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

Jianguo Yuan[#,1], Jing Sun[#,1], Bing Liu[2], Shilin Li[2], Yujia Li[2], Xiaoqiong Duan[2], Chunhui Yang[2], Peibing Zeng[2] and Limin Chen[*,2,3]

[1]Sichuan Mingri Pharmaceutical Research Institute of New Technology, Chengdu, Sichuan 610100, China
[2]Institute of Blood Transfusion, Chinese Academy of Medical Sciences and Peking Union Medical College, Chengdu, Sichuan 610052, China
[3]Toronto General Research Institute, University Health Network, University of Toronto, Toronto, ON, M5G 1L6 Canada
*Corresponding author: limin_chen_99@yahoo.com or limin.chen@utoronto.ca Phone: +86-28-61648530
[#]These authors contributed equally to this manuscript

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 underlie 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 ubiqutin-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/USP15 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 millon 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 [2]. 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[3]. With the addition of direct acting antivirals (DAAs) targeting specific HCV polymerase or protease as“ triple therapy“, 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 develope 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[3]. The HCV life cycle starts with receptor-mediated viral particle endocytosis. Several receptors or co-receptors have been identified, including CD81 [10], the scavenger receptor class B type I (SR-BI) [11], claudin-1 [12], occludin [13], DC-SIGN and L-SIGN [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[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) [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[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[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[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 (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[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 PegIFNα/RBV[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) [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 [22,23], and have been validated prospectively in a larger cohort of chronic HCV patients prior to initiating antiviral treatment[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 titers above.

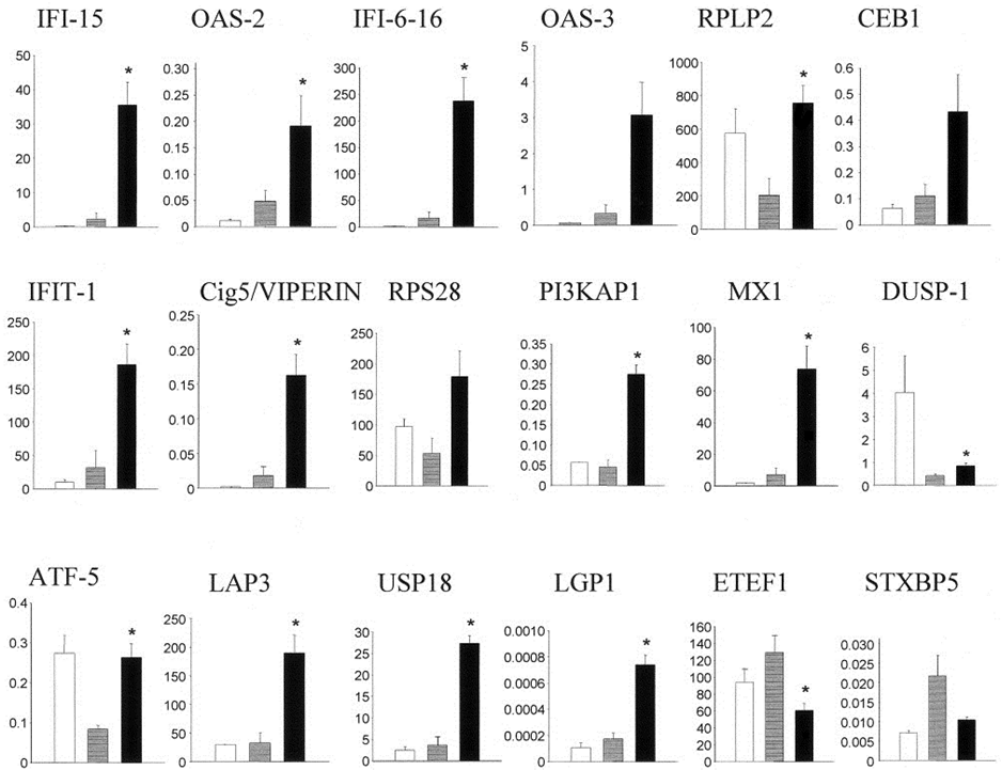

Fig. 1 Real-time PCR verification of genes predicted to be altered by microarray. Real-time PCR verification was performed as described in Materials and Methods. In all cases, 4 genotype 1 R samples were compared with 4 genotype 1 NR samples and 3 normal liver samples (*white*, normal; *gray*, R; *black*, 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.

