<td>1</td>
</tr>
<tr>
<td>High</td>
<td>23 (41%)</td>
<td>10 (45%)</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>38 (68%)</td>
<td>13 (59%)</td>
<td>0.84</td>
</tr>
<tr>
<td>F</td>
<td>18 (32%)</td>
<td>9 (41%)</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Viral Load</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low &lt; 8x10^6 IU</td>
<td>32 (57%)</td>
<td>17 (77%)</td>
<td>0.55</td>
</tr>
<tr>
<td>High &gt; 8x10^6 IU</td>
<td>24 (43%)</td>
<td>5 (23%)</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NR</td>
<td>21 (38%)</td>
<td>2 (9%)</td>
<td>0.09</td>
</tr>
<tr>
<td>SVR</td>
<td>25 (45%)</td>
<td>17 (77%)</td>
<td>0.22</td>
</tr>
<tr>
<td>Relapsers</td>
<td>10 (17%)</td>
<td>3 (14%)</td>
<td>1</td>
</tr>
</tbody>
</table>
Predictive value of an 18-gene “responder” signature:

We first sought to confirm the phenotypes described in our earlier study. After gene expression profiling on the 78 liver biopsies (31 from the original study,16 and 47 additional ones) we asked whether the eighteen-gene signature described in our earlier study segregated samples by treatment response. We performed a hierarchical cluster analysis, independent of patient identifiers. As shown in Figure 3-1, two clusters resulted: one that was highly enriched for treatment responders, the other for nonresponders.

We next asked whether expression levels for the 18 genes could classify patient samples as “responder” or “nonresponder.” We used four different families of classifiers, all validated in previous microarray classification studies:28 k-nearest neighbor (KNN), diagonal quadratic (DQDA) and linear discriminant analysis (DLADA), and classification and regression trees (CART). Misclassification rates for each classifier were estimated over 100 runs using a 2:1 sampling scheme based on random divisions of learning and test sets (2/3 and 1/3 of the data, respectively). All four classifiers had very similar results (Table 3-2). In all cases there was a better prediction rate for R (PPV 0.96) than for NR (NPV 0.58). Overall, these results describe a predisposition: patients with the “low ISG” responder phenotype are much more likely to respond to treatment than those with the “high ISG” nonresponder phenotype.
Table 3-2: Response signature validation based on gene expression data from cDNA microarray

<table>
<thead>
<tr>
<th>Methods</th>
<th>Sensitivity</th>
<th>specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNN</td>
<td>0.78 +/- 0.08</td>
<td>0.81 +/- 0.14</td>
<td>0.93 +/- 0.05</td>
<td>0.56 +/- 0.09</td>
</tr>
<tr>
<td>DQDA</td>
<td>0.75 +/- 0.10</td>
<td>0.91 +/- 0.10</td>
<td>0.97 +/- 0.04</td>
<td>0.55 +/- 0.08</td>
</tr>
<tr>
<td>DLDA</td>
<td>0.73 +/- 0.08</td>
<td>0.92 +/- 0.09</td>
<td>0.97 +/- 0.04</td>
<td>0.53 +/- 0.08</td>
</tr>
<tr>
<td>CART</td>
<td>0.84 +/- 0.08</td>
<td>0.87 +/- 0.16</td>
<td>0.96 +/- 0.05</td>
<td>0.66 +/- 0.13</td>
</tr>
</tbody>
</table>

PPV: positive prediction value; NPV: negative prediction value; KNN: K-nearest neighbour; DQDA: Diagonal Quadratic Discriminant Analysis; DLDA: Diagonal Linear Discriminant Analysis; CART: Classification and Regression Trees.
Hierachial clustering analysis of 78 HCV chronically infected liver samples based on expression levels of 18 previously-defined signature genes.

Figure 3-1. Hierachial clustering analysis of 78 HCV liver samples based on expression levels of 18 previously defined signature genes. Hierarchial clustering analysis was performed based on the expression levels of 18 previously defined genes. The analysis broadly separates patients into 2 groups: responders (R) and nonresponders (NR).
Multivariate analysis of microarray expression data:

The above results suggest that there is a persistent difference in the viral/host response, manifest at the level of gene expression, influencing the response to treatment. We next asked whether this difference was influenced by or was independent from the clinical factors that are used to predict treatment responses (genotype, viral load, fibrosis). We performed a multivariate ANOVA analysis on the genes identified as being consistently altered by CHC infection when compared to uninfected, normal liver tissue. Taking a $p$ value of 0.001 and a fold change (vs normal liver tissue) of $\geq1.5$, a total of 123 genes was altered in CHC liver tissue. The full gene list can be accessed from our lab server at http://142.150.56.35/~LiverArrayProject2/home.html. (user name: lab; Password: samatalw41)

We first considered the influence of gender, viral load, genotype, disease activity, and fibrosis on gene expression. Restricting the analysis to these five factors we could ascribe differences in expression of 33 genes to single factors. Differences in viral genotype (genotype 1 vs genotype 2/3) were correlated with altered gene expression levels of the highest number of genes (17) (Figure 3-2A). The genes most influenced by genotype 1 infection included DUSP1, OAS3, LAP3, and RPS28, all genes that we previously identified as predictors of treatment response (Table 3-3A). Real-time PCR confirmed the accuracy of these results (data not shown).
Figure 3-2. Multivariate ANOVA analysis of genes altered by chronic HCV infection

(A) Response status excluded: The bar graph shows the number of genes significantly associated with each factor (genotype/disease \([D]\), gender \([G]\), viral load \([V]\), fibrosis \([F]\), and inflammation \([I]\)). Genotype was associated with the most genes (17). The box plots below show the distribution of fold differences in gene expression between the levels of each factor for significant genes associated with each factor. (B) Response status included: In this analysis, response status \((R)\) was considered in addition to the 5 previous factors. Response status was associated with the most genes (24).
Table 3-3A: Genes affected by viral genotype (‘response to treatment’ excluded)

<table>
<thead>
<tr>
<th>cloneID</th>
<th>LOCUS</th>
<th>Symb</th>
<th>geneName</th>
<th>Hepc/Nor</th>
<th>p.value</th>
<th>p.genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>5725681</td>
<td>81671</td>
<td>VMP1</td>
<td>vacuole membrane protein 1</td>
<td>0.58</td>
<td>1.00E-04</td>
<td>0.01521314</td>
</tr>
<tr>
<td>5221374</td>
<td>51561</td>
<td>IL23A</td>
<td>IL23A interleukin 23, alpha subunit p19</td>
<td>1.57</td>
<td>1.00E-04</td>
<td>0.020740392</td>
</tr>
<tr>
<td>26063</td>
<td>432395</td>
<td>C4B</td>
<td>complement component 4B, telomeric</td>
<td>0.63</td>
<td>1.00E-04</td>
<td>0.039356361</td>
</tr>
<tr>
<td>108690</td>
<td>EST</td>
<td>EST</td>
<td>EST</td>
<td>1.67</td>
<td>1.00E-04</td>
<td>0.020127457</td>
</tr>
<tr>
<td>428195</td>
<td>51614</td>
<td>SDBCAG84</td>
<td>serologically defined breast cancer antigen 84</td>
<td>1.48</td>
<td>1.00E-04</td>
<td>0.003643768</td>
</tr>
<tr>
<td>52905</td>
<td>1843</td>
<td>DUSP1</td>
<td>dual specificity phosphatase 1</td>
<td>0.52</td>
<td>1.00E-04</td>
<td>0.015530487</td>
</tr>
<tr>
<td>471667</td>
<td>6772</td>
<td>STAT1</td>
<td>signal transducer and activator of transcription 1, 91kDa</td>
<td>1.77</td>
<td>1.00E-04</td>
<td>0.027999208</td>
</tr>
<tr>
<td>754047</td>
<td>23097</td>
<td>CDC2L6</td>
<td>cell division cycle 2-like 6 (CDK8-like)</td>
<td>1.73</td>
<td>1.00E-04</td>
<td>0.038349041</td>
</tr>
<tr>
<td>487534</td>
<td>51056</td>
<td>LAP3</td>
<td>leucine aminopeptidase 3</td>
<td>1.45</td>
<td>1.00E-04</td>
<td>0.000593948</td>
</tr>
<tr>
<td>4277</td>
<td>9246</td>
<td>UBE2L6</td>
<td>ubiquitin-conjugating enzyme E2L 6</td>
<td>1.54</td>
<td>1.00E-04</td>
<td>0.000806251</td>
</tr>
<tr>
<td>176650</td>
<td>441618</td>
<td>RPS28</td>
<td>40S ribosomal protein S28</td>
<td>1.85</td>
<td>1.00E-04</td>
<td>0.004459306</td>
</tr>
<tr>
<td>324284</td>
<td>4940</td>
<td>OAS3</td>
<td>2'-5'-oligoadenylate synthetase 3, 100kDa</td>
<td>1.96</td>
<td>1.00E-04</td>
<td>0.002096342</td>
</tr>
<tr>
<td>502921</td>
<td>83666</td>
<td>PARP9</td>
<td>poly (ADP-ribose) polymerase family, member 9</td>
<td>1.87</td>
<td>1.00E-04</td>
<td>0.007749409</td>
</tr>
<tr>
<td>485859</td>
<td>64108</td>
<td>IFRG28</td>
<td>28kD interferon responsive protein</td>
<td>1.52</td>
<td>1.00E-04</td>
<td>2.08E-05</td>
</tr>
<tr>
<td>491243</td>
<td>3627</td>
<td>CXCL10</td>
<td>chemokine (C-X-C motif) ligand 10</td>
<td>2.73</td>
<td>1.00E-04</td>
<td>0.010216357</td>
</tr>
<tr>
<td>5474956</td>
<td>6181</td>
<td>RPLP2</td>
<td>ribosomal protein, large P2</td>
<td>2.74</td>
<td>1.00E-04</td>
<td>0.005944298</td>
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<tr>
<td>485870</td>
<td>55601</td>
<td>FLJ20035</td>
<td>hypothetical protein FLJ20035</td>
<td>2.37</td>
<td>1.00E-04</td>
<td>0.00042658</td>
</tr>
</tbody>
</table>

Hepc/Nor, ratio of the gene expression level in CHC-infected liver to normal liver; P value, P value of Hepc/Nor; P genotype, P value for the viral factor genotype.

NOTE. Response to treatment excluded.
In the next multivariate ANOVA model analysis, we included “response to treatment” as a variable. In this analysis we could link 54 genes to individual variables. As presented in Figure 3-2B, “response to treatment” became the single most predominant influencing variable (24 genes). At the same time, the effect of genotype was markedly diminished (9 genes). Genes uniquely influenced by response to treatment included several that we have previously identified as being important predictors of treatment response, including IFIT1, DUSP1, GIP3, and LAP3 (Table 3-3B).
### Table 3-3B: Genes affected by response status

<table>
<thead>
<tr>
<th>cloneID</th>
<th>LOCUS</th>
<th>Symb</th>
<th>gene Name</th>
<th>Hepc/Nor</th>
<th>p.value</th>
<th>p.response</th>
</tr>
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<tr>
<td>754047</td>
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<td>CDC2L6</td>
<td>cell division cycle 2-like 6 (CDK8-like)</td>
<td>1.73</td>
<td>1.00E-004</td>
<td>2.30E-007</td>
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EST, expressing sequence tag; Hepc/Nor, ratio of the gene expression level in CHC-infected liver to normal liver; 
P value, P value of HepC/Nor; P response, P value for the factor response.
“Genotype” is a surrogate for a viral/host response:

The above results suggest that as a variable, “response to treatment” identifies two underlying viral/host responses, manifest at the level of gene expression, that influence treatment outcomes. If so, then “genotype” may be a surrogate for “response status,” and the genes that we found to be influenced by “genotype” should segregate genotype 1 patients into “responder” and “nonresponder” samples. An unbiased approach to this question is to perform a hierarchical cluster analysis of all genotype 1 samples using the 17 genes identified in the first ANOVA analysis, above. As shown in Figure 3-3, this analysis results in two clusters: one is significantly enriched for responder patients (30/40) and the other for nonresponder patients (11/16) (p<0.005, Fisher’s exact test). These results support the contention that genotype is a surrogate for an underlying host/viral response associated with treatment outcomes.
Figure 3-3. Hierarchical cluster plot of CHC genotype 1 patients segregated by the 17 genes specific to “genotype”.

Figure 3-3. Hierarchical cluster plot of genotype 1 patients segregated by “genotype”-specific genes. The 17 genes shown on the right of the plot were significantly associated with “genotype/disease” using the ANOVA multivariate model. There are 2 main clusters, with nonresponders being significantly enriched in one and responders in the other (see Results section).
ISG15 protein expression defines cell-specific activation in nonresponder vs responder liver tissue:

Having determined that the “high ISG” nonresponder phenotype is a robust and independent determinant of a CHC patient’s likelihood to respond to treatment, we wanted to define the cellular source of the phenotype. We asked whether the “high ISG” pattern derived from hepatocytes, indicating a close interaction with the virus, or immune cells, which might indicate a deficiency in the immune response. We examined the cellular expression of ISG15, one of the genes highly predictive of treatment response, even alone. ISG15 mRNA is consistently upregulated in the liver tissue of nonresponder patients, in our lab and others,17-19 and one of the regulators of ISG15 protein conjugation, USP18, plays an important role in the anti-HCV effect of IFN.29 Thus, ISG15 protein expression may reveal important aspects of the host response to CHC infection.

