HHS Public Access

Author manuscript

Liver Int. Author manuscript; available in PMC 2020 May 17.

Published in final edited form as:

Liver Int. 2013 August ; 33(7): 991–998. doi:10.1111/liv.12148.

IL28B genotype and the expression of ISGs in normal liver

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Abstract

Background & Aims: Both polymorphisms in the IL28B gene locus and ISG expression levels are associated with the outcome of hepatitis C virus (HCV) infection. The two are also interrelated, although the mechanism is unknown. Favourable CC genotype at rs12979860 expresses lower baseline ISG levels and responds better to treatment than unfavourable CT and TT genotypes. Little is known about this relationship in normal, uninfected liver. This study sought to explore this relationship.

Methods: Normal human liver specimens (64) and HCV positive human liver specimens (95) were genotyped for IL28B rs12979860 C > T. mRNA levels of ISGs and other relevant genes were studied by qPCR.

Results: Most studied ISGs had significantly different expression by IL28B genotype in normal liver. CC genotype expressed the highest levels, CT intermediate and TT the lowest. This is opposite to the pattern seen in HCV patients. Principal component analysis of IL28B genotype and ISG expression further revealed a distinct set of genes correlated with the C allele (ISG15, HTATIP2, LGALS3BP, IRF2 and BCL2) and T allele (IFNα, β, γ, λ3 and CD80).

Conclusion: A subset of ISGs was found to be differentially expressed in normal liver by IL28B genotype. This suggests a relationship between IL28B genotype and gene expression before HCV infection.

Keywords

gene expression; hepatitis C virus; IL28B; interferon-stimulated genes

While major advances have been made recently in the hepatitis C virus (HCV) field, many unsolved mysteries remain. Even with the advent of protease inhibitors and the introduction

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Conflict of Interest: The authors have no conflict of interest to report.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

of genotypic tests with high predictive value in determining treatment response, outcomes are still far from certain. The current treatment standard of pegylated interferon (IFN) and ribavirin produces only a 50% response rate in genotype 1 individuals. In addition, interferon treatment is expensive and many patients cannot tolerate the serious side effects, which may prevent the completion of the treatment course. Although age, gender, body mass index (BMI) and viral load are associated with treatment response, finding a marker that predicts treatment outcome has been difficult. Recently, a polymorphism in the interleukin 28B (IL28B) gene was found to be associated with treatment response across ethnicities (1). IL28B codes IFNλ3, an endogenous antiviral cytokine that is similar to type I IFNs but with higher tissue specificity. Expression of λ IFN receptors is largely limited to cells of epithelial origin, whereas type I IFN receptors are expressed ubiquitously (2). Like type I IFNs, λ IFNs act through the JAK/STAT pathway to induce a set of interferon-stimulated genes (ISGs), which are induced by viral infection and protect against HCV *in vitro* (3). IFNλ is currently in clinical trials for the treatment of chronic HCV (4). Phase I trials have been encouraging and support the idea that IFNλ induces comparable antiviral activity to IFNα, but without the adverse effects that derive from the ubiquitous expression of IFNα receptors (4, 5). The polymorphism of the IL28B gene is located at rs12979860, 3 kilobases upstream of the transcription start site (1). Genotype CC of rs12979860 has been associated with both increased rates of treatment response and spontaneous viral clearance (1, 6). It has been suggested that the variable prevalence of the favourable genotype among different racial populations may explain differential response rates across ethnicities (1). Although initially identified in HCV genotype 1 patients, the predictive power of the polymorphism has held true for genotypes 2 and 3 as well (7, 8). IL28B genotype can be incorporated with other variables into a prediction model to predict treatment outcome.

The mechanism by which the IL28B polymorphism influences treatment response is unknown. The polymorphism has no obvious effect on gene function, and there is conflicting data concerning the effect on IL28B levels. Some studies have found a correlation between IL28B genotype and IL28B mRNA expression (9, 10), but others have not supported this finding (11, 12).

Recently, there has been interest in the relationship between ISG expression and IL28B. ISG levels are associated with HCV treatment response. Low pretreatment ISG levels predict sensitivity to IFN treatment (13, 14). Although the mechanism is unknown, it has been suggested that in nonresponsive patients, ISG expression is already maximally induced prior to treatment, whereas in responsive patients, lower baseline ISG levels remain capable of responding to exogenous IFN. IL28B genotype has been strongly associated with intrahepatic ISG expression, with the unfavorable genotypes (CT and TT) expressing higher baseline ISG levels compared with the favourable CC genotype patients (11, 15). These findings provide at least a partial functional explanation of the association between IL28B genotype and response to interferon therapy.