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[25]. 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[23]. 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[22]. 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 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[29].

Although a few specific targeted antiviral therapies for hepatitis C (STAT-C), such as NS3/4A protease and NS5B polymerase inhibitors, are being used in the clinic in some countries, viral mutations that are resistant to these modalities often occur[30]. 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.

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[31]. 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 [32-34], 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)[35], then an E2 conjugating enzyme (UbcH8, UbcH6)[36,37], and finally an E3 ligase (EFP, CEB1/Herc5) [38,39]. 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[40].

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[41]. 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[42,43]. 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[44]. Several lines of evidence suggest that ISG15 conjugation also plays an important role in the innate antiviral response[45] : 1) ISG15 is targeted by other viruses: non-structural protein (NS-1B) of Influenza B virus binds to the free form ISG15, preventing ISGylation [35]; 2) ISG15 inhibits HIV release (but not virus replication) [46]; 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[47] ; 4) Mice lacking ISG15 deconjugation enzyme (USP18/UBP43), resulting in increased ISGylation, are resistant to LCMV and VSV infection[45].

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[46]. 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[47]. 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[48]. 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 [40,49,50], IFN signaling is intact in ISG15 knock-out mice[51]. 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[52] and ISG15 null mice have an increased susceptibility to Sindbis, Influenza, and HSV-1 virus infections[53], there was no difference in the replication of VSV and LCMV in ISG15 null and wild type mice[51,54]. 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 [55]. Quite surprisingly, we and others have found that ISG15 promotes viral production in a cell culture model [56].

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[40]. 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 [57]. 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) [45]. 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 [51] or its E1 activating enzyme Ube1L in mice[58] 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 [59]. 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 [56]: 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 infectionchen. 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 down-stream 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.

Acknowledgements The study was partially support by Sichuan Provincial Science and Technology department (2011SZ001 to Dr. L Chen, 2013JY0048 to Dr. S Li through the Institute of Blood Transfusion, Chinese Aademy of Medical Sciences/Peking Union Medical College and 2013SZ0066 to Dr. J Yuan throug Sichuan Mingri Pharmaceutical Research Institute of New Technology) is gratefully acknowledged.

References

[1] Lindenbach BD, Rice CM. Unravelling hepatitis C virus replication from genome to function. *Nature*. 2005; 436: 933–938.

[2] Choo Q-L, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science*. 1989; 244:359–362.

[3] Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med*. 2002;347(13):975-82.

[4] Poordad F, McCone J Jr, Bacon BR, Bruno S, Manns MP, Sulkowski MS, Jacobson IM, Reddy KR, Goodman ZD, Boparai N, DiNubile MJ, Sniukiene V, Brass CA, Albrecht JK, Bronowicki JP; SPRINT-2 Investigators. Boceprevir for untreated chronic HCV genotype 1 infection. *N Engl J Med.* 2011;364(13):1195-206.

[5] Alter M.J. Epidemiology of hepatitis C virus infection.World J. *Gastroenterol*. 2007;13:2436–2441.

[6] Alter MJH, Margolis HS, Krawczynski K, Judson F,Mares A, Alexander WJ, Hu P-Y, Miller JK, et al. The natural history of community-acquired hepatitis C in the United States. *N. Engl. J. Med.* 1992; 321: 1494–1500.

[7] Gerlach JT, Diepolder HM, Zachoval R, Gruener NH, Jung MC, Ulsenheimer A, Schraut WW, Schirren CA, Waechtler M, Backmund M, Pape GR. Acute hepatitis C: high rate of both spontaneous and treatment-induced viral clearance. *Gastroenterology*. 2003; 125: 80-88.