Immunohistochemical studies of the 31 CHC pre-treatment liver biopsies used in our original description of the “high ISG” phenotype16 were performed using anti-ISG15 pAb (Methods and Materials). Hepatocyte, macrophage and lymphocyte staining was evaluated for all biopsies as outlined above. Hepatocytes and macrophages were identified by their typical morphological appearance. While there were no differences in lymphocyte staining across R and NR samples (data not shown), there was a consistent and marked difference in hepatocyte and macrophage ISG15 staining. Treatment nonresponders had more hepatocellular staining, treatment responders had more macrophage staining (Figure 3-4A and 3-4C); a similar trend was observed in studies of immunostaining for MxA - another ISG that we found to be differentially upregulated in NR
liver tissue (Figure 3-4B). There was no association between degree of fibrosis and ISG (ISG15, MxA) distribution (data not shown).
Figure 3-4: ISG activation in different cell types in the pretreatment livers of R and NR

A

** P<0.0001

* P<0.005

NR R

[Graph showing quantitation of hepatocyte and macrophage expression of ISG15 protein in pretreatment liver biopsies of R (n=15) and NR (n=16) patients. All slides were scored in a blinded fashion from 0 (no cell staining) to 3 (all cells staining). The Y-axis is the average score for a given response status (R or NR) presented as mean ± SD.]

B

** P<0.001

* P=0.0036

NR R

[Graph showing quantitation of hepatocyte and macrophage expression of MxA protein in pretreatment liver biopsies of R (n=15) and NR (n=16) patients. All slides were scored in a blinded fashion from 0 (no cell staining) to 3 (all cells staining). The Y-axis is the average score for a given response status (R or NR) presented as mean ± SD.]
Chen et al. Figure 3-4C

3-4C. representative ISG15 immunohistochemistry. A. Representative liver biopsy from NR patient immunostained for ISG15 showing predominantly hepatocellular pattern of immunoreactivity (magnification 50x); B) Liver biopsy from same NR patient as in A), immunostained for ISG15, showing detail of hepatocellular staining at high power (magnification 400x); C) Representative liver biopsy from R patient, immunostained for ISG15, showing macrophage pattern of immunoreactivity (magnification 50x); D) Liver biopsy from same R patient as in C), immunostained for ISG15, showing detail of macrophage staining in high power (magnification 400x).
3-4D. Macrophage-specific staining.

A) Liver biopsy from R patient, immunostained for ISG15, showing typical macrophage pattern of immunoreactivity in sinusoidal lining cells (magnification 400x);

B) Liver biopsy from the same patient as in A), immunostained for SMA, showing no reaction in sinusoidal lining cells (magnification 400x);

C) Liver biopsy from same patient, immunostained for CD68, showing immunoreactivity in sinusoidal lining cells similar in morphology and location to the ISG15 immunoreactive cells in A) and confirming that the ISG15 immunostaining is specific to macrophages (magnification 400x).
In order to eliminate the possibility of nonspecific ISG15 antibody staining we performed a quenching experiment. The binding of anti-ISG15 pAb to recombinant ISG15 protein was quenched when the antibody was pre-incubated with recombinant ISG15 (Figure 3-5A). Incubation of purified ISG15 protein (2μg/ml) with antibody specific to MxA had no effect on the binding of MxA to CHC liver tissue (Figure 3-5B). However, purified ISG15 protein eliminated binding of ISG15-specific antibody to CHC liver tissue, confirming the specificity of our findings (Figure 3-5B).
Figure 3-5. ISG15-specific immunohistochemical staining:

5A

Recombinant ISG15 protein attenuates ISG15 antibody binding \textit{in vitro} in a dose-dependent manner. Shown is a Western blot for ISG15: 10ng of purified human ISG15 in each of the 6 lanes was separated on SDS-PAGE gel and transferred onto nitrocellulose membrane. The membrane was then cut into 6 individual lanes and incubated with a mixture of mouse anti-human ISG15 polyclonal antibody (1:300) and ISG15 purified protein (ng/ml) 0 (lane1), 2 (lane 2), 20 (lane 3), 200 (lane 4), 2000 (lane 5), and 4000 (lane 6) for 2 hrs. Note that ISG15 signal was successfully attenuated (quenched by) the higher amounts of purified ISG15 protein.
5B. Confirmation of ISG15-specific staining in immunohistochemical analysis

A) Liver biopsy from R patient, immunostained using mAb for MxA, showing typical macrophage pattern of immunoreactivity (magnification 200x);

B) Liver biopsy from the same R patient as in (A), first quenched with ISG15 protein (2μg/ml) and then immunostained with the MxA antibody, showing persistence of immunoreactivity and indicating no cross-reaction between MxA antibody and ISG15 protein (magnification 200x);

C) Liver biopsy from NR patient, immunostained for ISG15, showing typical hepatocellular pattern of immunoreactivity (magnification 200x);

D) Liver biopsy from same NR patient as in C), first quenched with ISG15 protein(2μg/ml) and then immunostained for ISG15, showing loss of immunoreactivity and indicating no cross-reaction between the ISG15 antibody and MxA or other protein (magnification 200x).
We next confirmed the cell types identified above. Resident cells within the liver sinusoidal spaces are generally Kupffer cells (macrophages) and endothelial cells, but activated stellate cells may be confused for Kupffer cells. We therefore performed IHC using CD68 as a macrophage marker, and smooth muscle actin (SMA) as a marker for activated stellate cells. As shown in Figure 3-4D, cells that reacted with antibody specific to ISG15 conformed in morphology and location to cells that stained with antibody specific to CD68, but did not express SMA. These results confirm that Kupffer cells are a source of the ISG15 signal.
Discussion:

Although the molecular mechanisms involved in IFN resistance in CHC patients who do not respond to treatment are not well understood, the interaction between viral and host factors plays a critical role. In this study we present evidence that chronic infection with HCV leads to two different states at the level of hepatic gene and protein expression: one, the “high ISG” nonresponder phenotype, is associated with high level ISG expression in hepatocytes, the other, the “low ISG” responder phenotype, has more ISG expression in tissue macrophages. Far from being a surrogate of clinical or viral factors, our data suggest that this pattern is a strong and independent predictor of treatment outcome, that the principal source of the “high ISG” signal in the nonresponder phenotype is the hepatocyte, and that CHC infection is best conceived of in terms of two patterns of cellular activation giving rise to two different patterns of gene expression.

Using a larger cohort of patients with CHC than previously,\textsuperscript{16} we could perform classifier analyses and seek influences of individual variables on gene expression levels. The similarity in results obtained with different classifiers (Table 3-2) suggests real biological differences that are independent of the method used for classification. The original 18-gene set is quite accurate in predicting who will respond (e.g. positive prediction value for CART, PPV=0.96), but performs less well for no response (e.g. negative prediction value for CART, NPV=0.66). Similar rates of prediction were observed using real-time PCR data for the levels of expression of the 18 genes (data not shown). Taken together, we have observed similar predictive results across two independent platforms (microarray, real-time PCR) and four independent classifier analyses,
arguing that the ISG-dominated gene signature reflects real biological differences between responder and non-responder groups. In fact, the nonresponder signature was a more powerful predictor of treatment outcomes than any clinical variable included in our study, even in an integrative logistic regression model (data not shown).

A similar influence was found in our multivariate analysis of the effect of individual variables on gene expression values. We performed two analyses: first without and then with “response to treatment” as a variable (a full description of results and methodology is found on our server: http://142.150.56.35/~LiverArrayProject2/home.html (user name: lab; Password: samatalw41). Viral genotype influenced the greatest number of genes in the first analysis, but “response to treatment” influenced more genes in the second analysis with a diminished effect of genotype. When all genotype 1 samples were sorted by hierarchical cluster analysis, using the genes most influenced by “genotype” in the first analysis, two clusters resulted: one was significantly enriched for nonresponders, and one for responders. In fact, we could not identify any consistent differences between genotype 2/3 patients and genotype 1 patients who responded to treatment: at the level of gene expression they behave similarly (data not shown). Considered together, these data suggest that hepatic gene expression is not a surrogate for genotype, but that there is an independent and consistent difference in how patients who are predisposed to treatment failure respond to the chronic infection at the level of gene expression.

The fact that the viral genotype is not directly causal of response is supported by other studies that implicate the host in this process. For example, single nucleotide polymorphisms (SNPs) in
the interferon-stimulated genes for IFNγ, SOCS3, OAS, MxA IL10 and, most-recently, IL28B, have been linked to treatment response.\textsuperscript{14,31,32,33} In the virus, mutations in the interferon sensitivity-determining region (ISDR) of the NS5a protein correlate with viral load even within individual viral subtypes.\textsuperscript{34} In a recent study increased levels of hepatic SOCS3 expression correlated with nonresponder status regardless of genotype (genotype 1 responders had similar levels of SOCS3 expression as genotype 2/3 responders).\textsuperscript{35} Pretreatment hepatic SOCS was the most powerful predictor of sustained viral response, even when compared to genotype, by logistic regression analysis. Since SOCS3 is an ISG, this data is consistent with our own, in which increased expression of a subset of ISGs predicts treatment response and is independent of genotype alone (Ref 16 and current study). One conclusion of these combined observations is that the responder “state of cellular activation” is the most-likely outcome of infection with genotypes 2 and 3 and a less-likely outcome of genotype 1 infection.

A number of the genes that differ between Genotype 1 R and NR patients are ISGs, such as OAS3, LAP3, DUSP1, and RPS28. These genes are part of our previously identified predictive gene set for discriminating SVR and NR.\textsuperscript{16} Although these results suggest a difference in IFN response, we were unable to find any consistent differences in a survey of IFN-signaling related gene expression, such as IFNs, their receptors, Jak1, TLR3, A20, and IRF3 (real-time PCR, data not shown). Other groups have described changes in some of the signaling molecules important to IFN signaling. Asahina \textit{et al} examined the baseline expression levels of cytoplasmic viral sensors and related regulators involved in innate immunity in 74 CHC liver samples. Up-regulation of RIG-I and MDA5, and down-regulation of Cardif, were associated with NR patients.\textsuperscript{36} However, these differences in molecules relevant to IFN signaling may not explain the
differences we and others have observed in ISG expression in CHC liver tissue, where IFN levels are variable but generally low.\textsuperscript{37}

Our immunohistochemical data describes both the source of the ISG “signal” in the nonresponder phenotype, and identifies a new pattern of cellular activation that correlates with treatment response: hepatocellular ISG15 and MxA expression in treatment nonresponders, macrophage ISG15 and MxA expression in treatment responders. This result corroborates previous work demonstrating a predominantly hepatocellular pattern of staining for the MxA in treatment nonresponders.\textsuperscript{26} Our finding that ISG15 and MxA ISG expression are more often expressed in the macrophages of treatment responders than nonresponders suggest that macrophages may be more strongly activated in responders. When normal liver tissue is compared to pretreatment liver biopsies from 10 genotype 1 responders and 10 genotype 1 nonresponders, there is a trend to increased mRNA expression for TNFα, IFNγ, IL1α, IFNα1, IFNAR2, and IFNβ in responder liver tissue (Supplemental Data). CHC infection leads to discrete patterns of cellular activation that are reflected in gene expression profiles and that have profound implications for treatment response.

The different patterns of cell activation identified in this report define a new way of looking at chronic HCV: as a two-state condition defined by relative macrophage and hepatocyte activation. The mechanisms underlying this differential state are unclear, but are likely not due to STAT1 signaling. In a recent study pretreatment NR liver tissue had only weak hepatocyte phospho-STAT1 staining.\textsuperscript{38} Post-treatment, phospho-STAT1 levels were increased in macrophages, but
not consistently in hepatocytes. By contrast, responder liver tissue had little-to-no phospho-STAT1 staining pre-treatment, but strong hepatocyte staining post-treatment. If this study is validated, it would suggest that STAT1 is not the driving force behind gene expression prior to treatment.

On the other hand, most of the ISGs that we have found to be differentially regulated in the liver tissue of nonresponders\textsuperscript{16} are linked by virtue of IRF3 sites in their promoter regions (bioinformatics analysis, not shown). Intriguingly, a similar pattern of IRF3-dependent ISG expression was seen in response to herpes simplex virus replication in human embryonic lung cells.\textsuperscript{39} Whether HCV replication has a similar ability to stimulate IRF3 in nonresponders should be the focus of future work.

Overall, our data suggests that tissue macrophages in nonresponders are relatively impaired. A number of studies have described aberrant macrophage function in chronic HCV, possibly resulting from direct interaction with viral particles. Peripheral blood monocytes from HCV-infected patients preferentially express IL10 when stimulated by recombinant HCV particles – the IL10 may suppress T cell responses and inhibit responses to TLR3 and TLR4 ligands.\textsuperscript{40,41} HCV core protein can inhibit TLR4-induced production of IL12 by macrophages.\textsuperscript{42} Although viral loads are equivalent in the responders and nonresponders in our study, different patterns of HCV replication in the infected liver might contribute to different patterns of macrophage activation and functional impairment.
In summary, R and NR patients can be distinguished based on the gene expression profiles. These profiles are independent of viral genotype or any other viral or clinical factor examined, and do not reflect, as we predicted previously, graded expression of these genes in a single cell type, but rather markedly different patterns of cellular activation in liver tissue chronically infected by HCV. The data strongly suggest that treatment responders and nonresponders differ fundamentally in their response to chronic HCV infection. The majority of HCV research is directed at defining how HCV affects a single cell (usually the hepatocyte); future work should be directed at defining how the hepatocyte and macrophage cell types in the liver interact in chronic HCV and during treatment of the infection.
Supplementary Data: Real-time PCR analysis of responders vs nonresponders:

Introduction:

Our data suggests that tissue (liver) macrophages play an important role in mediating immune responses that impact on response to treatment. To test this hypothesis, we performed a real-time PCR analysis that examined the mRNA expression of selected inflammatory and immune modulators in the pre-treatment liver tissue of responders and nonresponders. Liver biopsies from 10 R and 10 NR pre-treatment liver biopsies were evaluated; all patients were infected with HCV genotype 1.