Little is known about the relationship between IL28B and ISG expression in normal liver. Considering that ISGs are the likely effectors of IL28B genotypic difference, it is important to understand the interplay between these two components in a normal setting, so we can better understand how they behave in a disease setting.

Patients and methods

Populations studied

Sixty-four normal liver specimens were obtained from the University of Kansas Liver Center Tissue Bank. Consent was obtained from all patients according to a protocol approved by the Institutional Review Board in accordance with the 1975 Declaration of Helsinki. These specimens were obtained from normal donor livers at the Liver Transplant Program at the University of Kansas Hospital. Donors with diagnostic abnormalities were excluded. Ninety-five liver specimens positive for HCV were also studied. Inclusion criteria for these patients were as follows: chronic hepatitis C patients older than 18 years and positive for anti-HCV antibody and serum HCV RNA. The following patients were excluded: positive for hepatitis B virus surface antigen, primary biliary cirrhosis, autoimmune hepatitis, Wilson's disease, haemochromatosis, and co-infection with human immunodeficiency virus. Patients treated with antiviral or immunosuppressive agents within 6 months of when the liver tissue was obtained were also excluded.

Laboratory tests

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (TBILI), alkaline phosphatase (ALP), total cholesterol (CHOL), triglyceride (TRIG), and fasting plasma glucose were obtained from patients' charts and all tests were performed within 3 months of liver biopsy. The HCV genotype was determined by sequencing using the TRUGENE HCV 5′NC Genotyping Kit.

Pathological examination

Haematoxylin and eosin-stained as well as Masson's trichrome-stained liver sections were used for diagnosis by pathologists at KUMC. The degrees of inflammation and fibrosis were evaluated according to the criteria proposed by Ishak *et al.* Steatosis was graded based on the percentage of hepatocytes involved: mild (≤ 10%), moderate (11–32%), or severe (≥ 33%).

Hepatic mRNA quantification

Hepatic RNA was extracted using TRIzol reagent (Ambion, Grand Island, NY, USA). cDNA was generated using High Capacity Reverse Transcription Kit (Life Technologies, Grand Island, NY, USA). Real-time quantitative PCR experiments were performed using Applied Biosystems (Grand Island, NY, USA) 7900HT Fast Real-time PCR System using SYBR Green technology (Life Technologies). Gene expression was studied using RNA extracted from individual, not pooled, RNA samples. The studied genes and primers are listed in Table S1. Data were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger RNA (mRNA) level.

IL28B genotyping

A total of 95 HCV positive patients and 64 normal donor livers were genotyped for the rs12979860 single nucleotide polymorphism using TaqMan SNP genotyping assay (Life Technologies). Primers and probes were as follows: TGCCTGTCGTGTACTGAACCA and GAGCGCGGAGTGCAATTC and TaqMan probes VIC-TGGTTCGCGCCT TC-MGB and

6FAM-CTGG-TTCACGCCTTC-MGB. Automated allele calling was performed using SDS software from Applied Biosystems. Positive and negative controls were used in each genotyping assay.

Statistical analysis

Student's *t*-test was used for gene expression comparison between two groups. Delta delta Ct method was used to analyze qPCR data, with GAPDH used for normalization. Chi-squared test was used to compare genotypic frequencies. Principal component analysis was used to identify the relationship between IL28B genotype and gene expression. $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

Demographic information and clinical data of the 64 normal donors and 95 HCV positive patients are summarized in Table 1.

IL28B rs12979860 allelic frequency

Although a clear shift away from the C allele and towards the T allele was present in HCV-infected patients compared with non-infected donors, the trend did not reach significance (Pearson $\chi^2 = 0.089$) (Figure S1). This is expected as the C allele has been linked to spontaneous resolution and the T allele to persistent infection (6, 16, 17). All alleles are in Hardy–Weinberg equilibrium.

Relationship between IL28B genotype and hepatic gene expression by principal component analysis in normal liver

All data were standardized before principal component analysis (PCA) analysis to avoid bias caused by variation in gene expression. Included in the analysis were 22 donor livers for which all data were available, mRNA expression levels of 34 ISG and ISG-related genes, IL28B genotype and donor characteristics (age, gender, ethnicity and BMI). Score plot analysis indicated IL28B genotype as the component with the largest possible variance, meaning that it accounts for more of the variance in gene expression than any other analyzed criterion. Score plot analysis (Fig. 1A) showed a strong correlation between Component 1 and IL28B genotype indicating that the gene expression pattern was distinct between TT and CC genotype, with CT falling in between. Loading plot (Fig. 1B) showed a distinct set of genes correlated with the T and C alleles. IFNα, β, γ and λ3 as well as CD80 expressions were correlated with the T allele, whereas ISG15, HTATIP2, LGALS3BP, IRF2 and BCL2 expressions were correlated with the C allele (Fig. 2).