[8] Micallef JM, Kaldor JM, Dore GJ. Spontaneous viral clearance following acute hepatitis C infection: a systematic review of longitudinal studies. *J. Viral. Hepat*. 2006; 13: 34-41.
[9] Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. *Lancet. Infect. Dis.* 2005;5: 558-567.
[10] Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ,Houghton M, Rosa D, Grandi G, Abrignani S. Binding of hepatitis C virus to CD81. *Science*. 1998;282:938-941.
[11] Scarselli E, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A. The human scavenger receptor class B type I is a novel andidate receptor for the hepatitis C virus. *EMBO J*. 2002; 21: 5017-5025.
[12] Evans MJ, von Hahn T, Tscherne DM, Syder AJ, Panis M, Wolk B, Hatziioannou T, cKeating JA, Bieniasz PD, Rice CM. Claudin-1 is a hepatitis C virus co-receptor required or a late step in entry. *Nature* . 2007; 446: 801-805.
[13] Ploss A, Evans MJ, Gaysinskaya VA, Panis M, You H, de Jong YP, Rice CM. Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature.* 2009; 457:882-886.
[14] Pöhlmann S, Zhang J, Baribaud F, Chen Z, Leslie G, George Lin G, Granelli-Piperno A, Doms R, Rice CM, Jane A, McKeating J. Hepatitis C Virus Glycoproteins Interact with DC-SIGN and DC-SIGNR. *J Virology.* 2003;77(7):4070-4080
[15] Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med*. 2002;347(13):975-82.
[16] Thimme R, Lohmann V, Weber F. A target on the move: innate and adaptive immune escape strategies of hepatitis C virus. *Antiviral Research.* 2006; 69:129-141
[17] Marinho RT, Pinto R, Santos ML, Lobos IV, Moura MC. Effects of interferon and ribavirin combination therapy on CD4+ proliferation, lymphocyte activation, and Th1 and Th2 cytokine profiles in chronic hepatitis C. *J Viral Hepat.* 2004;11(3):206-16.
[18] Shirren CA, Zachoval R, Gerlach JT, Ulsenheimer A, Gruener NH, Diepolder HM, Baretton G, Schraut W, Rau HG, Nitschko H, Pape GR, Jung MC. Antiviral treatment of recurrent hepatitis C virus (HCV) infection after liver transplantation: association of a strong, multispecific, and long-lasting CD4+ T cell response with HCV-elimination. *J Hepatol.* 2003;39(3):397-404.
[19] Amaraa R, Mareckova H, Urbanek P, Fucikova T. Immunological predictors of different responses to combination therapy with interferon alpha and ribavirin in patients with chronic hepatitis C. *J Gastroenterol.* 2003;38(3):254-9.
[20] 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)
[21] Chen L, Borozan I, Feld J, Sun J, Tannis LL, Coltescu C, et al. Hepatic gene expression discriminates responders and nonresponders in treatment of chronic hepatitis C viral infection. *Gastroenterology*. 2005;128:1437–1444.
[22] Feld JJ, Nanda S, Huang Y, Chen W, Cam M, Pusek SN, et al. Hepatic gene expression during treatment with peginterferon and ribavirin: identifying molecular pathways for treatment response. *Hepatology* . 2007;46:1548–1563.
[23] Asselah T, Bieche I, Narguet S, Sabbagh A, Laurendeau I, Ripault MP, et al. Liver gene expression signature to predict response to pegylated interferon plus ribavirin combination therapy in patients with chronic hepatitis C. *Gut.* 2007 57(4):516-624.