Real-Time Polymerase Chain Reaction

Two-step real-time polymerase chain reaction (PCR) was performed after reverse transcription of 2µg of total RNA with 2 µg pd(N)6-random hexamer primer (Amersham, Oakville, Ontario, Canada). The resulting complementary DNA was used as a template for real-time PCR quantification with the QuantiTect SYBR PCR Kit (Qiagen), and real-time PCR (normalized to -actin) was performed using the DNA Engine Opticon 2 cycler (MJ Research, Reno, NV). For primers, see Table 3-4.
Table 3-4: real-time PCR primers

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Results:

As shown in Figure 3-6, genes for inflammatory mediators, such as TNFα, IL-1 and IFNγ, tended to be more highly expressed in the liver tissue of treatment responders than nonresponders. This data corroborates the immunohistochemical impression that macrophage activation is more pronounced in the liver tissue of responders than in nonresponders. While this trend will need to be validated in further studies, it does support the finding that macrophage activation is more pronounced in the liver tissue of eventual responders than in nonresponders, even within Genotype 1 patients. The finding that IFNAR2 is increased more in responders than nonresponders adds to previous reports that demonstrated that relative to responders, nonresponders have decreased expression of this component of the IFN receptor.¹²
Figure 3-6: Real-time PCR for inflammatory mediators in normal, R and NR livers

Real-time PCR of indicated genes in normal liver tissues (n=10, blank), pretreatment liver tissues from HCV treatment responders (n=10, grey), pretreatment liver tissues from HCV treatment nonresponders (n=10, black). All HCV liver biopsies were taken prior to initiation of treatment with PegIFN/Rib. Responder and nonresponder liver tissue had similar viral loads, and all HCV biopsies were obtained from Genotype 1 patients.
References for supplementary data:


References for the main text:


Chapter 4: Microarray screen identifies in an unbiased way the ISG15/USP18 pathway to be involved in IFN resistance in HCV infected patients

From 19,000 genes tested, I found a few hundred genes whose expression levels were altered in HCV chronically-infected liver tissues, and 18 genes that showed differential expression between Responders (R) and Non-responders (NR). 3 out of these 18 genes are involved in the ISG15/USP18 ubiquitin-like signaling pathway (only 5 genes in this pathway). Therefore, this pathway might play an important role in IFN resistance in those patients who do not respond to treatment.

To further explore the role of the ISG15/USP18 pathway in HCV production, I used the HCV in vitro culture model to address the effects of USP18 and ISG15 on HCV production.

Chapter 4-1: The role of USP18 in HCV production

The work “Silencing of USP18 Potentiates the Antiviral Activity of Interferon Against Hepatitis C Virus Infection” was published in Gastroenterology 2006;131(5):1584-1591

My role in this study: participating in experimental design, performing part of the experiments, acquisition of the data, summarizing/analyzing data I generated and writing part of the paper

Silencing of USP18 Potentiates the Antiviral Activity of Interferon Against Hepatitis C Virus Infection

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Abstract

**Background & Aims:** Modulation of the host innate immune response is an attractive means of inhibiting hepatitis C virus (HCV) replication. Having previously determined that expression of the interferon-sensitive gene (ISG)15 protease USP18 is increased in the liver biopsy specimens of patients who do not respond to interferon (IFN)-alfa therapy, we hypothesized that USP18 might hinder the ability of IFN to inhibit HCV replication. **Methods:** The role of USP18 in IFN antiviral activity was examined using an in vitro model of HCV replication that reproduces the full viral life cycle. USP18 was silenced specifically using small inhibitory RNAs (siRNAs), and the dose response of HCV replication and infectious virus production to IFN-alfa was measured. **Results:** The siRNA knockdown of USP18 in human cells consistently potentiated the ability of IFN to inhibit HCV-RNA replication and infectious virus particle production by a factor of 1–2 log₁₀. USP18 knockdown also resulted in a number of cellular changes consistent with increased sensitivity to IFN. Decreasing USP18 expression led to increased cellular protein ISGylation in response to exogenous IFN-alfa, prolonged tyrosine phosphorylation of signal transducer and activation of transcription (STAT1), and a general enhancement of IFN-stimulated gene expression. **Conclusions:** These data suggest that USP18 modulates the anti-HCV type I IFN response, and is a possible therapeutic target for the treatment of HCV infection.
Abbreviations:

EC50, median effective concentration; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; ISG, interferon-sensitive gene; PCR, polymerase chain reaction; siRR, irrelevant small inhibitory RNA; siRNA, small inhibitory RNA
Introduction

Hepatitis C virus (HCV) is a serious public health problem. More than 170 million persons worldwide are infected by the virus, which can lead to end-stage liver disease and hepatocellular carcinoma.\(^1\) The best antiviral therapy at present is a combination of pegylated interferon (IFN)-2-alfa and ribavirin, but almost half of all patients do not respond to this costly and often quite morbid treatment.\(^2,3\) Given the widespread nonresponse to exogenous IFN-alfa, novel therapeutic approaches that either augment or complement the antiviral activity of IFN-alfa would be very beneficial.

We recently showed that patients who do not respond to pegylated IFN-2-alfa and ribavirin show a consistent alteration in their pretreatment hepatic gene expression.\(^4\) When compared with responder liver tissue, nonresponders have increased expression of a number of IFN-sensitive genes. The expression of a subset of 8 genes accurately predicted treatment response in more than 90% of patients independent of viral load, genotype, or hepatic fibrosis. Two of the 8 genes, USP18 and IFN-sensitive gene (ISG)15, are linked biochemically. ISG15 is a ubiquitin-like molecule that is posttranslationally attached to the lysine residues of more than 150 target proteins.\(^5,6\) Protein targets include other IFN-stimulated genes, in addition to proteins in a diverse set of unrelated pathways. The function of ISG15 conjugation remains elusive. USP18/UBP43 is a ubiquitin-specific protease that cleaves the ubiquitin-like (and IFN-induced) ISG15 protein from its cellular targets in vitro.\(^7\) Together these data suggested that nonresponder patients have a disordered host IFN response, and correlate the up-regulation of USP18 with nonresponse to IFN treatment.
Multiple lines of evidence previously have linked ISG15, USP18, and the cellular response to IFN. Both genes are induced by type I IFN in many cell types,\(^8\)-\(^10\) and cells isolated from USP18 knockout mice are hypersensitive to IFN, with prolonged Jak–Stat signaling.\(^11\) These proteins also have been linked to the innate immune response to viruses. Overexpression of ISG15 enhances the antiviral activity of IFN against human immunodeficiency virus and Sindbis virus replication in vitro.\(^12\)-\(^14\) Influenza B virus NS1 protein binds ISG15 and prevents host protein ISGylation, a function that correlates with influenza B resistance to IFN.\(^15\) Based on these data, we hypothesized that USP18 may be a critical determinant of the human IFN antiviral response to HCV.

Until recently, hypotheses about mechanisms of HCV immunity have been difficult to address because a reliable cell culture model for the propagation of HCV was not available. However, we recently developed an in vitro HCV model that reproduces the complete HCV replication cycle.\(^16\) The viral genome is a chimeric HCV genotype 2a sequence. Its replication is robust (\(\sim 10^5\) infectious units/mL) and is neutralized by antibodies to the viral glycoprotein E2, or a soluble form of a cellular receptor for HCV, CD81. Importantly, replication is sensitive to a number of viral inhibitors, including IFN. By using this model, we were able to address the role of USP18 in the antiviral functions of IFN against HCV.

**Materials and Methods**

**Cells and HCV FL-J6/JFHVirus**

Huh-7.5 cells are a subline derived from Huh-7 hepatoma cells.\(^17\) HCV FL-J6/JFH is a full-length genotype 2a sequence that produces the full replication cycle in cell culture.\(^16\) It is a
chimera containing the JFH 5' nontranslated region,\textsuperscript{18} the J6 core through NS2 genes,\textsuperscript{19} and the JFH NS3 through 3' nontranslated region. Cells were maintained in Dulbecco’s modified Eagle media supplemented with nonessential amino acids and 10% fetal bovine serum. They were maintained at 37°C in 5% CO\textsubscript{2}.

**RNA Interference Assay**

USP18 small inhibitory RNAs (siRNAs) were obtained as follows (for each, the sense strand sequence is described): 4 USP18 siRNAs: siUSP18-a, 5′-GGAAUUCACAGACGAGAAAUU; siUSP18-b, 5′GGAAGAAGACAGCAACAUGUU; siUSP18-c, 5′GGGAAGACAUCCAGUGUACUU; and siUSP18-d, 5′CCAGGAGUUAAACACCCUGGUU. The irrelevant siRNA (siIRR) is 5′-ggcgcuuguggacauucugTT. Chemically synthesized RNA oligos were prepared as recommended by the manufacturer (Dharmacon, Lafayette, CO) and transfected as described previously.\textsuperscript{20-22} One nanomole of RNA duplexes in annealing buffer (100 mmol/L potassium acetate, 30 mmol/L HEPES-KOH pH 7.4, 2 mmol/L magnesium acetate) was electroporated into 2.5 × 10\textsuperscript{6} Huh-7.5 cells (5 pulses of 900 V for 99 μs, with 1-s intervals on a BTX electroporator [Harvard Apparatus, Inc, Holliston, MA]). Thirty hours after electroporation, cells were treated with IFN-alfa (0–1000 U) for 15 hours, washed, infected with HCV FL-J6/JFH for 6 hours, rinsed, and overlaid in media lacking IFN-alfa. RNA and protein were harvested for analysis of ISG expression. Two days after infection, cells were harvested for assessment of HCV replication (RNA and infectious particles). The effect of USP18 siRNAs on IFN activity against previously infected Huh-7.5 cells was tested as follows. Huh-7.5 cells were infected for 6 hours with 2 multiplicities of infection of J6-JFH HCV, then electroporated with irrelevant or USP18 siRNAs as per the standard protocol. Cells were seeded into 96 wells,
maintained, and then treated with indicated amounts of IFN from 24 to 48 hours after infection. IFN-containing media then was replaced with Dulbecco’s modified Eagle media + 10% fetal bovine serum. Cellular RNAs were harvested 72 hours after infection for quantification of HCV replication.

Quantification of Infectious HCV

Virus titers were determined by limiting dilution analysis as described previously.\textsuperscript{16} Supernatants from infected cells were collected and diluted 10-fold in media. Eight thousand Huh-7.5 cells were inoculated with virus titrations in 96-well plates (8 wells/dilution) and maintained for 3 days. Cells were washed in phosphate-buffered saline (PBS), fixed in methanol at −20°C, and blocked in PBS/1% bovine serum albumin/2% milk followed by 3% H\textsubscript{2}O\textsubscript{2}. Cells were incubated with monoclonal antibody to HCV NS5A (9E10) at 1:200, followed by mouse-horseradish peroxidase (Vector Impress, Burlington, ON, Canada). Cells then were exposed to 3,3′-diaminobenzidine tetrahydrochloride reagent (DAKO, Carpinteria, CA). HCV-positive wells were counted and the 50% infectious dose was calculated by the method of Reed and Muench.\textsuperscript{23}

RNA Quantification

Total RNA was harvested and purified with 96-well RNA-easy columns as recommended by the manufacturer (Qiagen, Mississauga, ON, Canada). For cellular RNAs, 2\(\mu\)g of DNaseI-treated total RNA was reverse transcribed with Superscript II (Invitrogen, Carlsbad, CA) for 2 hours at 42°C. Complementary DNA (cDNA) synthesis was primed with AnCT primer (Invitrogen). The reverse transcriptase was heat inactivated at 95°C for 5 minutes. A total of 1/15 of the cDNA mix was mixed with an equal volume of 2\(\times\) Sybr Green master mix (Qiagen). Polymerase chain
reaction (PCR) was performed using the following forward and reverse primers: ISG15: 5′-CGCAGATCACCAGAAGATT and 5′-GCCCTTGTATTCTCACA, IFN-induced protein with tetratricopeptide repeats: 5′-GCAGCCAAGTTTTTACCGAAG and 5′-GCCCTATCTGGTATGCAGT; and 2′-5′-oligoadenylate synthetase 3: 5′-GTCAAAACCAAGCCACAAGT and 5′-GGGCGAATGTTCACAAAGTT. Values were normalized to that of β-actin, amplified with forward and reverse primers 5′-TGGACTTCGAGCAAGAGATGG and 5′-GGAAGGAAGGCTGGAAGTG. PCR conditions were as follows: 94°C for 10 minutes, (94°C for 45 s, 56°C for 45 s, 72°C for 1 min) × 45 cycles. For HCV-RNA quantitation, 50 ng of total RNA was reverse transcribed and amplified with HCV-specific primers 5′-TGA GTG TCG TAC AGC CTC CA and 5′-ACG CTA CTC GGC TAG CAG TC using Platinum Quantitative reverse-transcription PCR Thermoscript One-step System (Invitrogen, Life Technologies). A 100-ng aliquot of total RNA was used to quantify HCV-specific RNA levels using an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). PCR conditions were as follows: 50°C for 2 minutes, 50°C for 30 minutes, 95°C for 10 minutes (95°C for 15 s, 50°C for 40 s, 72°C for 30 s) × 50 cycles. Glyceraldehyde-3-phosphate dehydrogenase detection mix from Applied Biosystems (VIC-MGBNFQ) was amplified as follows: 50°C for 2 minutes, 50°C for 30 minutes, 95°C for 10 minutes, (95°C for 15 s, 60°C for 1 min) × 40 cycles and used for normalization. Results were analyzed with SDS 1.9 software from Applied Biosystems.

**Statistical Analysis**

HCV replication data were analyzed with Prism software (San Diego, CA). Values from the indicated number of replicates for each sample were entered and a dose-response curve was
generated by using the nonlinear sigmoidal dose-response parameter. The median effective concentration (EC$_{50}$) values were calculated from these generated curves. The statistical significance of the different curves then was generated using an F test, which is appropriate for testing the goodness-of-fit of 2 models. This is assessed by the sum of squares, adjusting for difference in the number of degrees of freedom. For Figures in which the $P$ value is discussed, the siIRR data set contains 23 values, whereas the siUSP18 data set contains 22 values. The resulting $P$ value was less than the lower limit ($P < .0001$).

**Immunoblot Analysis**

At various times after virion exposure, Huh-7.5 cells were detached, washed once in PBS, then resuspended in 1× sodium dodecyl sulfate lysis buffer (50 mmol/L Tris pH 6.8, 2% sodium dodecyl sulfate, .1% bromphenol blue dye, 10% glycerol, and 100 mmol/L β mercaptoethanol) and heated to 95°C for 5 minutes. Lysates were passed through a 20G needle 10 times. Lysates prepared from 100,000 cells were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). Blots then were probed with the indicated antibodies. Antibodies used in this study include monoclonal anti-ISG15 antibodies (the kind gift of Dr. E. Borden, Scripps Institute, La Jolla, CA), monoclonal anti-phospho-STAT1 (Tyr701) (9172; Cell Signaling, Danvers, MA), and monoclonal anti-STAT1 (9172; Cell Signaling).

