High-throughput gene expression profiling identified a novel signalling pathway involved in hepatitis virus resistance to interferon-based therapy

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Microarray technology, one of the high-throughput gene expression profiling tools, has unique advantages in examining tens of thousands of gene transcript changes at the whole genomic level. Hepatitis C Virus (HCV) infects 170 million people worldwide, leading to chronic persistent infections, liver fibrosis, cirrhosis and even hepatocellular carcinoma (HCC). Current treatment with pegylated interferon and Ribavirin clears the virus in 50% of patients. The cure rate is increased up to 70% with the addition of the newly developed direct acting antivirals (DAA). Although much progress has been made in the understanding of the viral life cycle, the molecular mechanisms underlying interferon resistance in almost 50% of HCV patients remain to be determined. Using cDNA microarray technology, we identified an 18-gene signature that differentiates treatment responders and non-responders[1]. Based on the expression levels of these 18 host hepatic genes, we can accurately predict who will respond to interferon-based therapy with an accuracy higher than 96% before treatment initiation[2]. Furthermore, a novel ubiquitin-like pathway (ISG15/USP18) was identified to be involved in HCV resistance to interferon therapy[3]. Activation of the endogenous interferon signalling and the ISG15/USP18 pathway leading to increased expression of the baseline ISG15 and USP18 not only stimulates HCV production but also blunts interferon anti-HCV activity[4]. Interestingly, activation of this ISG15/USP18 pathway is also found in the patient liver tissues chronically infected with hepatitis B virus (HBV) who do not respond to interferon therapy, suggesting the association of the activation of ISG15/USP18 signaling pathway with interferon resistance is not virus-specific[5,6]. Novel anti-virals may be developed through targeting ISG15/USP18 ubiquitin-like pathway, opening a whole new avenue of hepatitis virus research.

Keywords Hepatitis C virus (HCV); microarray; ISG15/USP18 ubiquitin-like pathway; interferon resistance; Hepatitis B virus (HBV)

1. Introduction

HCV is a blood-borne virus that infects over 170 million people worldwide[1,2]. Although antibody is successfully induced in many patients infected, up to 85% fail to clear the virus and chronic infection is established[3]. Chronic HCV infection often leads to liver fibrosis, cirrhosis, and even hepatocellular carcinoma (HCC). Current treatment with pegylated interferon (IFN) and Ribavirin (Rib) clear the virus in about 50% of patients[1]. With the addition of direct acting antivirals (DAAs) targeting specific HCV polymerase or protease as triple therapy[4], the sustained virological response (SVR) was increased up to 75%[4]. Still there are a considerable portion of patients who do not respond to this treatment regime, therefore a prognostic tool that predicts who will and who will not respond to current treatment is needed in the clinic. Furthermore, understanding the molecular mechanisms of IFN resistance in those patients who do not respond to IFN-based therapy is necessary to develop more effective antiviral agents. In this chapter, we will first introduce the basics of HCV virology followed by current approaches to predict treatment response. Then we will discuss how to use microarray as a high-throughput gene expression profiling tool to identify a set of differentially expressed genes in the livers of treatment responders (Rs) and non-responders (NRs) as the basis for genomic approach to predict response. A newly-identified ubiquitin-like signaling pathway revealed from our microarray study will also be discussed in the context of IFN resistance of HCV.

2. HCV virology

Hepatitis C virus (HCV) is the only member of the genus Hepacivirus in the family Flaviviridae[5,6]. 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[1,2]. 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[7,8]. HCV chronic infection frequently results in progressive fibrosis, cirrhosis and an increased risk of hepatocellular carcinoma[9]. As such, HCV is a significant health burden and the leading indicator for liver
transplantation in the US and Western Europe\cite{3}. The HCV life cycle starts with receptor-mediated viral particle endocytosis. Several receptors or co-receptors have been identified, including CD81\cite{10}, the scavenger receptor class B type 1 (SR-B1)\cite{11}, claudin-1\cite{12}, occludin\cite{13}, DC-SIGN and L-SIGN\cite{14}. 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\cite{15}. 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)\cite{3}.

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.

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\cite{16}. 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\cite{10}.