[24] Chen L, Bronzan I, Sun J, Anand N, Heathcote EJ, Edwards A, et al. Validation of a gene signature predicting response to treatment in chronic hepatitis C virus infection. *Hepatology.* 2007;46:256A.
[25] Hayashida K, Daiba A, Sakai A, Tanaka T, Kaji K, Inaba N, et al. Pretreatment prediction of interferon-alfa efficacy in chronic hepatitis C patients. *Clin Gastroenterol Hepatol* . 2005;3:1253–1259.
[26] Lempicki RA, Polis MA, Yang J, McLaughlin M, Koratich C, Huang DW, et al. Gene expression profiles in hepatitis C virus (HCV) and HIV coinfection: class prediction analyses before treatment predict the outcome of anti-HCV therapy among HIVcoinfected persons. *J Infect Dis.* 2006;193:1172–1177.
[27] Feld J, Fried MW, Liang TJ. Gene expression in liver during interferon therapy in humans. In: American association for the study of liver diseases, hepatitis single topic conference, interferon and ribavirin in hepatitis C virus infection: mechanisms of response and non-response, 2003 March 1–3, Chicago, IL.
[28] Gerotto M, Dal Pero F, Bartoletto G, Reoldan S, Ferrari A, Baccato S, et al. PKR gene expression and response to pegylated interferon plus ribavirin therapy in chronic hepatitis C. *Antivir Ther.* 2004;9:763
[29] Taylor MW, Tsukahara T, Brodsky L, Schaley J, Sanda C, Stephens MJ, et al. Changes in gene expression during pegylated interferon and ribavirin therapy of chronic hepatitis C virus distinguish responders from nonresponders to antiviral therapy. *J Virol.* 2007;81:3391–3401.
[30] Shimakami T, Lanford RE, Lemon SM. Hepatitis C: recent successes and continuing challenges in the development of improved treatment modalities. *Curr Opin Pharmacol.* 2009 ;9(5):537-44.
[31] Zuki F, Arase Y, Suzuki Y, Tsubota A, Akuta N, Hosaka T, et al. Single nucleotide polymorphism of the MxA gene promoter influences the response to interferon monotherapy in patients with hepatitis C viral infection. *J Viral Hepat.* 2004;11:271–276.
[32] 32.Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem* .1998;67:227–64.
[33] Sen GC, Ranshoff RM. Interferon-induced antiviral actions and their regulation. *Adv Virus Res.* 1993;42:57–102.
[34] Martensen PM, Justesen J. Small ISGs coming forward. *J Interferon Cytokine Res.* 2004;24:1–19.
[35] Yuan W and Krug RM. Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. *EMBO J.* 2001; 20: 362–371
[36] Zhao C, Beaudenon SL, Kelley ML, Waddell MB, Yuan W, Schulman BA, Huibregtse JM and Krug RM. The UbcH8 ubiquitin E2 enzyme is also the E2 enzyme for ISG15, an IFN- /ß-induced ubiquitin-like protein. *Proc. Natl. Acad. Sci. USA.* 2004;101: 7578–7582
[37] Kim KI, Giannakopoulos NV, Virgin HW and Zhang DE. Interferon-inducible ubiquitin E2, Ubc8, is a conjugating enzyme for protein ISGylation. *Mol. Cell. Biol.* 2004;24: 9592–9600
[38] Zou W, Zhang DE. The interferon-inducible ubiquitin-protein isopeptide ligase (E3) EFP also functions as an ISG15 E3 ligase. *J Biol Chem*. 2006;281(7):3989-94.