**Results**

**USP18 Silencing Enhances the Antiviral Activity of IFN-Alfa in an Infectious HCV Cell Culture System**
We have shown previously that both viral (HCV) and cellular (lamin A/C and CD81) RNAs can be silenced efficiently in Huh-7.5 cells with siRNAs. By using these conditions, we tested the role of USP18 in IFN-alfa signaling and antiviral function in an in vitro HCV cell culture system. siRNAs were first introduced to establish silencing, after which IFN-alfa was added and the effects of IFN-alfa treatment were determined. USP18 expression was highly induced by IFN in Huh-7.5 cells treated with irrelevant siRNAs. This induction was dose dependent and is consistent with USP18 being an IFN-stimulated gene. A pool of 4 USP18-specific siRNAs (siUSP18) abrogated this effect, resulting in 75%–90% silencing over all doses of IFN-alfa (Figure 4-1-A). Each individual USP18 siRNA, targeting a distinct sequence, was effective at reducing USP18 RNA levels (Figure 4-1-B).
Inhibition of USP18 expression by USP18 siRNAs. (A) Huh-7.5 cells were transfected with siIRR (□) or USP18-specific (■, siUSP18) siRNAs, maintained for 30 hours, and then treated with the indicated IU of IFN/mL for 24 hours. RNA was purified and quantified by real-time PCR. USP18 RNAs were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) RNA levels and the fold induction of USP18 RNA in response to IFN is represented. Data are the mean ± SD for duplicate samples and are representative of 3 independent experiments. (B) Huh-7.5 cells were treated with siIRR, or 4 individual USP18 siRNAs (siUSP18-a–d) for 45 hours. RNA was purified and USP18 and GAPDH RNAs were quantified. Data were normalized for GAPDH RNA levels and are represented as a ratio of USP18/GAPDH relative to siIRR-treated cells. Data are the mean ± SD of duplicate samples.
We then tested the effects of IFN-alfa with or without USP18 silencing on HCV replication and infectious virus production in vitro. Huh-7.5 cells were transfected with an irrelevant siRNA, or USP18 siRNAs (pooled and specific), and treated with IFN-alfa. After IFN-alfa treatment, cells were infected with J6-JFH HCV and maintained for 2 days. Infectious virus particles were quantified from cell supernatants (Figure 4-1-2B), while intracellular RNA was extracted and HCV-RNA levels were determined (Figure 4-1-2A). In irrelevant siRNA-treated cells, HCV replication was inhibited at an EC$_{50}$ concentration of 1.6 IU/mL IFN-alfa. This was comparable with the EC$_{50}$ in cells that were not treated with siRNAs (.9 IU/mL IFN-alfa, data not shown). In cells treated with USP18 siRNAs, HCV replication was inhibited at significantly lower IFN-alfa conditions. The EC$_{50}$ concentration in USP18 siRNAs was .1 IU/mL IFN-alfa (a 1.2-log$_{10}$ enhancement of IFN-alfa anti-HCV activity). This change in EC$_{50}$ was significant by F test ($P$ < .0001).
Figure 4-1-2. USP18 silencing augments the antiviral effects of IFN against HCV infection. Huh-7.5 cells were transfected with siIRR (■) or siUSP18 (▲), maintained for 30 hours, and treated with the indicated concentrations of IU IFN/mL. After 15 hours, the cells were infected with HCV for 8 hours, then washed and maintained in media lacking IFN for 2 days, and HCV replication was measured. (A) Intracellular RNAs were purified and GAPDH and HCV RNAs were quantified by real-time PCR. (B) Infectious HCV in the supernatants of cells from A was quantified by limiting dilution assay and the 50% infectious dose/mL was calculated. The percentage of inhibition in response to IFN relative to untreated cells is shown. Data are the mean ± SD of (A) quadruplicate and (B) triplicate samples. The data are representative of 4 independent experiments.
The dramatically enhanced IFN-alfa inhibition of HCV replication in USP18-silenced cells correlated with decreased production of infectious HCV particles. Infectious virus particles in cell supernatants were quantified as described in the Materials and Methods section. Viral titers ranged from 20,000 50% infectious doses/mL in untreated cells, to undetectable viral titers in cells treated with high levels of IFN. USP18-silenced cells consistently showed enhanced antiviral activity of IFN-alfa, with a 1.3-log\textsubscript{10} enhancement in IFN-alfa anti-HCV activity (Figure 4-1-2B). The effects of IFN-alfa on the production of infectious HCV were similar to, if not slightly greater than, the effects on HCV-RNA replication. We confirmed that this effect was specific to USP18 by testing 4 different USP18 siRNAs. All USP18 siRNAs tested consistently augment the antiviral activity of IFN-alfa (Figure 4-1-3). Despite minor variations in the basal sensitivity to IFN, each USP18 siRNA had the effect of shifting the IFN dose response and EC\textsubscript{50} inhibition of HCV replication. The fact that all USP18 siRNAs show similar phenotypes strongly argues that the observed enhancement of IFN-alfa antiviral activity is specific to USP18.
Figure 4-1-3. Multiple USP18 siRNAs enhance the antiviral effects of IFN against HCV. Cells were treated with siIRR (■), or 4 individual USP18 siRNAs ( △, ▼, ●, ● siUSP18-a–d, respectively) for 30 hours, then with indicated IU IFN/mL for 15 hours. Cells were rinsed, inoculated with HCV for 8 hours, rinsed again, and maintained for 2 days in media lacking IFN, and HCV replication was measured. (A) HCV RNA was quantified by real-time PCR and normalized to GAPDH levels. (B) Infectious HCV was quantified by limiting dilution assay and the 50% infectious dose/mL was calculated. The percentage of inhibition relative to untreated cells then was calculated for each. Data are the mean ± SD of (A) quadruplicate and (B) duplicate samples and are representative of 2 independent experiments.
IFN-alfa is a cytokine that can establish an antiviral state in both naive and virally infected cells. The previous experiments tested the effect of treating naive cells with USP18 siRNAs and IFN before infection. We next examined whether USP18 siRNAs altered the antiviral activity of IFN in cells that already are infected with HCV. Huh-7.5 cells were infected with HCV at a multiplicity of infection of 2, then transfected with USP18 siRNAs, followed by treatment with IFN-alfa. Viral replication then was quantified as described previously (Figure 4-1-4). IFN-alfa inhibited ongoing HCV replication in irrelevant siRNA-treated cells (EC$_{50}$ = .06 IU/mL IFN-alfa). The antiviral activity was enhanced approximately 30-fold in the presence of USP18 siRNAs (EC$_{50}$ = .002 IU/mL IFN-alfa). Thus, USP18 siRNAs enhance the antiviral activity of IFN-alfa cells that are infected before treatment, in addition to priming the antiviral state in uninfected cells.
Figure 4-1-4. USP18 silencing augments the antiviral effects of IFN in cells previously infected with HCV. Huh-7.5 cells were infected with HCV at a multiplicity of infection of 2 for 6 hours. Infected cells then were transfected with siIRR (■) or siUSP18 (▲), maintained for 18 hours, and treated with the indicated concentrations of IU IFN/mL. After 24 hours, the cells were washed and maintained in media lacking IFN for 2 days, and HCV replication was measured. Intracellular RNAs were purified and GAPDH and HCV RNAs were quantified by real-time PCR. The percentage of inhibition in response to IFN relative to untreated cells is shown. Data are the mean ± SD of triplicate samples. The data are representative of 2 independent experiments.
Efficient Silencing of USP18 in IFN-Alfa–Treated Huh-7.5 Cells Enhances Jak1-STAT1 Signaling and ISG Expression

We next investigated the mechanism of enhanced IFN antiviral activity in USP18 silenced human hepatoma cells. Because USP18 is an ISG15-specific protease, decreasing USP18 expression should result in increased ISGylation of cellular proteins. In irrelevant siRNA-treated cells, free ISG15 increased in response to IFN-alfa treatment, and ISG15–protein conjugates accumulated in cells treated with high doses of IFN-alfa (Figure 4-1-5A). By contrast, USP18-silenced cells had abundant ISG15-protein conjugates that accumulated at lower concentrations of IFN-alfa, and increased unconjugated ISG15. This phenotype is similar to that described for mouse cells with a USP18 knockout, and is consistent with decreased USP18 ubiquitin protease activity and increased ISG15 expression. The low levels of ISG15-protein conjugates in irrelevant siRNA-treated cells and their enhanced accumulation in USP18 siRNA-treated cells confirms that the ISGylation pathway is functional in Huh-7.5 cells and suggests that USP18 negatively regulates protein ISGylation.
Figure 4-1-5. USP18 silencing enhances protein ISGylation and ISG15 induction by IFN. Huh-7.5 cells were treated as before with either siIRR or siUSP18, then with the indicated IU of IFN/mL. After 24 hours of IFN treatment, (A) protein lysates were collected, separated, and probed for ISG15 expression, or (B) RNA was purified and ISG15 and actin RNAs were quantified. RNA levels were normalized and presented as in Figure 4-1-1; the data are representative of 3 independent experiments. (B) □, siIRR; ■, siUSP18.
The increased ISG15 protein accumulation in USP18-silenced cells may reflect a general enhancement in ISG expression. This is supported by the observation that USP18 knockout mice are hypersensitive to IFN-alfa.\textsuperscript{11,24} As shown in Figure 4-1-5\textit{B}, USP18 knockdown resulted in a substantial (3- to 5-fold) increase in the IFN-alfa induction of ISG15 transcription as determined by real-time PCR. This effect was not limited to ISG15. IFN-alfa induction of 4 other ISGs—2′-5′-oligoadenylate synthetase 3, IFN-induced protein with tetratricopeptide repeats 1, Viperin, and myxovirus resistance 1—also was enhanced after silencing of USP18 (Figure 4-1-6). These genes were examined because (1) they are ISGs and (2) we previously showed that their expression is increased in the liver tissue of patients with chronic HCV.\textsuperscript{4} These data are consistent with a general enhancement of IFN-alfa–stimulated gene expression in USP18-silenced cells.
Figure 4-1-6. General enhancement of ISG induction in USP18-silenced cells. Huh-7.5 cells were treated with either siIRR (□) or siUSP18 (■) for 30 hours, followed by the indicated IU of IFN/mL for 24 hours. RNA was purified and quantified by real-time PCR: (A) 2′-5′-oligoadenylate synthetase 3, (B) interferon-induced protein with tetratricopeptide repeats 1, (C) Viperin, (D) myxovirus resistance 1. RNA levels were normalized to β-actin RNA levels and expressed as in Figure 4-1-1; the data are representative of 2 independent experiments.
The enhanced IFN-alfa–stimulated gene expression suggests that regulation of IFN-alfa signaling has been altered in USP18-silenced cells. We next examined the activation of a critical component of IFN-alfa signaling, STAT1. On engagement of the IFN-alfa–receptor subunits IFNAR1 and IFNAR2, the receptor-bound proteins Tyk2 and Jak1 are phosphorylated. This in turn leads to phosphorylation of STAT1 and STAT2 and their assembly with interferon regulatory factor (IRF)9 into the transcriptional activation complex ISGF3, leading to activation of ISGs. We examined the effects of USP18 silencing on the activation of this pathway, and specifically on STAT1 phosphorylation. In cells transfected with siIRR, STAT1 phosphorylation is induced within 1 hour of IFN treatment and returns to basal levels by 4 hours. In USP18-silenced cells, STAT1 is phosphorylated within 1 hour of IFN-alfa treatment; however, phosphorylated STAT1 remains 15 hours after treatment. Total STAT1 increases after IFN-alfa treatment (Figure 4-1-7), as would be expected for an ISG. These results indicate that IFN signaling is initiated normally in USP18-silenced cells, but that there is a defect in the subsequent negative regulation of IFN signaling. As such, it implicates a role for USP18 in the negative feedback of IFN signaling, with an outcome of limiting the anti-HCV activity of IFN-alfa in human hepatoma cells.
Figure 4-1-7. USP18 silencing prolongs STAT1 activation after IFN treatment. Huh-7.5 cells were transfected with siIRR or siUSP18, maintained for 24 hours, and treated with 100 IU of IFN/mL. Protein lysates were harvested at the indicated time, separated electrophoretically, and probed for STAT1-phospho701. Blots then were stripped and probed for total STAT1 expression.
Discussion

In a recent study, we found that expression of USP18 was disordered in the pretreatment liver tissue of patients with chronic HCV who do not respond to subsequent treatment with pegylated IFN-2-alfa and ribavirin. Specifically, increased USP18 expression correlated with lack of response to combination therapy. In the present study, we show that USP18 plays an important role in the anti-HCV–IFN response using an in vitro model of HCV replication that reproduces the full viral life cycle. The silencing of USP18 altered the cellular response to IFN-alfa, resulting in more protein ISGylation, prolonged STAT1 activation, and increased expression of ISGs. The increased biochemical effect of INF-alfa was mirrored by a potentiated inhibition of HCV RNA and infectious particle production in vitro. The congruence between our in vivo and in vitro results suggests that USP18 plays an important role in HCV pathophysiology.

USP18 may have a broader role in human disease. Increased ISG15 and/or USP18 expression correlates with chronic HCV infection and with the clearance of HBV infection in vivo. In animal models, USP18 knockout mice are resistant to otherwise fatal intracerebral infection by lymphocytic choriomeningitis virus and vesicular stomatitis virus. This effect was associated with decreased viral replication and increased cellular ISGylation by ISG15. However, USP18 may not modulate the activity of IFN against all viral infections. In preliminary studies, USP18 silencing in Hela cells led to increased protein ISGylation after treatment with IFN-alfa, but did not alter measles virus replication (Dr. C. Richardson, unpublished observations).