Hepatic ISG expression in normal liver by IL28B genotype

A subset of ISGs with predictive power in determining HCV treatment response was selected for qPCR analysis in normal hepatic tissue genotyped for IL28B (Fig. 3A). All of the selected ISGs showed significant differences in mRNA level for TT vs. CC liver. Most (RSAD2, ISG15, LGALS3BP, OAS3, IFI6, HTATIP2, IFIH1) had significant differences in

mRNA levels in TT vs. non-TT genotype (CT and CC) livers. Another subset of ISGs identified recently by two publications as having putative activity against HCV *in vitro* are shown grouped together (Fig. 4A). Four of these genes (IRF1, IRF7, IRF27 and IFI6) had significantly lower expression level in TT than non-TT genotype livers. The most significant difference was found in IRF1, which was shown to be the gene with the most potent activity against HCV *in vitro* (18).

Hepatic ISG expression in normal vs. HCV-infected liver

The same subset of ISGs was analyzed in HCV-infected livers (Figs 3B and 4B). There was a dramatic upward trend by genotype displayed by most ISGs, with the highest levels in TT genotype tissue, which was consistent with literature's findings (11). The pattern is opposite to that observed in normal tissue, where ISG expression is significantly lower in TT genotype tissue. As expected, these genes are much more highly induced (up to 100-fold) in HCV-infected livers as compared with normal livers. Additionally, while in normal liver tissue expression tends to group according to TT vs. non-TT, in infected liver the unfavourable T allele's presence is more striking, and the expression pattern tends toward CC vs. non-CC.

Discussion

Previous reports have shown that ISG expression and IL28B genotype are strongly linked post-HCV infection. Although the mechanism is poorly understood, this relationship can be used to predict treatment response. Here, we show that a relationship exists in normal uninfected liver and that this relationship is opposite to that seen in HCV positive liver. It is known that in HCV-infected liver, TT genotype is linked with the highest levels of ISG expression, and CC with the lowest. Our data indicate that this relationship is reversed in normal liver, where TT genotype expresses significantly lower levels of ISGs than CC genotype tissue.

Principal component analysis using donor characteristics, ISG mRNA levels, and IL28B genotype revealed that IL28B genotype is the most important of the studied components in accounting for variance among the normal population. This indicates that ISG expression and IL28B are highly interrelated even before HCV infection. The PCA loading plot revealed two distinct clusters of genes with high expression in C (ISG15, HTATIP2, LGALS3BP, IRF2 and BCL2) and T alleles (IFNα, β, γ, λ3 and CD80).

Of the group of genes associated with the C allele, four (ISG15, HTATIP2, LGALS3BP and IRF2) are ISGs, and the first three of these have been identified as predictors of treatment response in HCV patients (15, 19). Interferon, alpha-inducible protein (clone IFI-15K) (ISG15) is a ubiquitin-like protein that, upon induction by IFN, is conjugated to a wide range of intracellular targets, including other ISGs, thus greatly expanding the effects of IFN α/β (20, 21). HIV-1 Tat interactive protein 2 (HTATIP2) is a pro-apoptosis, anti-angiogenesis ISG that acts as a tumor suppressor and is down-regulated in many cancers (22). Its role in HCV infection is unknown. Lectin, galactoside-binding, soluble 3 binding protein (LGALS3BP) is involved in promoting integrin-mediated cell adhesion as well as viral immune response associated with natural killer cells (23). It has long been known that

elevated serum LGALS3BP level in HCV patients is predictive of both treatment response (19) and disease severity (24). Interferon regulatory factor 2 (IRF2) is a repressor of the IFN system and acts to competitively inhibit IRF1-mediated transcriptional activation of IFN α/β (25). However, IRF2 also functions as a transcriptional activator of certain genes through ISRE-like sequences (26–28). It also positively regulates RIG-1 expression (29). Notably, IRF2 was recently identified as one of a small subset of ISGs capable of inhibiting HCV replication (30). Of this subset, only IRF1 and IRF2 hepatic mRNA expression is significantly decreased in human HCV patients (unpublished data). B cell lymphoma 2 (BCL2) is perhaps the best characterized anti-apoptosis proto-oncogene, long known to have a role in cancer promotion. B cell disorders such as cryoglobulinemia and non-Hodgkin's lymphoma are frequently associated with HCV, which implies a critical role for B cells in viral infection. Increased expression of BCL2 has been reported in HCV patients with both these disorders (31, 32). Additionally, activated B cells in HCV patients are resistant to apoptosis, likely mediated through elevated BCL2 levels (33, 34). The association of these genes with the favourable C allele in normal liver may indicate their importance in resolving acute HCV infection or preventing it altogether.