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\cite{17-19}. 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 (eEVR) – no detectable virus at 12 weeks of treatment – have a 68% chance of being cured; however, <70% of genotype 1 patients achieve an eEVR. The negative predictive value is only about 50% for both\cite{20}. 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.

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.

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. cDNA microarray was used to study pre-treatment liver biopsy specimens taken from patients with chronic HCV who were subsequently treated with combination therapy with PegIFN/ribavirin\cite{21}. Gene expression levels were compared among 15 non-responder, 16 responder, and 20 normal liver biopsy specimens. A discrete set of 18 genes whose expression differed consistently between responders and non-responders (p < 005) was identified (Figure 1)\cite{21}. Many of these 18 genes were IFN stimulated (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\cite{22,23}, and have been validated prospectively in a larger cohort of chronic HCV patients prior to initiating antiviral treatment\cite{24}. Using four different methods for classification accuracy (KNN, DQDA, DLDA, CART), the authors 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 titer above.

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 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 IF127 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 the previous observation made by Toronto group that ISGs are more highly expressed in the pre-treatment liver tissue of non-responders than responders [Gastro 2005]. 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.

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[26]. 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[21,27]. 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[28].

Taylor et al. studied gene expression profiles in PBMC 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 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.

5. ISG15/USP18 signaling pathway is involved in IFN resistance in patients chronically infected with HCV

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.
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. 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, 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.

ISG15, 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). 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.

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. 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.

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 cancer.

Several lines of evidence suggest that ISG15 conjugation also plays an important role in the innate antiviral response: 1) ISG15 is targeted by other viruses: non-structural protein (NS-1B) of Influenza B virus binds to the free form ISG15, preventing ISGylation; 2) ISG15 inhibits HIV release (but not virus replication); 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; 4) Mice lacking ISG15 deconjugation enzyme (USP18/UBP43), resulting in increased ISGylation, are resistant to LCMV and VSV infection.

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 ISG5 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.

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 [60].

5.3. ISG15 stimulates HCV replication and inhibits IFN anti-HCV activity

ISG15 is one of the most abundantly expressed (induced) genes following type I IFN stimulation or viral infection. Microarray gene expression profiling identified increased ISG15 in the pretreatment non-responder liver tissues of HCV chronically infected patients [21]. Functional studies using HCV in vitro culture system indicated that increased expression of ISG15 plays two roles in HCV infection and response to type I IFN treatment [50]. First, ISG15 stimulates HCV RNA replication and increases HCV virion particle secretion in the absence of IFN. Secondly, ISG15 inhibits IFN anti-HCV activity. These two effects work together to render patients not responding to IFN therapy.

5.4. USP18 inhibits type I IFN signaling to blunt IFN anti-HCV activity

USP18 knock out mice are resistant to LCMV and VSV lethal infection. This indicates too much USP18 which we observed in the pretreatment non-responder liver tissues of HCV infected patients may play a role in treatment non-response. To investigate the role of USP18 in HCV replication and response to IFN, we inhibited USP18 expression by specific siRNA and found that silencing USP18 potentiates IFN anti-HCV activity by 40-100 fold through activation of the Jak/STAT signaling pathway as indicated by the prolonged activation of STAT1 phosphorylation and increased expression of the downstream anti-viral ISGs [61].

6. Conclusion

High-throughput gene expression profiling identified a few hundred hepatic genes whose expression levels were altered in the HCV chronically infected liver tissues compared to normal livers. The expression levels of 18 genes were significantly and statistically different between treatment responder and non-responder pretreatment liver tissues. Based on expression levels of these 18 genes, a prognostic tool that predicts who will respond to IFN therapy with an accuracy of 95% was developed. A lot of these 18 genes are interferon stimulated (sensitive) genes that are more highly expressed in the NR livers, indicating pre-activation of the endogenous IFN signaling leading to increased expression of these ISGs contributes to treatment non-response. 3 out of those 18 genes are involved in the same ubiquitin-like ISG15/USP18 signaling pathway. Functional studied using HCV in vitro culture system demonstrated that increased ISG15 and USP18 not only promotes HCV replication but also inhibits IFN anti-HCV activity, leading to treatment failure. Targeting ISG15/USP18 pathway maybe a good alternative for the development of new antiviral agents for HCV infection.

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