The mechanism by which USP18 modulates the antiviral activity of IFN remains unclear. Decreased USP18 expression results in IFN hypersensitivity, and resistance to viral infection in human cells in this study and in knockout mice. Although this correlates with an increase in
ISG15 protein conjugates, this phenotype is apparently ISG15 independent. Mice that lack ISG15 have an intact IFN system and respond normally to viral challenge.\textsuperscript{30} Similarly, in preliminary results we have found that siRNAs targeting ISG15 did not alter the anti-HCV activity of IFN (data not shown). Quite recently, 2 lines of double knockout mice have been examined: USP18 and ISG15\textsuperscript{−/−} and USP18 and Ube1L\textsuperscript{−/−}. Both mice had similar IFN and antiviral sensitivity as the parental USP18\textsuperscript{−/−} mice. This suggests that the function of USP18 in regulating the sensitivity to IFN is ISG15 independent.

In this study we have shown that STAT1 activation is altered in USP18-silenced cells, producing a phenotype similar to USP18\textsuperscript{−/−} mice. Specifically, STAT1 appears to be activated (phosphorylated) normally in USP18-silenced cells, but remains active for extended periods of time compared with normal Huh-7.5 cells. This suggests a defect in the negative regulation of IFN signaling. Because USP18 is itself induced by IFN-alfa, it appears to be a critical regulator in a classic negative feedback loop. USP18 would appear to regulate a process upstream of STAT1 phosphorylation, such as IFN-alfa–receptor turnover or the interactions between the receptor and signaling molecules. Prolonged STAT1 activation is associated with a general increase in IFN-stimulated gene expression in USP18-silenced cells. The specific anti-HCV effectors modulated by USP18 remain to be identified. We have shown that the IFN induction of at least 5 ISGs is enhanced by USP18 silencing. In this light, it is interesting to note that one of these, IFN-induced protein with tetratricopeptide repeats 1, has been shown previously to play a role in inhibiting HCV replication in a replicon model.\textsuperscript{31}

We believe that the question of how USP18 contributes to IFN-alfa antiviral activity in human HCV is a critical one. Not only is IFN-alfa the major component of antiviral therapy for acute
and chronic HCV infection, but nonresponse to IFN-alfa is an important and unresolved issue in HCV treatment. IFN-alfa has been shown to target multiple stages of viral life cycles, notably translation, but also viral entry and capsid assembly. The HCV infectious cell culture system used in this study recapitulates the entire viral life cycle, and therefore provides the most advanced system for studying the antiviral activity of IFN-alfa against HCV. USP18 silencing in this system produced a robust and consistent enhancement of the ability of IFN-alfa to inhibit HCV RNA and infectious virus production.

In conclusion, this study extends our observations that USP18 is a predictor of successful IFN-alfa therapy by showing that USP18 plays a critical role in the antiviral activity of IFN-alfa against HCV infection in vitro. Currently one of the chief limitations of HCV treatment is the fact that a large fraction of compliant patients do not respond to exogenous therapy in a sustained manner. Enhancing the antiviral activity of IFN by modulation of USP18 may represent a strategy for improving responses to HCV treatment.

Acknowledgement:

The authors thank Victoria Kramer for technical assistance, and Dr. David Grant, University Health Network, for critical insight with the manuscript. The authors also acknowledge the work of Grant Welstead in Dr. Chris Richardson’s laboratory, University of Toronto, for assistance with our preliminary measles virus studies.
References:


Chapter 4-2: The role of ISG15 in HCV production

ISG15 has been recognized as an anti-viral protein. My gene expression study indicated that elevated pretreatment ISG15 expression in the liver is associated with treatment non-response even though ISG15 would be predicted to have anti-HCV activity.

In order to clarify the role of ISG15 in HCV infection, I modulated ISG15 expression and then tested the effect of these modulations on HCV RNA replication and viral particle production using the in vitro HCV culture model.


My role in this study: experimental design, performing experiments, summarizing/analyzing the data and writing up the paper

Reprinted from *J Gen Virol.* 2010 Feb;91(Pt 2):382-8
ISG15, a ubiquitin-like interferon stimulated gene, promotes Hepatitis C Virus production in vitro: Implications for chronic infection and response to treatment

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Keywords: Interferon stimulated gene 15Kda (ISG15), Hepatitis C virus (HCV), ISG15 conjugation (ISGylation), Ubiquitin-E1 like (Ube1L) activating enzyme, siRNA

Running title: ISG15 stimulates HCV production

Grant support: This work was funded by grants from the Canadian Institute of Health Research (No. 62488 to I.D.M). L.C. was supported by the National Canadian Research Training Program in Hepatitis C (NCRTP-HepC) and Canada Graduate Scholarship (CGS) from the Canadian Institute of Health Research.

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Abbreviations:

Ube1L: ubiquitin E1 like
HCV: Hepatitis C virus
ISG15: interferon stimulated gene 15
pegIFN/rib: pegylated interferon/ribavirin
MOI: multiplicity of infection
SUMMARY

**Background & Aims:** Up-regulation of interferon stimulated genes (ISGs), including interferon stimulated gene 15 (ISG15) and other members of the ISG15 pathway, in pre-treatment liver tissue of Hepatitis C virus (HCV) chronically-infected patients is associated with subsequent treatment failure (pegylated interferon-α/ribavirin, pegIFN/rib). Here, we study the effect of ISG15 on HCV production in vitro. **Methods:** The levels of ISG15 and of its conjugation to target proteins (ISGylation) were increased by plasmid transfection, or ISGylation was inhibited by siRNA directed against the E1 activating enzyme Ube1L in Huh7.5 cells. Cells were infected with HCV J6/JFH1 virus, and HCV RNA and viral titers determined. **Results:** Levels of both HCV RNA and virus increased when levels of ISG15 and ISGylation were increased, and decreased when ISGylation was inhibited. The effects of ISGylation on HCV are independent of upstream IFN signaling: IFNα-induced ISG expression is not altered by Ube1L knockdown. The effect is also not likely secondary to a cytokine effect: treatment of cells with purified ISG15 does not inhibit HCV production. **Conclusions:** Although ISG15 has antiviral activity against most viruses, ISG15 promotes HCV production. HCV might exploit ISG15 as a host immune evasion mechanism, and this may in part explain how increased expression of ISGs, especially ISG15, correlates with subsequent interferon-based treatment failure.
INTRODUCTION

HCV is adept at evading host antiviral mechanisms and is often resistant to the current standard of care - combination treatment with PegIFN and Ribavirin. This regimen eradicates the virus in only 50% of cases. A number of mechanisms contribute to evasion and treatment resistance, including cleavage of the RIG-I adaptor protein IPS1/MAVS/cardif by the HCV NS3/NS4A protease and modulation of the host response by the HCV core protein (Li, et al., 2005; Loo, et al. 2006). However, none of these mechanisms have been consistently demonstrated to play a role in the clinical disease and thus cannot explain the ability of the virus to escape the host response in patients.

Response to treatment can be predicted by levels of expression of interferon stimulated genes (ISGs) in the liver prior to initiation of PegIFN/Rib treatment. Non-responders have increased expression of a number of ISGs (Chen, et al.,2005; Feld, et al., 2007; Asselah, et al., 2008; Asahina, et al., 2008; Sarasin-Filipowicz, et al., 2008). Three of these ISGs are components of the ISG15 ubiquitin-like pathway. ISG15 was the first ubiquitin-like protein to be described and, like its homologue ubiquitin, is conjugated to proteins in a tightly regulated process called ISGylation. The ISG15 E1-activating protein, Ube1L, coordinates with the E2-conjugating enzyme (UbcH8) and the E3-ligase (CEB1) to join the C-terminus of ISG15 to a wide variety of proteins (Loeb, et al., 1992). ISG15 can be removed from its target proteins by USP18, an ISG15 protease (Malakhov, et al. 2002). ISG15, CEB1, and USP18 are upregulated in the liver tissue of patients infected with HCV who then did not respond to treatment with PegIFN/Rib (Chen, et al. 2005). A functional association between this pathway and the regulation of HCV production has been established: knockdown of USP18 increases the anti-HCV potency of
IFNα (Randall, et al., 2006), and ISG15 protein expression is highly upregulated in the hepatocytes of treatment nonresponders, but is increased in the macrophages of treatment responders. The ISG15 pathway is likely important to clinical HCV disease and to determining treatment outcomes.

ISGylation is implicated in different cellular processes, but its role in viral biology is the one best established (Dao, et al., 2005). ISG15 is targeted by a number of viruses in animal model and cell culture systems. For example, non-structural protein 1B of Influenza B virus binds to the free form of ISG15, preventing ISGylation (Yuan, et al., 2001). Over-expression of ISG15 in IFN-α/β-R knockout mice protects them from Sindbis virus-induced lethality and decreases Sindbis virus replication in multiple organs (Lenschow, et al., 2005). ISG15/- mice are more susceptible to influenza A and B, HSV1, Sindbis virus, and murine gammaherpesvirus68 infection; for Sindbis virus this effect is dependent on ISGylation (Lenschow, et al., 2007). While these studies suggest a general role for ISG15 as an antiviral agent, a recent report found that ISG15 can inhibit IFN responses after infection by NDV virus (Kim, et al., 2008a ). ISGylation of the antiviral RIG-I enzyme inhibited IFN signaling in MEF cells (Kim, et al., 2008b ). Thus, ISG15 inhibits viral production for many viruses, but it may promote production for some.

In this study we examined the role of ISG15 and ISGylation in HCV production in vitro, using the J6/JFH HCV infectious model. Unexpectedly, increasing the level of ISG15/ISGylation promoted HCV production, and blocking ISGylation decreased both HCV RNA and viral titers. This work therefore suggests a new context for the host ISG response to HCV: some aspects of the host ISG response to HCV foster viral production, rather than inhibit it.
MATERIAL and METHODS

Cells and HCV FL-J6/JFH Virus

Huh7.5 cells and HCV FL-J6/JFH were kindly provided by Dr. Charles Rice (Rockefeller University) (Lindenbach, et al., 2005). Cells were maintained in Dulbecco’s modified Eagle media (DMEM) supplemented with nonessential amino acids/10% fetal bovine serum/100 u/ml ampicillin/100 μg/ml streptomycin at 37°C in a 5% CO₂ humidified incubator.

ISG15 expression plasmid

Human full-length ISG15 was generated using pOTB7-ISG15 plasmid DNA (MGC clones, Open Biosystems) as template, and the resulting PCR product was cloned into pcDNA4/HisMax TOPO TA expression vector (Invitrogen). Primers used were: forward 5’ ATGGGCTGGGACCTGACGGTG 3’; reverse 5’TTAGCTCCGCCCAGGCTC 3’. Plasmid DNA for transfection studies was prepared using Plasmid Maxiprep Kit (Qiagen).

ISG15 Transfection and detection of ISG15 expression by western blot

8μg of ISG15 plasmid DNA or blank vector was transfected into Huh7.5 cells (2.5x10⁵/ml, 5ml per 6 cm culture dish) with Lipofectamine 2000 (Invitrogen) following kit protocol. 48 hours later the transfected cells were harvested, washed (PBS), and lysed in 200ul lysis buffer (50mM HEPES pH7.8, 500mM NaCl, 1% Triton x-100,1mM EDTA, protease inhibitor cocktail (Sigma)). Proteins were separated by NuPAGE 4-12% Bis-Tris Gel and transferred onto nitrocellulose membrane by Trans-Blot SD Semi-dry transfer cell (Bio-Rad). ISG15 expression was assessed by using a polyclonal anti-ISG15 antiserum raised in rabbits against purified human ISG15 protein – (Cedarlane). Blots were developed with OptiBlaze WESTfemtoLUCENT kit (G Biosciences).
For studies of the effect of ISG15/ISGylation on HCV production, 0.3μg of ISG15 plasmid DNA or blank vector was transfected into Huh7.5 cells in each well of the 96-well plate for 48 hours, following which the cells were treated in the absence or presence of IFNα (0-1 U/ml) for 16 hours followed by infection with HCV FL-J6/JFH virus (MOI=0.3) for 6 hours rinsed, and overlaid with fresh media. Two days post infection, cells were harvested for assessment of HCV replication (RNA and infectious particles, see below).

**Ube1L SiRNA knock down**

Four Ube1L small inhibitory RNAs (siRNAs) were designed as follows (the sense strand sequence is described): siUbe1L#1, 5’-CAUCUUUGCUAGUAUCUA; siUbe1L#2, 5’-CGAAUUGUGGGCCAGAUUA; siUbe1L#3, 5’- AUAGAGCGCUCCAAUCUCA; and siUbe1L#4, 5’-GCAUGGAGUUUGCUUUCUG. The irrelevant siRNA (siIrr) is 5’-GGCGCUUGUGGACAUUCUGTT. Chemically synthesized RNA oligos were prepared as recommended by the manufacturer (Dharmacon, Lafayette, CO). One nanomole of RNA duplexes was electroporated into 2.5 × 10^6 Huh-7.5 cells as previously described. Thirty hours after electroporation, cells were treated with IFN-α (0–1 U/ml) for 15 hours, then either harvested for determining siRNA knock down efficiency (Ube1L mRNA by real-time PCR) or washed, infected with HCV FL-J6/JFH (MOI=0.3) for 6 hours, rinsed, and overlaid with fresh media. 48 hours post infection, cells were harvested for assessment of HCV production (RNA and infectious particles). Another similar experiment was performed using higher dosages of IFN-α (0-1000 U/ml) to investigate whether silencing Ube1L and/or decreased ISGylation has any effect on IFN down-stream ISG mRNA expression (real time PCR).

**Quantification of Infectious HCV virion and HCV RNA**
Viral titers were determined by limiting dilution analysis of culture supernatants as previously described. For HCV RNA quantification, total cellular RNA was harvested and purified with 96-well RNA-easy columns (Qiagen, Mississauga, ON, Canada), reverse transcribed (Superscript II, Invitrogen), cDNA was constructed (AnCT primer, Invitrogen), and real-time PCR performed using Sybr Green mix and either the primers listed in Table 4-2-1 (Supplementary data) or HCV-specific primers 5'-TGA GTG TCG TAC AGC CTC CA and 5'-ACG CTA CTC GGC TAG CAG TC (Platinum Quantitative reverse-transcription PCR Thermoscript One-step System, Invitrogen, Life Technologies) as described previously (Randall, et al., 2006).