Of the group of genes associated with the T allele, CD80 was the only non-IFN gene. CD80 is a cell surface co-stimulatory molecule important for T cell activation. It is up-regulated by type I and II IFNs through IRF1 (35). HCV envelope protein E2 increases the expression of CD80 on dendritic cells (36, 37). It was recently reported that CD80 expression on plasmacytoid dendritic cells in HCV patients is significantly higher in individuals who achieve SVR (38).

All tested IFNs (α, β, γ and λ3) also separated into the T allele group. This is striking as interferons control the expression of ISGs, and most ISGs had significantly lower expression levels in non-CC genotype patients. This may indicate some dysregulation in the interferon pathway even in normal livers when the T allele is present.

The general pattern of ISG expression in normal liver is neatly stepwise by genotype, with CC expressing the highest levels, CT the next highest, and TT the lowest. This pattern is dramatically reversed after HCV infection, when ISG levels rise sharply and TT genotype patients express the highest levels of ISGs. As the mechanism of IL28B's effect on treatment response remains unknown, it is unclear how the relationship between IL28B and ISGs pre- and post-infection fit together.

Although it seems counterintuitive that the protective CC genotype expresses the highest levels of ISGs in normal liver, this genotype may in fact have adverse effects in other contexts. In NAFLD patients, CC genotype has been associated with more severe inflammation and fibrosis (39). Our findings may help to explain this, as ISGs and ISG-related genes have roles in inflammation and the development of fibrosis. These findings may also be important in a transplant setting, as the only positive manifestation of the CC donor genotype may be in achievement of viral response.

An important caveat to this study is the use of donor livers. Although these livers are histologically normal, and have transaminase and bilirubin levels largely within normal

range, it is important to note that they all came from brain dead donors. It is possible, even likely, that this had an effect on the IFN pathways in these livers. However, it seems unlikely that this had an impact on the differences between IL28B genotypes. Physical size of the samples prevented us from doing confirmatory protein studies, although these are a crucial component of future research.

In conclusion, these data provide novel insights into the relationship between IL28B genotype and ISG and related gene expression in normal hepatic tissue without HCV infection. Normal hepatic tissue displays a gene expression pattern that is stepwise by genotype, with CC expressing the highest levels and TT the lowest. This is opposite from the gene expression pattern seen after HCV infection, when TT genotype expresses the highest levels of ISGs. The identification of this relationship pre-infection has implications for understanding the still unclear mechanism behind the IL28B genotype, and more immediate implications in the context of transplantation. Furthermore, examination of the mechanism underlying the IL28B polymorphism and its effect on ISG levels, in normal as well as HCV-infected liver, is still urgently needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank patients, physicians, and nurses for their contributions to the KU Liver Center Tissue Bank.

Abbreviations

ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	serum aspartate aminotransferase
BCL2	B-cell CLL/lymphoma 2
BMI	body mass index
CD80	CD80 molecule
CHOL	total cholesterol
GAPDH	glyceraldehyde 3-phosphatase dehydrogenase
HCV	hepatitis C virus
HTATIP2	HIV-1 Tat interactive protein 2
IFI6	interferon, alpha-inducible protein 6
IFI27	interferon, alpha-inducible protein 27
IFN	interferon

IL28B	interleukin 28B
IRF1	interferon regulatory factor 1
IRF2	interferon regulatory factor 2
IRF7	interferon regulatory factor 7
ISG15	interferon, alpha-inducible protein (clone IFI-15K)
ISG	interferon-stimulated gene
LGALS3BP	lectin, galactoside-binding, soluble 3 binding protein
mRNA	messenger RNA
PCA	principal component analysis
TBILI	total bilirubin
TRIG	triglyceride