[39] 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
[40] Malakhov MP, Malakhova OA, Kim KI, Ritchie KJ, Zhang DE. UBP43 (USP18) specifically removes ISG15 from conjugated proteins. *J Biol Chem*. 2002 ;277(12):9976-81
[41] Yeh ET, Gong L, Kamitani T. Ubiquitin-like proteins: new wines in new bottles. *Gene*. 2000;248:1–14.
[42] MalakhovMP, Kim KI, Malakhova OA, Jacobs BS, Borden EC, Zhang DE. High-throughput immunoblotting. Ubiquitin-like protein ISG15 modifies key regulators of signal transduction. *J Biol Chem.* 2003;278:16608–13.
[43] Zhao C, Denison C, Huibregtse JM, Gygi S, Krug RM. Human ISG15 conjugation targets both IFN-induced and constitutively expressed proteins functioning in diverse cellular pathways. *Proc Natl Acad Sci ,USA*. 2005;102(29):10200-5..
[44] Dao CT, Zhang DE. ISG15: Ubiquitin-like Enigma. Regulation of ISG15 Expression and Conjugation. *Front Biosci.* 2005;10:2346-2365.
[45] Ritchie K J, Hahn CS, Kim KI, Yan M, Rosario D, Li L, de la Torre JC, Zhang DE. Role of ISG15 protease UBP43 (USP18) in innate immunity to viral infection. *Nat. Immunol*. 2004;10:1374-1378.
[46] Kumura G, Lu I, Pitha-Rowe, Pitha PM. Innate antiviral response targets HIV-1 release by the inducion of ubiquitin-like protein ISG15. *2006 Jan 31;103(5):1440-5. Epub 2006 Jan 24*
[47] Lenschow DJ, Giannakopoulos NV, Gunn LJ, Johnston C, O'Guin AK, Schmidt RE, Levine B, Virgin IV HW. Identification of interferon-stimulated gene 15 as an antiviral molecule during Sindbis virus infection in vivo. *J. Virol.* 2005;79:13974–13983
[48] Recht M, Borden EC,Knight E Jr. A human 15-kDa IFN-induced protein induces the secretion of IFN-gamma. *J. Immunol.* 1991;147:2617-2623
[49] Ritchie KJ , Malakhov MP , Hetherington CJ , Zhou L , Little MT , Malakhova OA , Sipe JC , Orkin SH , Zhang DE. Dysregulation of protein modification by ISG15 results in brain cell injury. *Genes Dev*. 2002;16: 2207–2212
[50] Malakhova O, Malakhov M, Hetherington C, Zhang DE. Lipopolysaccharide activates the expression of ISG15-specific protease UBP43 via interferon regulatory factor 3. *J Biol Chem.* 2002;277:14703–11.
[51] Osiak AO, Utermöhlen S, Niendorf I, Knobeloch KP. ISG15, an interferon-stimulated ubiquitin-like protein, is not essential for STAT1 signaling and responses against vesicular stomatitis and lymphocytic choriomeningits virus. *Mol. Cell. Biol.* 2005;25:6338-6345.
[52] Lenschow DJ, Giannakopoulos NV, Gunn LJ, Johnston C, O'Guin AK, Schmidt RE, et al. Identification of interferon-stimulated gene 15 as an antiviral molecule during Sindbis virus infection in vivo. *J Virol.* 2005;79:13974–83.
[53] Lenschow DJ, Lai C, Frias-Staheli N, Giannakopoulos NV, Lutz A, Wolff T, et al. IFN-stimulated gene 15 functions as a critical antiviral molecule against influenza, herpes, and Sindbis viruses. *Proc Natl Acad Sci USA*. 2007;104:1371–6.
[54] Knobeloch KP, Utermohlen O, Kisser A, Prinz M, Horak I. Re-examination of the role of ubiquitin-like modifier ISG15 in the phenotype of UBP43-deficient mice. *Mol Cell Biol.* 2005;25:11030–4.
[55] Kim, M.J., Hwang, S.Y., Imaizumi, T., Yoo, J.Y. Negative feedback regulation of RIG-I-mediated antiviral signaling by interferon-induced ISG15 conjugation. *J Virol.* 2008;82, 1474-83
[56] Chen L, Sun J, Meng L, Heathcote J, Edwards A, McGilvray I. ISG15, a ubiquitin-like interferon stimulated gene, promotes Hepatitis C production in vitro: Implications for chronic infection and response to treatment. J Gen Virol. 2010 Feb;91(Pt 2):382-8.
[57] Malakhova OA , Yan M , Malakhov MP , Yuan YZ , Ritchie KJ , Kim KI , Peterson LF , Shuai K , Zhang DE. Protein ISGylation modulates the JAK–STAT signaling pathway. *Genes Dev.* 2003;17: 455–460
[58] Kim KI, Yan M, Malakhova O, Luo JK, Shen MF, Zou W, de la Torre JC, Zhang DE. Ube1L and protein ISGylation are not essential for alpha/beta interferon signaling. *Mol Cell Biol.* 2006;26(2):472-9.
[59] Malakhova OA, Kim KI, Luo JK, Zou W, Kumar KG, Fuchs SY, Shuai K, Zhang DE. UBP43 is a novel regulator of interferon signaling independent of its ISG15 isopeptidase activity. *EMBO J.* 2006 ;25(11):2358-67
[60] Duex JE, Sorkin A. RNA interference screen identifies Usp18 as a regulator of epidermal growth factor receptor synthesis. *Mol Biol Cell.* 2009;20(6):1833-44.
[61] Randall G, Chen L, Panis M, Fischer AK, Lindenbach B, Sun J, Heathcote J, Rice CM, Edwards AM, McGilvray ID. Silencing of USP18 potentiates the antiviral activity of interferon against hepatitis C virus infection. *Gastroenterology.* 2006; 131(5):1584-91