Statistics:
Where appropriate, Student’s t test was used to compare 2 categorical values and one-way ANOVA was used to compare more than 2 categorical values. For western blot studies the presented work was repeated at least three times.
RESULTS:

Increasing and decreasing ISGylation in Huh7.5 cells:

In order to test whether ISG15 conjugation plays a role in HCV replication/production, we developed ways of increasing and decreasing ISG15 conjugation (ISGylation). Inducing ISGylation can be difficult in certain cells: for example, in Hela cells ISGylation could only be induced by overexpression of ISG15 in combination with its E1 activating enzyme Ube1L and its E2 conjugating enzyme UbcH8 (Zhao, et al. 2005). However, in Huh7.5 cells over-expression of ISG15 alone led to pronounced protein ISGylation in Huh7.5 cells (Figure 4-2-1a). Combining over-expression of ISG15 with over-expression of Ube1L and/or UbcH8 did not appreciably increase protein ISGylation beyond that observed with over-expression of ISG15 alone (data not shown). In order to inhibit ISGylation, the ISG15 E1 Ube1L enzyme was knocked down with siRNA. Ube1L mRNA was successfully knocked down even in the presence of increasing concentrations of IFNα (0-1 U/ml) (Figure 4-2-2). This maneuver abolished ISGylation even in the presence of high levels of IFNα (100 IU/ml) (Figure 4-2-1b). Thus, in Huh7.5 cells ISGylation can be increased and decreased relatively easily.
Fig. 4-2-1. Modulation of ISGylation in Huh7.5 cells

(a) Western blot for ISG15 after transfection of Huh7.5 cells with ISG15 or empty vector. U, Untreated; V, empty vector. The presence or absence of IFN-α (100 U ml⁻¹, 24 h) is indicated.

(b) Comparison of protein ISGylation (Western blot for ISG15) in the presence or absence of IFN-α (100 U ml⁻¹, 24 h) following electroporation of irrelevant siRNA (siIrr) or siRNA specific to Ube1L (siUbe1L). Molecular mass markers are shown on the left (kDa).
Fig. 4-2.2. Ube1L mRNA expression was silenced by siRNA.

Fig. 4-2.2. Ube1L mRNA expression was silenced by siRNA. Huh7.5 cells were electroporated with irrelevant siRNA (shaded bars) or siRNA specific to Ube1L (filled bars). Thirty hours after electroporation, cells were treated with increasing amounts of IFN-α for 15 h before being harvested to determine the levels of Ube1L mRNA by real-time PCR (normalized to β-actin). Data represent the means±SD of three replicates; the results shown are representative of three similar experiments. *P<0.05 (versus irrelevant siRNA).
HCV RNA and virus are increased in parallel with ISGylation:

We next asked whether ISGylation (and ISG15) modulates HCV production. As seen in Figure 4-2-3, increasing ISGylation by over-expression of ISG15 significantly increased the production of both HCV RNA and virus, even in the presence of increasing IFNα, suggesting that increased ISG15/Isgylation blunts IFNα anti-HCV activity (Figure 4-2-4) in J6/JFH HCV in vitro culture system. By contrast, silencing of the ISG15 Ube1L E1 enzyme decreased levels of HCV RNA and virus both at baseline (in the absence of IFNα) and in the presence of IFNα, an effect that was more pronounced for HCV viral titers (Figure 4-2-5). To ensure that the siRNA were selective, we tested the effects of four individual Ube1L siRNAs and compared these to the effects of pooled Ube1L siRNA. As shown in Figure 4-2-6, all four individual siRNAs had a similar inhibitory effect on HCV viral particle secretion when compared to the pooled siRNA. These data argue that the effect we observed is specific to the knockdown of Ube1L. Taken together, these data suggest that ISGylation is important for baseline HCV production.
Figure 4-2-3. ISG15 promotes HCV production in vitro

Fig. 4-2-3. ISG15 promotes HCV production *in vitro*. Huh7.5 cells were transfected with empty vector (V) or ISG15 plasmid DNA for 48 h before the cells were infected with FL-J6/JFH as described in Methods. HCV RNA was quantified by real-time PCR (a), and HCV virus particle titres were assessed by serial dilution of culture supernatants (b). Data represent the means±SD of three replicates; the results shown are representative of three similar experiments. U, Untreated control. ***P<0.001; *P<0.05 (versus empty vector).
Figure 4-2-4. Increased ISG15/ISGylation decreases IFNα anti-HCV activity

Fig. 4-2-4. Increased ISG15/ISGylation decreases IFN-α anti-HCV activity. Huh7.5 cells were transfected with empty vector (shaded bars) or ISG15 plasmid DNA (filled bars) for 48 h before the cells were treated with increasing amounts of IFN-α, as indicated, for 16 h followed by infection with FL-J6/JFH as described in Methods. HCV RNA was quantified by real-time PCR (a), and HCV virus particle titres were assessed by serial dilution of culture supernatants (b). Data represent the means±SD of three replicates; the results shown are representative of three similar experiments. *P<0.05 (versus empty vector).
Figure 4-2-5. Silencing Ube1L inhibits HCV production.

Fig. 4-2-5. Silencing Ube1L inhibits HCV production. Huh7.5 cells were electroporated with irrelevant siRNA (shaded bars) or Ube1L siRNA (filled bars) and the cells were treated with different concentrations of IFN-α as indicated. HCV RNA (a) and the number of infectious particles (b) were determined as described in Methods. Data represent the means±SD of three replicates; the results shown are representative of three similar experiments. *P<0.05 (versus silrr-transfected cells).
Figure 4-2-6. Comparison of individual vs pooled Ube1L siRNA

Fig. 4-2-6. Comparison of individual versus pooled Ube1L siRNAs. siRNA was electroporated into Huh7.5 cells, after which the cells were infected with FL-J6/JFH as described in Methods. Viral particles were titrated 48 h after infection. Data represent the means±SD of three replicates; the results shown are representative of three similar experiments.
Silencing Ube1L does not affect upstream IFNα signaling

Increased ISGylation can prolong STAT1 phosphorylation, suggesting that ISGylation might play an important role in IFN signaling (Malakhova, et al. 2003). To test this hypothesis in the HCV model, we assessed the effect of decreasing ISGylation by Ube1L knockdown on downstream ISG expression in the presence of IFNα. As shown in Figure 4-2-7, the expression of a number of ISG transcripts was not affected following Ube1L SiRNA knock down in the presence of IFNα.
Figure 4-2-7. IFNα-induced ISG expression following Ube1L siRNA knockdown

Huh7.5 cells were electroporated with irrelevant (shaded bars) or Ube1L (filled bars) siRNA, after which the cells were exposed to different concentrations of IFN-α as indicated. Cells were lysed 24 h after the introduction of IFN-α, and ISG expression was quantified using real-time PCR normalized to β-actin expression levels. Data represent the means±SD of three replicates; the results shown are representative of three similar experiments.
DISCUSSION:

ISG15 is one of the most abundant ISGs induced after virus infection and type I IFN treatment, and we and others have found that increased pretreatment ISG15 expression in the livers of HCV-infected patients predicts subsequent treatment failure (Chen, et al., 2005; Feld, et al., 2007; Asahina, et al., 2008; Asselah, et al., 2008; Sarasin-Filipowicz, et al., 2008). Although ISG15 is generally considered antiviral, we now present evidence that ISGylation promotes HCV production, blunts the anti-HCV effect of IFNα, and is particularly relevant for steps downstream of HCV RNA replication. Thus, aspects of the host ISG response favour, rather than inhibit, HCV persistence. ISG15 is a novel mechanism for viral persistence, and ISGylation is a possible target for therapy of HCV infection.

As noted in the Introduction, the effect of ISG15 on viral production may be specific to the virus. For example, ISG15 can be antiviral to Sindbis, influenza, HSV, HIV, and Ebola virus (Lenschow, et al., 2005; Okumura, et al., 2006; Lenschow, et al., 2007; Zhang, et al., 2007), but ISGylation does not contribute to murine susceptibility to LCMV and VSV (Knobeloch, et al., 2005), nor to Hepatitis B virus replication in ISGylation-deficient mice (Ube1L -/-) (Kim, et al., 2008). Although ISG15 may also promote viral production, by acting as a negative regulator of the innate immune response through its conjugating to RIG-I (Kim, et al., 2008), this mechanism is unlikely to contribute to our observed effects as Huh7.5 cells are deficient in RIG-I (Sumpter, et al., 2005). Our data suggest that HCV exploits the ISG15/ISGylation pathway to increase HCV production: over-expression of ISG15, which increases ISGylation in Huh7.5 cells.
(Figure 4-2-1), increased HCV RNA 3-fold and viral titers 2.2-fold (Figure 4-2-3). Blocking ISGylation by knockdown of Ube1L decreased HCV RNA but largely abolished production of infectious virus (Figure 4-2-5).

Another approach to examining the role of ISG15 in HCV production would be to decrease ISG15 mRNA using specific siRNA. We have not employed this method in the current study – in preliminary work we found that ISG15-specific siRNA was not able to consistently decrease ISG15 mRNA in Huh7/7.5 cells, particularly in the presence of IFNα (data not shown). However, others have demonstrated that knockdown of ISG15 in two HCV replicon models (Con1 and murine MH1 cells) resulted in decreased HCV production both with and without IFNα (Broering, et al., 2008). Although this study does not directly address the role of ISGylation, it adds evidence for the permissive role of the ISG15 pathway in the HCV lifecycle.

Our data provide mechanistic insight into how ISG15 affects HCV production. ISG15 exists in three forms: 1) a free, unconjugated intracellular protein, 2) conjugated to viral and/or host target proteins, and 3) an extracellular cytokine (Recht, et al., 1991; D’Cunha, et al., 1996a and 1996b; Lai, et al., 2009). All three forms could potentially affect HCV viral production. In other systems, the free form of ISG15 has been shown to inhibit the release of Ebola virus-like particles by interfering with the activity of Nedd4 (Malakhova, et al. 2008; Okumura, et al., 2008). ISGylation is critical to the effect of ISG15 on Sindbis virus, and ISGylation is targeted by the human influenza NS1 protein (Yuan, et al., 2001; Lenschow, et al., 2007). As a cytokine, purified ISG15 can activate NK and cytotoxic T-cells, stimulate IFN-γ production, and
induce dendritic cell maturation and neutrophil recruitment (Recht, et al., 1991). Our data argue that ISGylation is the predominant mechanism through which ISG15 affects HCV production.

Blocking ISGylation by Ube1L knockdown does not decrease free ISG15 but dramatically reduces HCV viral titers and significantly reduces HCV RNA. In order to test for a direct cytokine role of ISG15 we exposed Huh7.5 cells to high dose purified ISG15 (2μg/ml) for 36 hours before cells were infected with J6/JFH virus (MOI=0.3) as before. Although the dose we used is considerably higher than that used by D’Cunha et al. (100 ng/ml) to define the cytokine effect of ISG15 (D’Cunha et al., 1996b), we were unable to find any inhibition of HCV production, nor did we find any reduction in the ability of IFNα to stimulate ISG expression (data not shown).

The current study demonstrates that increasing ISGylation promotes HCV production and blunts that anti-HCV effect of IFNα. However, previous work from our group demonstrated that decreasing expression of USP18, the ISG15 protease, increases ISGylation yet potentiates IFNα anti-HCV activity (Randall, et al., 2006). These data at first blush are conflicting, but only if one assumes that USP18 and ISG15 work entirely through the same pathway. In fact, USP18 clearly has additional targets beyond ISG15, and manipulating USP18 expression has effects on protein expression that are independent of ISG15. For example, EGF receptor synthesis is regulated by USP18 (Duex, et al., 2009). USP18 has both protease-dependent and –independent functions (Malakhova, et al., 2006). Our preliminary data would support that USP18 has a role in HCV production that is independent of ISG15 and of ISG15 protease activity (Chen, et al., 2008). Taken together, the data from our group suggest that ISGylation is necessary but not sufficient for HCV production.
Blocking ISGylation enhances the anti-HCV effect of IFNα (Figure 4-2-5). This effect is not likely to be mediated at the level of IFN signaling, since Ube1L knockdown did not promote (or inhibit) IFN-dependent ISG expression, which is the readout of activation of the IFN pathway (Figure 4-2-7). Although ISGylation may play an important role in the regulation of the Jak-STAT pathway and IFN signaling in some cells (Malakhov, et al., 2002a and 2002b; Ritchie, et al., 2002), the IFN signaling pathway is intact in ISG15-/− and Ube1L-/− mice (Osiak, et al., 2005; Kim, et al., 2006). In our work, IFN signaling appears to be unaffected despite knockdown of Ube1L, and Ube1L knockdown inhibits HCV production even in the absence of IFNα. This data suggests that ISGylation of viral (or host) proteins is directly important for the viral life cycle. The conjugation of ISG15 to cellular or viral proteins might alter their function, or compete for ubiquitination. For example, in order for HIV virus to be secreted from infected cells the Gag protein must be ubiquitinated and then recruited to the endosomal transport complex. ISG15 conjugates to Gag, prevents its ubiquitination, and thus inhibits HIV release (Okumura, et al., 2006). In another example, ISG15 conjugation to interferon regulatory factor 3 (IRF3), a key signal-transducing factor for IFN-dependent immune responses, protects IRF3 from Ub-mediated degradation (Lu, et al., 2006). Thus, ISGylation of HCV proteins or of host proteins important for the HCV lifecycle may alter their function or protect them from degradation – the specific steps involved in the ISG15 effect remain to be defined.