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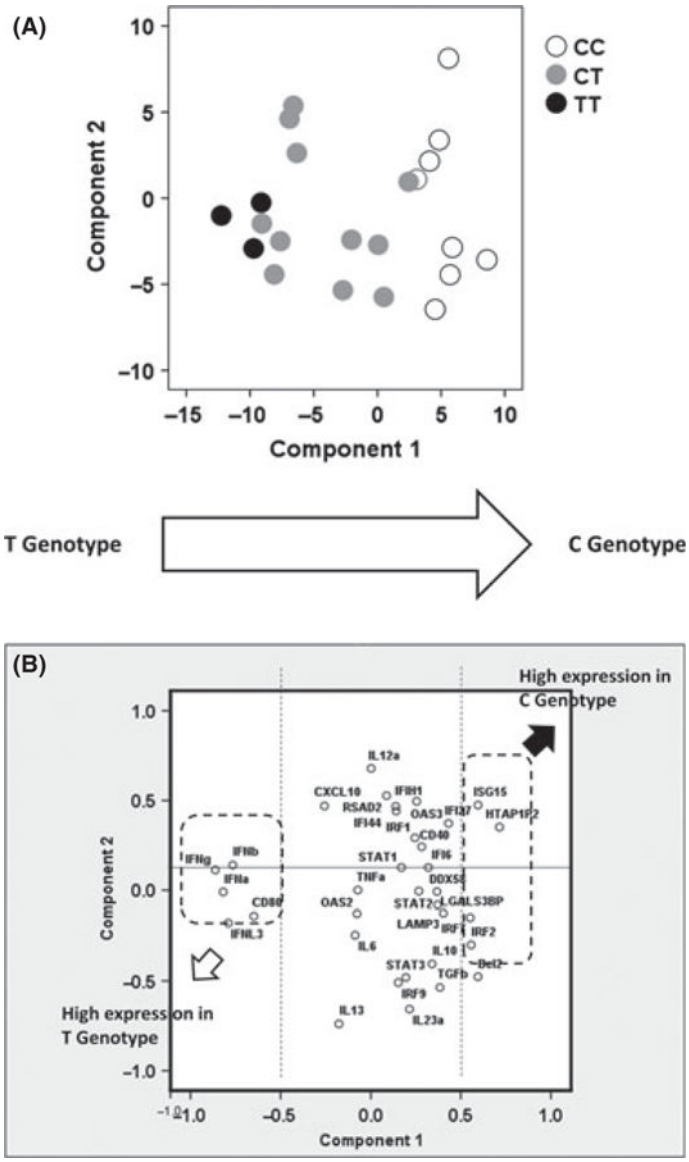

Fig. 1.

Principal component analysis of 34 ISG and ISG-related genes and donor characteristics in 22 normal liver samples for which all data was available. (A) Score plot (B) Loading plot of 34 ISGs

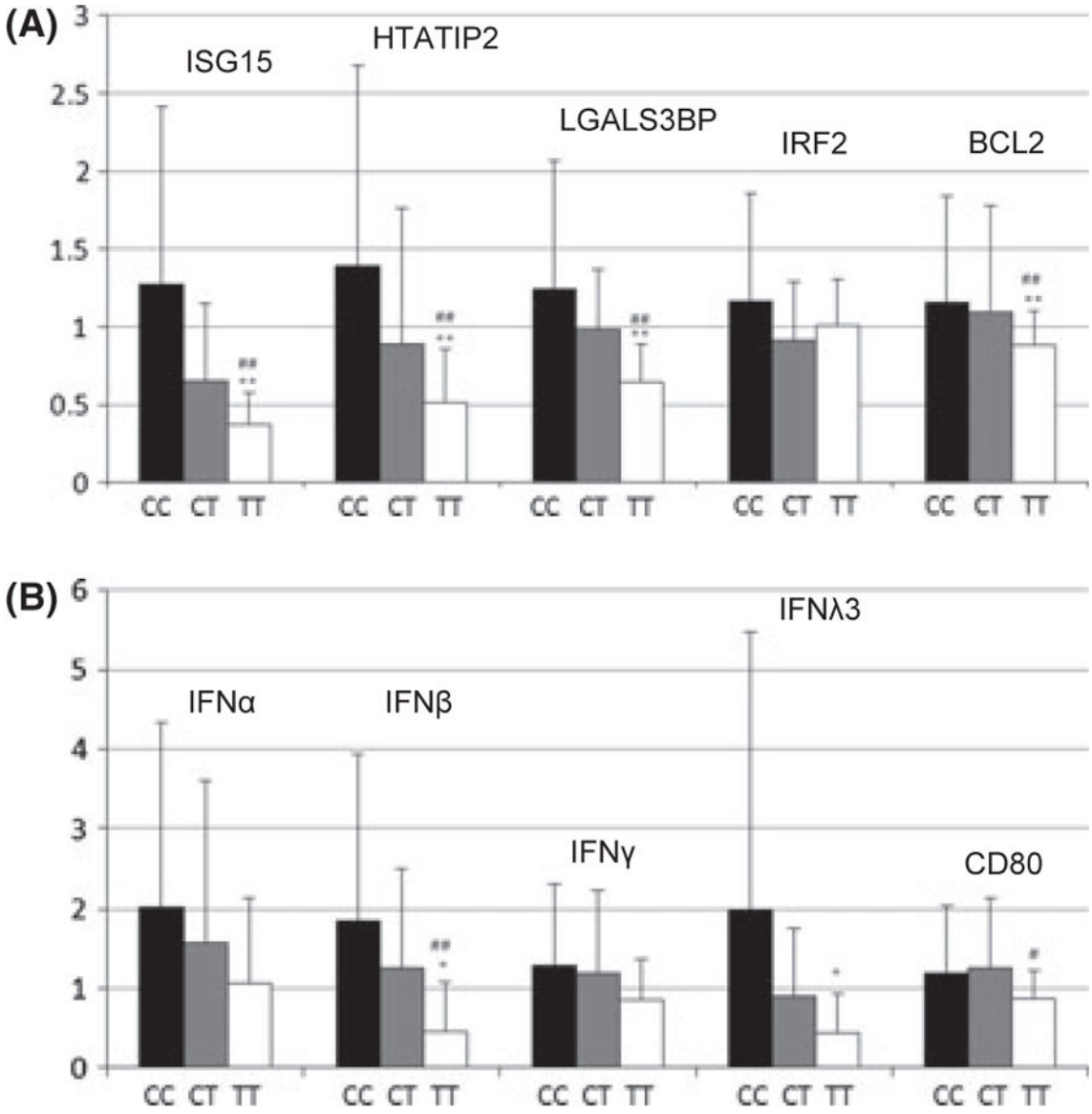

Fig. 2.

Hepatic mRNA expression of genes identified by PCA. (A) Genes with higher expression for C allele (B) genes with higher expression for T allele. $*P \leq 0.05$, $**P \leq 0.01$ to CC; $\#P \leq 0.05$, $\#\#P \leq 0.01$ to CT.

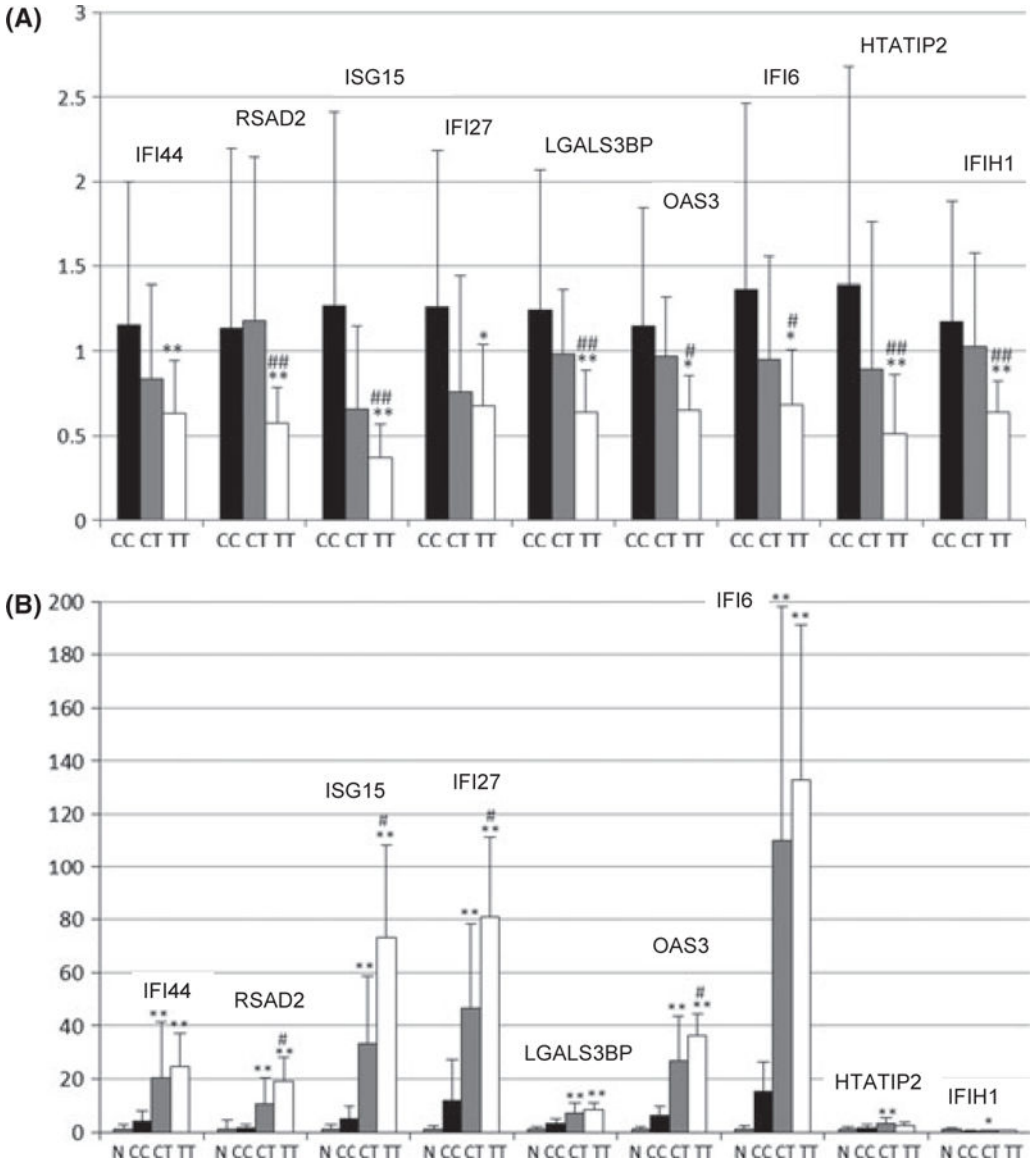

Fig. 3.

Hepatic predictive interferon-stimulated genes (ISGs) levels by IL28B genotype. (A) Normal liver tissue. Levels decrease by IL28B genotype with the lowest in TT genotype livers in normal hepatic tissue. CC, $n = 28$; CT, $n = 30$; TT, $n = 6$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ to CC; $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ to CT. (B) HCV infected liver tissue. There is a dramatic upward trend by genotype, with highest levels in TT tissue. This pattern opposes that seen in (A) in normal liver tissue. CC, $n = 29$; CT, $n = 50$; TT, $n = 16$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ to CC. All $P \leq 0.05$ to donor.

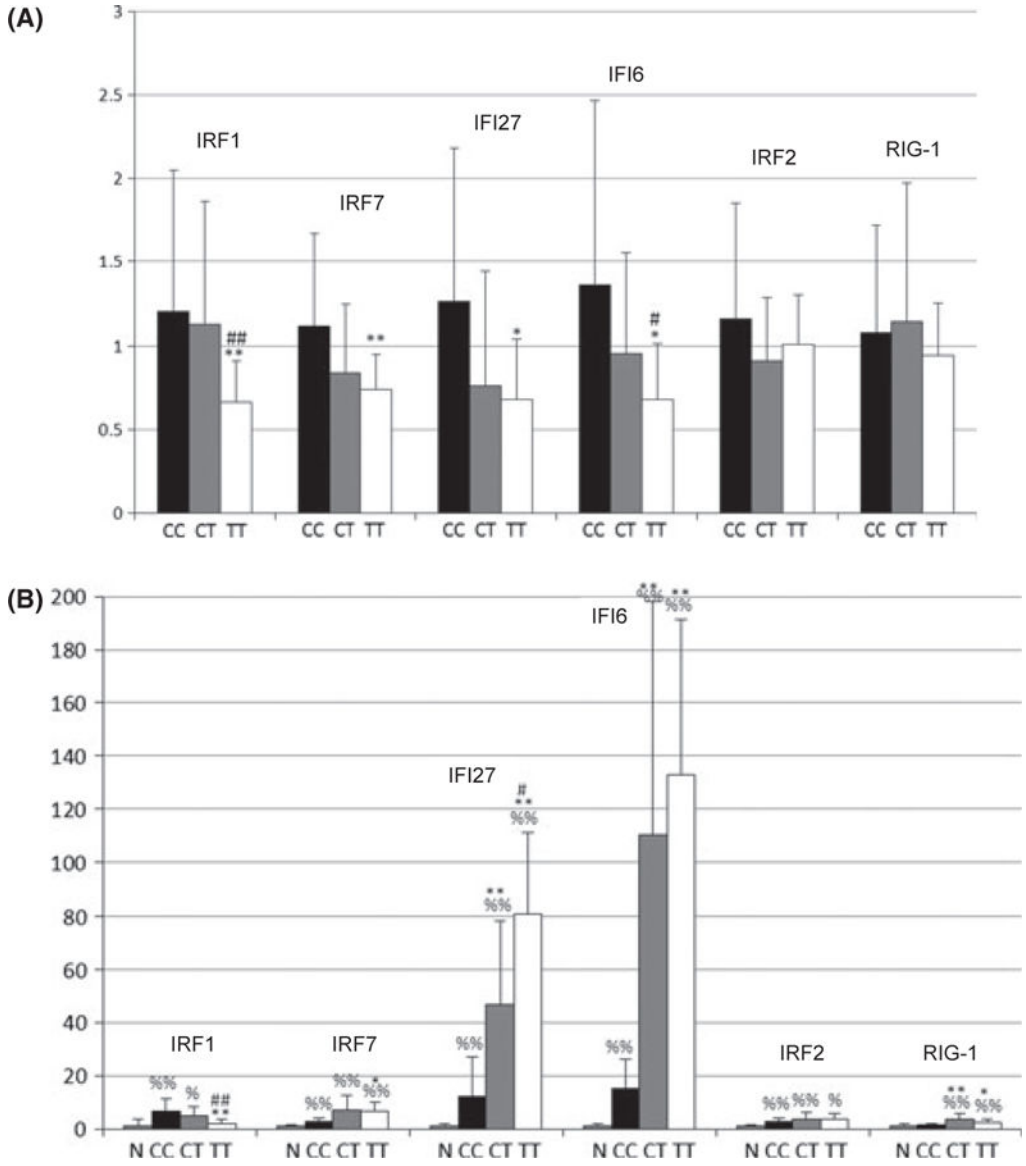

Fig. 4.

Hepatic anti-HCV ISG levels by IL28B genotype. (A) Hepatic mRNA levels of a small group of ISGs with putative activity against HCV in normal liver tissue. IRF1, IRF7, IFI27, and IFI6 are significantly lower in TT genotype livers in normal hepatic tissue. CC, $n = 28$; CT, $n = 30$; TT, $n = 6$. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ to CC; $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ to CT. (B) Hepatic mRNA levels of anti-HCV ISGs in HCV infected liver tissue. CC, $n = 29$; CT, $n = 50$; TT, $n = 16$. $\%P \leq 0.05$, $\%\%P \leq 0.01$ to donor, $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ to CC

Liver Int. Author manuscript; available in PMC 2020 May 17.

Table 1.

Population characteristics: (a) donor and (b) chronic hepatitis C patients

Group	Total	IL28B CC	IL28B CT	IL28B TT
(a)				
No.	64	28	30	6
Age (range)	46.3 ± 14.5 (17–80)	44.5 ± 13.7 (22–80)	48.3 ± 14.6 (17–73)	44.8 ± 17.5 (21–79)
Male/female	38/26	15/13	18/12	5/1
Ethnicity (Caucasian/African-American/Other)	57/4/2	28/0/0	26/3/1	3/1/2
BMI	28.1 ± 4.98	27.6 ± 4.6	28.5 ± 5.5	28.2 ± 3.9
AST	70.7 ± 88.7	55.3 ± 49.4	70.5 ± 73.6	135.1 ± 203.9
ALT	50.6 ± 53.8	43.7 ± 43.9	59.1 ± 63.1	42.7 ± 49.5
ALP	58.0 ± 18.5	55.6 ± 19.1	61.5 ± 19.0	52.3 ± 12.0
GGTP	38.7 ± 30.4	40.0 ± 30.4	42.0 ± 32.3	52.3 ± 12.0
Total bilirubin	0.85 ± 1.0	0.73 ± 0.40	0.72 ± 0.47	1.87 ± 2.8
(b)				
No.	95	29	50	16
Age (range)	53.2 ± 8.7 (30–84)	51 ± 10.1 (30–84)	53.9 ± 7.1 (41–75)	55.1 ± 8.2 (32–68)
Male/female	61/34	18/11	33/17	10/6
Ethnicity (Caucasian/African-American/Hispanic/Asian)	73/13/8/1	24/3/1/1	39/6/5/0	10/4/2/0
BMI (range)	29.45 ± 5.3	29.8 ± 5.45	29.3 ± 4.6	29.5 ± 6.77
HCV RNA level (log_{10})	6.1 ± 0.67	6.0 ± 1.2	6.3 ± 0.67	5.9 ± 0.8
Glucose	115 ± 36.8	113.4 ± 42.7	116 ± 31.6	115 ± 39.9
Total bilirubin	2.5 ± 2.5	1.53 ± 1.28	3 ± 3	2.3 ± 1.5
Platelet ($\times 10^9$)	141 ± 85.7	163.6 ± 63.5	134.9 ± 96.2	127.8 ± 77.6
AST	110.7 ± 225.7	89.9 ± 79.1	129.8 ± 302.2	88.7 ± 60.7
ALT	96.1 ± 159.5	96.3 ± 101.7	99.4 ± 201.6	86.3 ± 74.1
ALP	91.8 ± 38.77	89.6 ± 33.2	93.1 ± 40.7	91.9 ± 41.1
GGTP	77.7 ± 106.8	91.4 ± 102.3	84.1 ± 123.8	44.8 ± 25.1
Histological Activity (≤ 4/≥ 5)	25/34 *	7/13 *	15/14 *	3/7 *
Fibrosis (≤ 2/3–5/6)	29/25/18 *	5/14/6 *	20/8/8 *	4/3/4 *
Steatosis (≤ 10%/11–32%/≥ 33%)	56/6/8 *	20/2/3 *	28/3/3 *	8/1/2 *

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* Where pathology data was available.