In summary, our in vitro data strongly argue that ISG15, and specifically ISGylation, is important to the HCV life cycle in an infectious cell culture model of HCV. This study offers one explanation for how increased baseline expression of some ISGs, including ISG15, correlates
with treatment failure in HCV infected patients. Targeting ISGylation – or specific targets of ISGylation - may identify new anti-viral therapies for HCV.
Chen, et al. ISG15 promotes HCV production in vitro

Supplementary data

Table 4-2-1. Real-time RT-PCR primers

<table>
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<th>Gene name</th>
<th>Annotation</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon length (bp)</th>
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<td>USP18</td>
<td>Ubiquitin specific protease 18</td>
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<td>AGCTCATACTGCCCTCCAGA</td>
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<tr>
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</tr>
<tr>
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<td>Myxovirus (influenza virus) resistance 1</td>
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<td>CTGGTGATGAGCTGTAAGT</td>
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<tr>
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<td>Viperin</td>
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</tr>
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</table>
References:


Chapter 5: General Discussion

With over 170 million people chronically infected, HCV infection is a major health burden.\textsuperscript{1,2} The majority of the infected patients cannot clear the virus, leading to chronic infection and often progressing to liver cirrhosis and hepatocellular carcinoma. Current treatment with the combination therapy of pegylated IFNα/Rib clears the virus from at most 50% of all the patients and this therapy is associated with many side-effects.\textsuperscript{1} One goal of my thesis was to predict in advance which patients would benefit from therapy and which could be spared the treatment. Predicting who will respond to treatment before initiation of therapy would benefit patients in several ways. First, it would certainly boost the willingness of the patients to go through the full course of treatment if they are predicted to be responders; compliance is a serious issue. Second, it would avoid causing patients unnecessary side-effects and save the cost of treating those who are predicted to be non-responders. There is no diagnostic tool available in the clinic to predict treatment response in HCV infected patients before treatment starts. Therefore, developing a prognostic tool and studying the molecular mechanism of IFN resistance in those patients who do not respond to IFN therapy are of huge interest in HCV research.

5.1 Identification of an HCV response signature and its validation

Virus-host interactions play an important role in determining response status. Given the fact that different individuals infected with the same genotype of HCV have different outcomes, this indicates that differences in host factors might be involved. My PhD research focused on host factors that determine response status in HCV infected patients.
There are several approaches to study host response to a virus infection. Traditionally, effort would be focused on a few candidate genes that had been identified to be associated with virus infections previously, especially in the immune response, viral replication and apoptosis pathways. While this approach could certainly validate some previous findings, it is not likely to reveal any new gene/pathway that is involved in host response to a given viral infection. I used genome-wide approaches to screen for genes differentially expressed between virally infected and normal control tissues or between treatment responders (R) and non-responders (NR) in the HCV infected livers. Expression levels of more than ten thousands human genes were studied on the same microarray chip simultaneously. This approach, unbiased by any predetermined hypotheses, should reveal the true differences at the mRNA level between groups of samples studied and shed light on new genes/pathways that are likely involved in any particular disease state.

In order to develop a prognostic tool that could be used to predict treatment response to IFN combination therapy in HCV infected patients, I screened for hepatic gene expression differences between treatment responders (R) and non-responders (NR) using a cDNA microarray comprising 19,000 human genes. 31 HCV chronically infected patients whose liver biopsies were obtained prior to the onset of treatment were divided into responders (R, n=16) and non-responders (NR, n=15) based on their after-treatment status. I found a few hundred genes whose expression levels were different in the HCV infected livers compared to normal liver controls, and 18 genes whose levels of expression were consistently and statistically different between R and NR (page 55, Figure 2-1). Based only on the relative expression levels of these 18 genes, 30 out of 31 patients could be classified correctly into their treatment outcome group (page 56, Figure 2-2).
Because this response signature was generated in a retrospective study, I also initiated a prospective validation study by recruiting another 47 HCV chronically infected patients. Based on the expression levels of the 18 signature genes, the positive prediction value (PPV) reached 96%, as derived using 4 different predicting methods (Page 81, table 3-2). This indicates that this HCV response signature can be used to predict treatment response prospectively.

Although microarray technology is an important tool for biological research, it has not yet been employed in a clinical diagnostic setting. The technology has the reputation of being noisy: studies addressing the reproducibility and reliability of microarray data across different laboratories and platforms have often been inconsistent. There is general agreement that the variability inherent to DNA microarray technology is due to the variability in the biology, in the technology platforms and in the statistical treatment of the data.

There are a number of microarray platforms independently developed by industry and academia. Two major types of platforms have been developed: one is based on oligo nucleotides (50-mer or 70-mer) and the other is based on cDNA fragments (about 200 nucleotides). Different protocols are used by different laboratories for RNA preparation and labeling. Some labs use total RNA without amplification while others amplify the mRNA before reverse-transcription to integrate different labels into the generated cDNA (Probes). Different statistical and computational tools are used in the analysis of the microarray results. Indeed, a study by Irizarry et al. on microarray data reproducibility has demonstrated that disagreement observed in some of the studies may also be due to questionable statistical analysis. Biological variability is another factor contributing to the already complex inconsistency in microarray experiments.
Due to these differences it has proven challenging to extract reproducible, biologically meaningful information from different DNA microarray experiments that address the same, or very similar biological questions. In this regard, it is necessary to verify the gene list generated by high-throughput microarray studies using an orthogonal method, such as real-time PCR. As shown in Figure 2-1 (page 55), the differences of the mRNA expression levels of all these 18 genes have been successfully confirmed by real-time PCR.

Since its publication on *Gastroenterology*, this HCV response signature has gained wide acceptance in the HCV field. It has been validated prospectively by our group\(^{15}\) and confirmed by many other independent labs as well.\(^{16,17}\) One of the major characteristics of this response signature is the elevated expression of pretreatment hepatic ISGs in treatment non-responders (NRs). Feld et al.\(^{16}\) used a microarray-based approach to shed light on the mechanisms of action of combination PegIFNa/RBV therapy and they confirmed our previous observation that ISGs are more highly expressed in the pre-treatment liver tissue of non-responders than responders. Asselah et al. recently identified a two-gene signature (IF127 and CXCL9) that can accurately predict treatment response in 79% of patients being treated for their hepatitis C.\(^{17}\) Although the gene identities are different from ours, they all belong to ISGs. Taking all these results together, preactivation of the IFN pathway or other pathways that lead to increased expression of downstream ISGs correlates with NR.

### 5.2 Cell-type specific expression of ISGs underlies treatment response in HCV infected patients
My previous expression experiments using microarrays and real-time PCR were based on total RNA isolated from HCV infected liver, which contains mixtures of RNA from different cell types: hepatocytes, infiltrating lymphocytes, macrophages, and other cell types (Kupffer cells, stellate cells…). Because only 0.5-10% of hepatocytes are infected with virus in chronic HCV infected liver, it is very important to learn where the response signature comes from, in order to get at the underlying mechanism. The simplest hypothesis to explain the HCV response signature based on differential expression was to assume that the signatures all derived from hepatocytes, due to the abundance of this cell type in the liver. To test this hypothesis, I looked for cell-type specific expression using different antibodies in immunohistochemistry against the proteins encoded by the various dysregulated genes (ISG15, MxA, etc). ISG15 and MxA were selected as representative proteins because they are ISGs and the majority of the dysregulated genes in the 18-gene signature set were ISGs. ISG15 was also one of the major players in the ubiquitin-like signaling pathway that I identified was involved in IFN resistance. MxA protein expression had been seen before to be differentially expressed in liver tissues infected with HCV, which served as a control for my studies.

In collaboration with Dr. Maha Guindi at UHN, I used liver biopsies from patients chronically infected with HCV to see if hepatocytes or infiltrating lymphocytes are the major source of ISG-producing cells and to see if there exist distinct expression patterns in ISG-producing cells between treatment responders and non-responders.

Contrary to my previous hypothesis that the HCV response signature was all derived from hepatocytes, responders and non-responders have distinct expression patterns of ISG-producing
cells: hepatocyte staining was predominantly found in non-responders and macrophages were stained preferentially in responders. (Figure 3-4A and 4B, page 92-93)

These IHC data suggest that the HCV response signature we identified using cDNA microarrays was derived from different cell types in the HCV infected livers of treatment responders (R) and non-responders (NR). Considering that the majority of cells in the human livers are hepatocytes, restricted expression of ISG15 and MxA in hepatocytes of NR livers may help explain why there is increased expression of ISGs in the pretreatment NR livers. Similarly, the many fewer macrophages present in the liver also explain lower expression of ISGs in the R livers. Therefore, the differential baseline ISG expression between Rs and NRs revealed using cDNA microarrays is not due to the difference in absolute expression levels, but to the different cell types with different abundance in the liver. This intriguing finding suggests in general that cell-type specific gene expression patterns are under-explored parameters in tissue microarray studies.

Our findings of distinct expression pattern of ISG-producing cells in R and NR have at least three implications:

First, different ISG-expressing cell types in the liver of R and NR may allude to some novel mechanisms of HCV pathology and IFN resistance. For example, because HCV replicates primarily in hepatocytes, increased ISG expression in the hepatocytes of NR livers may stimulate HCV replication and virion production in the hepatocytes, because both ISG15 and USP18 stimulate HCV production in cell culture. As a result, more HCV may already be present in the livers of NRs before initiation of IFN treatment. Increased HCV within the hepatocytes before
treatment coupled with the blunting effect of USP18 and ISG15 on IFN anti-HCV activity following treatment may culminate in rendering the patients nonresponsive to IFN. On the contrary, increased ISG15 and MxA expression in the macrophages from R livers might have little effect on HCV replication because most HCV are present in hepatocytes, not in macrophages in the liver, of patients with chronic HCV infections.

Second, ISG15 and MxA expression in macrophages from R livers indicate that HCV may primarily activate these immune-related cells in Rs. Consequently, a stronger immune response in R might be induced to facilitate virus clearance. Consistent with this hypothesis, I found the expression levels of some immune-related and inflammation-involved genes are increased in the livers of Rs compared to NRs (figure 3-6, Page 107). Taking all these data together, the logical hypothesis for treatment nonresponse is either because the HCV infected hepatocytes developed mechanisms to counteract the action of IFN, or because there is a defect in macrophage activation in NR livers or both.

Third, the distinct expression patterns of ISG-producing cells in R and NR detected by IHC may be used to predict whether a given patient will or will not respond to treatment, without checking the expression levels of the 18 signature genes. This IHC procedure can be integrated easily into the routine assessment of liver biopsies for the degree of fibrosis and inflammation in HCV infected livers.

5.3 ISGs with pro-HCV roles

The innate immune response is the first line of defence against viral infections. It is initiated by the pattern recognition receptor (PRR), which senses viral genomes through the pathogen
associated molecular patterns (PAMPs), such as double-stranded RNA (dsRNA), single-stranded RNA (ssRNA) and unmethylated CpG motifs in DNA, and lead to the production of type I IFNs (IFNα and IFNβ).18-20 Binding to the surface receptor IFNAR1/2 heterodimer, type I IFNs induce the expression of a few hundred interferon stimulate genes (ISGs) through activation of the Jak/STAT signaling pathway.21 Although the functions of most of these ISGs are not known, some of them, such as PKR, MxA, and OAS2, etc, have anti-viral activity.22

While previous studies suggest a general role for ISG15 as an antiviral agent, a recent report found that ISG15 can inhibit IFN responses after infection by NDV virus.23 ISGylation of the antiviral RIG-I enzyme inhibited IFN signaling in MEF cells.23 Thus, ISG15 inhibits viral production for many viruses, but it may promote production for some.

In order to understand the role of ISG15 in HCV infection and viral resistance to IFN therapy, I examined the role of ISG15 and ISGylation in HCV production in vitro, using the J6/JFH HCV infectious model. Unexpectedly, increasing the level of ISG15/ISGylation promoted HCV production, and blocking ISGylation decreased both HCV RNA and viral titers. This work therefore suggests a new context for the host ISG response to HCV: some aspects of the host ISG response to HCV foster viral production, rather than inhibit it. The exact mechanism underlying this is not clear, but at least for ISG15, this does not result from perturbation of the type I IFN signaling pathway because modulation of ISG15 conjugation has little effect on down-stream ISG expression, a readout of type I IFN signaling pathway, as shown by real-time PCR (Figure 4-2-7, page 161).
USP18 also promotes HCV production in a cell culture model. Over-expression of both protease-active and -inactive mutant USP18 enhanced HCV RNA replication within cells and viral particle secretion into culture medium (manuscript in preparation). This also indicates that USP18 promotes HCV production independent of its ISG15 protease-specific activity.

Considering the fact that increased expression of ISG15, USP18 and a few other ISGs in the pretreatment liver tissues is correlated with treatment non-response (my microarray study), this enhanced ISG expression may play at least two roles within hepatocytes: 1) promoting HCV replication and secretion; and 2) blunting subsequent IFN anti-HCV activity. These two aspects may culminate in making patients not respond to the IFN based treatment.

5.4 USP18 and its role in HCV infection and viral resistance to IFN therapy

The specific identities of the ISGs identified in our microarray study suggest a possible mechanism for treatment nonresponse. Three of the genes that are overexpressed in non-responders, interferon stimulated gene 15 (ISG15), ubiquitin specific protease 18 (USP18/UBP43), and CEB1/Herc5 (a HECT domain ISG15 E3 ligase), are linked to a newly defined ubiquitin-like protein (Ubl)/ubiquitin specific protease (ISG15/USP18) pathway.

The ISG15/USP18 pathway is involved in post-translational modification of some proteins, and is related to the ubiquitination pathway. ISG15 is a ubiquitin-like protein that, like ubiquitin, conjugates to its cellular targets through a series of enzymatic steps. Conjugation involves first an E1 activating enzyme (Ube1L), then an E2 conjugating enzyme (UbcH8, UbcH6), and
finally an E3 ligase (EFP, CEB1/Herc5)\textsuperscript{27,28}. The C-terminal LRLRGG sequence of ISG15 is required for conjugation to the lysine residues of target proteins. ISG15 can be stripped from its target proteins by the USP18 isopeptidase.\textsuperscript{29}

The ISG15 conjugation process is reversible and controlled by USP18 (UBP43), an IFN-inducible cysteine protease of the ubiquitin-specific protease (USP) family.\textsuperscript{29} USP18 appears to counteract the effects of interferon; lack of USP18 results in enhanced and prolonged STAT1 phosphorylation, DNA binding, and increased induction of hundreds of ISGs.\textsuperscript{30} By contrast, USP18 knock out mice show greater resistance to the cytopathic effects of a number of viruses including lymphocytic choriomeningitis virus (LCMV), vesicular stomatitis virus (VSV), and Sindbis virus (SNV).\textsuperscript{31} USP18-deficient cells exhibit high levels of ISG15 modified proteins (ISGylation). Furthermore, they are hypersensitive to type I IFN and undergo apoptosis upon IFN stimulation.\textsuperscript{32} Thus, USP18 appears to be a negative regulator of IFN signaling.

Although ISG15 may play a role in the anti-HCV response, the ability of USP18 to regulate the anti-HCV interferon response may be independent of its ability to deconjugate ISG15. Ablation of ISG15\textsuperscript{33} or its E1 activating enzyme Ube1L in mice\textsuperscript{34} did not reverse the phenotype of the USP18 knockout, nor affect IFN-induced JAK/STAT signaling, indicating that neither ISG15 nor ISGylation is essential in JAK/STAT signaling. It was recently reported that USP18 negatively regulates JAK–STAT signaling independently of its isopeptidase activity.\textsuperscript{35} In this study, USP18 action was specific to type I IFN responses and achieved through a direct interaction between USP18 and the IFNAR2 subunit of the type 1 IFN receptor. Binding of exogenous and endogenous USP18 to IFNAR2 \textit{in vivo} interfered with the JAK-receptor
interaction and led to inhibition of the downstream phosphorylation cascade and other signaling events. Whether this is a cell- or species-specific mechanism remains to be determined.

Data from my study clearly show that USP18 modulates IFN anti-HCV activity. Increased pretreatment hepatic expression of USP18 mRNA may inhibit IFN activity against HCV. Indeed, silencing USP18 by specific siRNA potentiated IFN anti-HCV activity in cell culture (figure 4-1-2, page 126), and this effect may be mediated through the enhanced and prolonged activation of the Jak/STAT signaling pathway (figure 4-1-7, page 136), leading to the increased expression of down-stream anti-viral ISGs (figure 4-1-6, page 134). Additionally, over-expression of the USP18 gene in Huh7.5 cells promoted HCV replication/production and blunted IFN anti-HCV activity (manuscript in preparation). Taking these observations together, increased expression of USP18 in the pretreatment NR livers may play two roles that cause the patients to not respond to treatment: 1) it may stimulate HCV production within hepatocytes and, as a result, increase the pre-treatment viral load in livers of subsequent NR; 2) it may blunt IFN anti-HCV activity following IFN treatment.

In order to understand the mechanism by which increased USP18 stimulates HCV production, I over-expressed USP18 in Huh7 cells harboring the HCV replicon, in collaboration with Dr. Chris Richardson (Dalhousie University). We found that USP18 did not affect HCV RNA replication, suggesting that the effect of USP18 on HCV production may be mediated by a step prior to replication, such as viral entry. Interestingly, although I was unable to investigate the effect of USP18 on the expression of the HCV receptor, USP18 can modulate the expression of other cell surface proteins. Knocking-down Usp18 in several cell lines reduced expression levels
of EGFR by 50-80% while overexpression of Usp18 elevated EGFR levels in a manner requiring the catalytic cysteine of Usp18. In their study, analysis of metabolically radiolabeled cells showed that the rate of EGFR protein synthesis was reduced up to 4 fold in the absence of Usp18. Interestingly, this dramatic reduction occurred despite no change in the levels of EGFR mRNA. This suggests that depletion of Usp18 inhibited EGFR mRNA translation. Whether USP18 regulates the expression of HCV entry receptors remains to be determined.
References for chapter 5:


Chapter 6: Future work

Future effort should be directed at better understanding how the ISG15/USP18 signaling pathway modulates IFN anti-HCV activity and its role in virus resistance. It will also be important to explore the basis for cell-type specific gene expression patterns in Rs and NRs. With the new findings of a role for an IL28B SNP on viral clearance (both treatment induced and spontaneous clearance) in HCV infected patients by GWAS, HCV research should be focused on the dysregulated type III IFN pathway and preactivation of IFN signaling in viral resistance.

6.1. Identification of ISG15 and USP18 interacting proteins

Over-expression of ISG15 promoted HCV RNA replication and equally boosted virus particle production, while inhibition of ISG15 conjugation (ISGylation) by silencing its E1 activating enzyme Ube1L caused a more pronounced decrease in viral particle formation than HCV RNA replication. This indicates that ISGylation is important for the HCV life cycle and that ISG15 targets are involved after the replication step. In order to understand the mechanism by which ISG15 modulates IFN anti-HCV activity, it is essential to find out which host or viral proteins are involved in this process. To this end, I generated ISG15 constructs with triple-tags to identify ISG15 target proteins. Although I was unable to continue this work because of time, I would have used tagged ISG15 to identify HCV viral proteins that are linked to ISG15.

USP18 is a specific protease that cleaves ISG15 from its targets. Previous studies demonstrated that USP18 has a protease-independent function: binding to the type I IFN receptor IFNAR2 negatively regulates Jak/STAT signaling. More recently, USP18 has been found to be able to
modulate EGFR protein expression. My previous work also indicates that USP18 promotes HCV production and blunts IFN anti-HCV activity independently of its protease activity. Taken together, USP18 may interact with host or viral proteins to modulate the anti-HCV activity of IFN. USP18 interacting proteins identified from Tap-tagging/Mass spectrometry will shed light on the role of USP18 in IFN resistance in HCV infected patients.

6.2. Understanding the cell-type specific expression of ISG proteins in hepatocytes Vs macrophages:

It is very intriguing that differential gene expression in pre-treatment Rs and NRs derives from different cell types. Immunohistochemical staining of pretreatment liver biopsies from HCV infected livers with ISG15 and MxA antibodies indicated that the characteristic response signature - elevated pretreatment expression of ISGs in the liver tissues, including ISG15 and MxA -in NRs derived from hepatocytes while the reduced signal is came from macrophages in Rs. This finding may change our traditional way of looking at chronic HCV infection. Understanding why macrophage expression of ISGs correlated with R while ISG expression in hepatocytes is associated with NR will help us further understand the role of ISG expression on HCV replication/production and virus resistance to therapy.

6.3. Is there any link between the IL28B SNP and ISG expression in the liver?

Host genetic polymorphisms are an important factor in determining a patient’s response to treatment. In recent studies of patients with chronic HCV infections, SNP analysis was employed to identify variants linked to both disease progression and therapeutic response. Several studies have reported associations between SNPs in the promoter regions of MxA,
OAS-1 and PKR and treatment outcomes. In a recent study of two patient cohorts, Huang et al. demonstrated an important role between a polymorphism of IFN-γ (−764G, located in the proximal I IFN-γ promoter region) and treatment response. Yee et al. reported that two SNP variants of IL10 (−592A and −819T SNP) were more frequently observed among the patients with SVR compared to the non-responders to treatment with IFN/RBV. Although these studies did provide useful information in linking some SNPs to HCV treatment outcomes or disease progression, the sample sizes were quite small, and cross-validation and whole genome scanning is necessary.

Results from 4 different Genome-wide Association Studies (GWAS) indicated that a single-nucleotide change roughly 3kb upstream of the IL28b gene promoter region, which encodes a subtype of type III IFNλ3, is associated with both treatment-induced and spontaneous Hepatitis C Virus clearance. Ge et al scanned the DNA sequences from more than 1600 treatment-naïve HCV G1 infected patients and found that SNP rs12979860 is associated with SVR. The variation at this locus is either C or T. The authors then associated the C allele or T allele with SVR in 3 ethnic populations (European Americans, African Americans, and Hispanics) and found that if a given patient has the C allele, then the patient has a much higher chance of achieving SVR as indicated in all 3 patient ethnic populations. It has been well known that African Americans have a lower rate of SVR than European-Americans, so the authors compared the distribution of C or T alleles in these 2 populations in relation to SVR and found that the percentage of patients with the favourable C/C allele in African Americans is lower than in European Americans, which may partially explain the difference in SVR rates between these 2 groups. It is also interesting to note that African Americans with the favourable C/C allele have a higher SVR than European Americans with T/T, indicating that genetic background is more
important than race or ethnicity in determining the response rate. A similar finding was reported by another group, indicating that this SNP is also associated with spontaneous clearance of the virus.\textsuperscript{15}

It is of note that, although SNP rs12979860 is indeed the strongest hit that was found to be associated with treatment-induced or spontaneous HCV clearance from these GWAS, the authors did find that other SNPs were also associated, albeit the association was statistically weaker. From the top 100 SNPs that were associated with SVR, none of them replicated previous reported SNPs (MxA, OAS-1, PKR, IFNγ, and IL-10) that were associated with treatment response.\textsuperscript{18} Failure to replicate these previous findings suggests that either those associations were too weak to be picked up by GWAS or those previous SVR associated SNPs were insignificant. To support this latter notion, from 637 non-Hispanic Caucasian patients infected with genotype I, a study by Morgan \textit{et al.} indicated that none of the 8 previously reported treatment response-associated SNPs was found to be associated with SVR.\textsuperscript{19}

SNP rs12979860 was mapped roughly 3kb upstream of the IL28B gene promoter region, which encodes a type III IFNλ3. Type III IFN (also called IFNλ3), discovered 6 years ago\textsuperscript{20}, belongs to the IL-10 super family with type I IFN-like functions. Three subtypes of IFNλs have been reported: IFNλ1 is encoded by the IL29 gene, and IFNλ2 and IFNλ3 are encoded by IL28A and IL28B, respectively. IFNλ signals through the type III IFN receptor, which consists of a heterodimer of IFNλ specific IL28 receptor α-chain (IL28Rα) and IL10 receptor β chain( IL10R2). These interferons, like type I IFN, activate the Jak/STAT signaling pathway. Also like IFNα or IFNβ, the outcome of the activation by IFNλ is elevated expression of a set of
ISGs. Marcello et al.\textsuperscript{21} compared the effect of type I IFNs (IFNα or IFNβ) with type III (IFNλ) on HCV replication in cell culture. Although the same sets of ISGs were induced by these two types of IFNs and similar inhibitory effects were observed, the kinetics of ISG induction was different: ISGs induced by type I IFNα or IFNβ peaked earlier but died down quickly, while ISGs induced by type III IFNλ were delayed, but lasted longer. The mechanism and clinical significance of the delayed induction of ISGs by IFNλ is not clear.

Another major difference in the action of type I IFNα or IFNβ and type III IFNλ is the cell-type restricted expression of the IFNλ receptor. Unlike the ubiquitously expressed type I IFN receptors (IFNAR1 and IFNAR2), type III IFN specific receptor IL28Rα chain is only expressed abundantly on epithelial cells and on human hepatocytes (but not on murine hepatocytes). A study by Ank et al.\textsuperscript{22} demonstrated that most cell types expressed both types I and III IFNs after TLR stimulation or virus infection, whereas the ability of cells to respond to IFNλ was restricted to a narrow subset of cells, including plasmacytoid dendritic cells and epithelial cells. Taking all these observations together, IFNλ function appears cell-type specific.

The fact that IFNλ induces the same set of ISGs and demonstrates similar anti-viral activities to IFNα or IFNβ in cell culture and in various mouse models, and its action is restricted to a narrow range of cell types (primarily epithelial cells and human hepatocytes), suggests that IFNλ may be more relevant than IFNα or IFNβ for treatment of HCV infection and might have fewer adverse effects in the clinical setting.

The IL28b C/C genotype is associated with better response and spontaneous clearance, but it is also associated with higher baseline viral load.\textsuperscript{18} This is contradictory to the clinical observation that lower baseline viral load predicts better response. How can we reconcile these
two seemingly contradictory observations? One possible explanation for this might be derived from my previous studies on the HCV response signature. We and other groups found that increased baseline hepatic ISG expression is associated with non-response to treatment. The ISG expression in these NR livers is already maximized, and a much smaller elevation in expression of these genes is observed compared to Rs following IFN treatment. This suggests that the IFN or other related signaling pathways have already been activated in the NR livers following HCV infection, leading to the enhanced expression of ISGs in the pre-treatment liver tissues.

It is quite plausible that the IL28B polymorphism has a role in the regulation of intrahepatic or macrophage ISG expression, which alters viral load within those affected cell types and eventually affects the treatment response. Indeed, I found that ISG15, one of the most abundant ISGs induced by type I IFNs and viral infection, promotes HCV replication in cell culture.\(^1\) Similarly, another ISG, USP18, the deconjugating enzyme for ISG15 conjugation pathway, also stimulates HCV production and blunts IFN anti-HCV activity in vitro.\(^2\) These data indicate that preactivation of IFN signaling, possibly from activation of the type III IFN pathway, may be the downstream effect of the IL28B SNP. I hypothesize that patients with the favourable IL28 C/C allele have less activation of IFN\(\lambda\) signaling in the hepatocytes, leading to less elevation of the hepatic ISGs and causing treatment response. On the other hand, patients with the less favourable IL28 T/T allele have more profound activation of IFN\(\lambda\) signaling in the hepatocytes, leading to increased expression of hepatic ISGs and causing treatment non-response.

In summary, data from previous research and the recent GWAS studies indicate that both type I and type III IFN signaling pathways might be dysregulated by HCV infection. My previous
observation on the preactivation of these signaling pathways, leading to the enhanced expression of hepatic ISGs in NRs, might be a downstream effect of the IL28B polymorphism. To link these data together, functional studies detailing the mechanisms of the IL28B SNP association with treatment response is needed, and connecting the restricted action of type III IFN on epithelial cells and hepatocytes, together with the differential cell-type specific expression of ISGs between R and NR, is required. It is conceivable that the IL28B SNP affects IFNλ expression, leading to different activation states in hepatocytes while having no effects on macrophage cells due to the restricted IL28B receptor expression pattern. This may cause the cell-type specific ISG expression I observed that correlates with treatment response.
References for Chapter 6:


