Viral genotype correlates with distinct liver gene transcription signatures in chronic hepatitis C virus infection

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Keywords
- genotype – HCV – interferon – ISGs – transcriptomics

Abbreviations
- DAAs, direct-acting antivirals; gt1, genotype 1; gt3, genotype 3; HCV, hepatitis C virus; IFN, interferon; ISG(s), interferon-stimulated gene(s); RIN, RNA integrity number; RNA-Seq, RNA sequencing; SVR, sustained virological response; Q-PCR, quantitative PCR.

Abstract

Background: Chronic hepatitis C virus (HCV) infection of the liver with either genotype 1 or genotype 3 gives rise to distinct pathologies, and the two viral genotypes respond differently to antiviral therapy. Methods: To understand these clinical differences, we compared gene transcription profiles in liver biopsies from patients infected with either gt1 or gt3, and uninfected controls. Results: Gt1-infected biopsies displayed elevated levels of transcripts regulated by type I and type III interferons (IFN), including genes that predict response to IFN-α therapy. In contrast, genes controlled by IFN-γ were induced in gt3-infected biopsies. Moreover, IFN-γ levels were higher in gt3-infected biopsies. Analysis of hepatocyte-derived cell lines confirmed that the genes upregulated in gt3 infection were preferentially induced by IFN-γ. The transcriptional profile of gt3 infection was unaffected by IFNL4 polymorphisms, providing a rationale for the reduced predictive power of IFNL genotyping in gt3-infected patients. Conclusions: The interactions between HCV genotypes 1 and 3 and hepatocytes are distinct. These unique interactions provide avenues to explore the biological mechanisms that drive viral genotype-specific differences in disease progression and treatment response. A greater understanding of the distinct host–pathogen interactions of the different HCV genotypes is required to facilitate optimal management of HCV infection.

Hepatitis C virus (HCV) is a highly diverse human pathogen, which is classified into seven distinct genotypes that vary by ~30% at the nucleotide sequence level (1). The virus establishes a chronic infection within liver hepatocytes and is one of the leading causes of liver disease worldwide (2). The development of novel direct-acting antivirals (DAAs) with higher efficacy than interferon (IFN) treatment (3, 4), and the first prophylactic vaccines moving into human trials (5), raises the possibility of combating HCV infection on a global scale. However, presently licensed DAAs act on a limited range of viral genotypes and the vaccines under development target only genotype (gt)1 viral antigens. While gt1 is prevalent worldwide (46.2% of HCV cases), over half of all infections are caused by other genotypes (6). Among these other HCV genotypes, gt3 is the most prevalent (accounting for 54.3 million cases, 30.1%) (6). Achieving HCV eradication requires a better understanding of the host–pathogen interactions that influence the natural history of each viral genotype.
Viral genotype is an important clinical indicator in the management of HCV infection, affecting both disease progression and treatment options. Gt3 infection is associated with decreased peripheral cholesterol levels and an increased prevalence of liver steatosis, which resolve upon successful viral clearance (7). There is also evidence that gt3 infection is independently associated with faster liver fibrosis progression (8). From the perspective of antiviral management of HCV infection, affecting both disease

Microarray analysis of hepatic gene expression has yielded valuable information on host–pathogen interactions during chronic HCV infection. From initial studies in chimpanzees, persistently infected animals displayed elevated transcription of genes including the following: (i) antiviral interferon-stimulated genes (ISGs), (ii) modulators of immune cell activity and (iii) regulators of lipid metabolism (10, 11). The same approach for analysis of human liver biopsies has identified similarities in the patterns of gene expression during chronic infection (12–15). These human and chimpanzee studies have largely focused on gt1 infection with limited data analysis on other viral genotypes, including gt3.

In this report, we set out to explore distinct host responses induced by different HCV genotypes. We hypothesise that the observed clinical features of gt1 and gt3 infection are related to fundamental differences in the nature of the virus–host interactions within liver cells. To explore this hypothesis, we have analysed the transcriptional changes in infected liver tissue using high-throughput RNA sequencing (RNA-Seq). This technology offers a broader dynamic range than microarray analysis and enables analysis of novel unannotated RNA transcripts. Our study aimed to create a transcriptional profile of gt1 and gt3 infection, as a platform to understand factors influencing the genotype-specific progression of liver disease and the response to antiviral therapy.

### Materials and methods

#### Clinical samples

Clinical samples were used with informed consent, conforming to the ethical guidelines of the 1975 Declaration of Helsinki. Study protocols were approved by the West of Scotland Research Ethics Committee and the Tayside Tissue Bank Access Committee. Liver needle biopsies from patients with chronic HCV infection (gt1, *n = 9*; gt3, *n = 9*), carried out as part of routine clinical care, were divided, with half fixed for routine histological examination and the remaining half snap-frozen in liquid nitrogen. Seven of the HCV-infected individuals had previously failed non-pegylated interferon therapy (4 gt1 and 3 gt3; Table 1), but none were receiving interferon therapy at the time of liver biopsy.

As a control group, RNA was extracted from uninfected liver biopsy and resection samples (*n = 6*). These included liver samples from patients with conditions that are associated with hepatic disease (one each with genetic haemochromatosis, non-alcoholic steatohepatitis and psoriasis) as well as three normal samples obtained during liver resection for metastatic colorectal adenocarcinoma. Clinical data are detailed in Table 1. Q-PCR analysis of conserved sequences in the 5′ untranslated region of the viral genome (16) confirmed that only liver biopsies from infected patients had detectable HCV RNA, and no difference in intrahepatic viral RNA levels was found between either gt1- or gt3-infected patients (data not shown).

### Table 1. Clinical data for liver biopsies

<table>
<thead>
<tr>
<th></th>
<th>Genotype 1</th>
<th>Genotype 3</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td><em>n</em></td>
<td>9</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>Male:Female</td>
<td>5:4</td>
<td>6:3</td>
<td>2:1*</td>
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<td>Previous Treatment Failure (Y:N)</td>
<td>4:5</td>
<td>3:6</td>
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<tr>
<td>rs368234815 Genotype (TT/TT:TT:AG:AG/AG)†</td>
<td>2:5:1</td>
<td>6:3:0</td>
<td>3:2:1</td>
</tr>
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<td>Ishak Inflammation Score (1-18)</td>
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<td>4 (2-7)</td>
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<tr>
<td>Ishak Fibrosis Score (0-6)</td>
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<tr>
<td>METAVIR Fibrosis Score (0-4)</td>
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<td>0 (0-4)</td>
<td>0.5 (0-1)</td>
</tr>
</tbody>
</table>

n/a, not applicable.

All values are given as the median with the range in brackets.

*Gender was unavailable for 3 anonymised control samples.
†rs368234815 genotype was unavailable for one gt1-infected patient.
RNA extraction
Snap-frozen liver biopsies were homogenised in 3 ml buffer RLT (Qiagen, Manchester, UK), supplemented with 30 μl β-mercaptoethanol, using a hand-held power homogenizer (Polytron; Kinematica AG, Lucerne, Switzerland), and total RNA was extracted using RNeasy Minicolumns with on-column DNase digestion (Qiagen). RNA quality and quantity were assessed on a 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA).

Illumina RNA-Seq
Samples were prepared with the Illumina TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA) and sequenced on an Illumina Genome Analyzer IIx (Glasgow Polyomics, University of Glasgow, Glasgow, UK). Raw FASTQ sequencing files (73 base reads) were quality-filtered for low-quality bases and adaptor sequences with Trim Galore v0.2.4 (http://www.bioinformatics.bbsrc.ac.uk/projects/trim_galore/). The RNA-Seq analysis was performed using the Tuxedo protocol (17), with full details provided in the supplementary methods. Differential gene expression was considered significant when the observed fold change of reads (ΔFPKM(log2)) was ≥1.0 and FDR/q-value <0.05 between comparisons. Pathway analysis was carried out using IPA (Ingenuity Systems, Redwood City, CA, USA).

Quantitative real-time PCR (Q-PCR) and genotyping
Total RNA was reverse-transcribed with the SuperScript® VILO™ Kit (Life Technologies, Paisley, UK), and gene expression was measured by quantitative real-time PCR (Q-PCR) using TaqMan® assays (Life Technologies; Table S1). Statistical significance was calculated using the Kruskal–Wallis test in GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Genotyping of IFNL4 rs368234815 was performed on cDNA using the assay described previously (18) with Type-it Fast SNP Probe PCR Master Mix (Qiagen).

Microarray analysis of IFN-α- and IFN-γ-stimulated hepatocyte-derived cell lines
Human hepatocyte-derived cells (HuH-7 and HepaRG) were mock-treated or incubated with 100 IU of IFN-α (Merck, Kenilworth, NJ, USA) or IFN-γ (Roche, Basel, Switzerland) for 24 h (n = 2). Total cellular RNA was extracted as above, and 300 ng of RNA was hybridised onto modified Agilent 4 × 44K microarrays containing 201 additional probes, corresponding to 119 genes previously not found on the array (Agilent Technologies). Raw data were extracted using Feature Extraction software (Agilent Technologies) and analysed with ChipInspector (Genomatix Software, Munich, Germany). Fold change values were calculated for each gene in response to IFN-α and IFN-γ (mean signal value treatment/mean signal value mock), as well as the ratio of responsiveness to IFN-α versus IFN-γ.

Results
Viral genotypes induce distinct liver transcriptomic profiles
To explore viral genotype-specific changes in liver gene expression, five of each of the gt1- and gt3-infected specimens were selected for high-throughput sequence analysis, on the basis of low fibrosis scores (METAVIR fibrosis score ≤1) and RIN values >6.5 (Table S2). From among the available uninfected biopsies, 4 samples were selected such that the control group included conditions associated with liver disease as well as a nor-
mal liver sample (Table S2). This approach was adopted to bias the resultant hits towards those regulated exclusively by HCV rather than liver inflammation in general, although this possibly reduced the number of hits that may have been obtained if solely healthy liver samples were utilised as controls. RNA-Seq generated ~17–25 million reads per sample, of which 82–87% uniquely mapped to the human genome (Table S2). All filtered sequence libraries had high quality, with >90% of reads having a total mean Phred quality score ≥32 and a mean per base Phred quality score of >34 (Fig. 1).

We identified a total of 348 and 565 genes that were differentially regulated (upregulated or downregulated with ΔFPKM(log2) ≥1.0 and q-value <0.05 as detailed in the supplementary data and summarised in Figure 1) in the gt1- and gt3-infected samples, respectively, compared to controls. A total of 196 genes were common to both gene sets (56% of the total differentially regulated genes in the gt1 samples and 35% of the total differentially regulated genes in the gt3 samples).

To directly explore viral genotype-specific transcriptional profiles, we analysed the sequencing data to identify genes differentially regulated between the gt1- and gt3-infected biopsies. A total of 149 genes were differentially regulated between the gt1- and gt3-infected samples with ΔFPKM(log2) ≥1.0 and q-value <0.05 (64 upregulated in gt3, 85 upregulated in gt1; Figure S2 and supplementary data). The finding that a significant number of genes are differentially regulated between the viral genotypes, together with the low level of overlap between the transcriptional profiles generated comparing either gt1- or gt3-infected samples to controls, highlights that viral genotype has a large influence on the regulation of host genes during chronic HCV infection.

To validate the RNA-Seq results, several differentially regulated genes were examined by Q-PCR using all available 24 liver RNA samples. The selected targets included genes that were differentially expressed in both gt1- and gt3-infected samples versus controls (CXCL9, IFIT1, ISG15 and RSAD2), as well as genes that were differentially regulated only in gt1 infection (USP18) or gt3 infection (IDO1). The Q-PCR data corresponded closely to the sequencing read counts confirming the RNA-Seq results (Fig. 1B).

### Enriched canonical pathways differ between HCV gt1 and gt3 infections

To explore potential biological processes underlying the observed transcriptional changes, the differentially regulated genes identified were analysed for pathway enrichment. The top enriched canonical pathways in the gt1- and gt3-infected liver biopsies compared to the controls differed markedly, with only three shared pathways in the top 10 enriched pathways, paralleling the small proportion of differentially regulated genes common to both comparisons (Table S3). While innate immune pathways were evident in the gt1-infected liver biopsies (e.g. activation of IRF by cytosolic pattern recognition receptors and interferon signalling), pathways relating to adaptive immune responses were more apparent in the gt3-infected liver biopsies (e.g. altered T- and B-cell signalling in rheumatoid arthritis, calcium-induced T-lymphocyte apoptosis, iCOS-iCOSL signalling in T-helper cells, B-cell development and CD28 signalling in T-helper cells). Despite the clinical association of gt3 infection with increased prevalence of liver steatosis and decreased peripheral cholesterol levels (7), no enrichment of lipid or cholesterol metabolic pathways occurred in the gt3-infected samples.

Pathway analysis was also performed on the 149 genes identified as differentially regulated between gt1- and gt3-infected liver biopsies. Among the top enriched canonical pathways, the antigen presentation pathway had the highest enrichment in gt3-infected samples, with B-cell development, allograft rejection signalling and altered T- and B-cell signalling in rheumatoid arthritis, also enriched in the gt3-infected patients (Table 2). The differentially regulated genes within these pathways include HLA genes involved in presentation of antigen to T-helper cells (HLA-DQB2, HLA-DRB1, HLA-DMA, HLA-DRA) as well as CD74, also known as the HLA invariant chain, which acts as a chaperone to regulate antigen presentation. Conversely, the interferon signalling pathway was enriched in the gt1-infected liver biopsies (Table 2), due to the elevated expression of a number of known ISGs, such as OAS1, MX1 and IFIT1.

### Type II IFN expression is elevated in HCV gt3 infection

The RNA-Seq data indicated that both gt1 and gt3 induced transcription of a number of ISGs within the liver, and this ISG induction was particularly evident in gt1 infection. To explore potential upstream regulators of the ISG response, we profiled transcription levels of...
type I, type II and type III IFNs, including IFNA2, IFNB1, IFNL2 and IFNG.

We detected expression of IFNA2, IFNB1 and IFNL2 in fewer than half of the biopsy samples and could not reliably quantify expression levels (data not shown). This low level of type I and type III IFN expression was also observed in the RNA-Seq data (data not shown), indicating that elevated type I and type III IFN expression is unlikely to directly account for the transcriptional changes observed in gt1-infected liver biopsies. Likewise, the role of type I, type II and type III IFN receptors could also be excluded as they were stably expressed across all biological groups (data not shown). Of the 4 tested IFN genes, only IFNG was expressed at a level that allowed reliable detection in liver tissues. IFNG expression was significantly higher in gt3-infected patients but did not differ significantly in gt1 infection compared to the control group (Fig. 2A).

Several genes identified within the gt3-specific enriched pathways are induced by IFN-γ, suggesting that the observed increase in IFNG gene expression may contribute to upstream regulation of these gt3-specific cellular pathways.

Identification of IFN-α- and IFN-γ-regulated genes in human hepatocyte-derived cell lines

As the transcriptional changes induced by different types of IFN overlap significantly (19, 20), it is difficult to classify ISGs as exclusively responsive to a particular type of IFN. Many ISGs do however show a preferential response to either type I or type II IFN. To identify particular ISGs which are preferentially induced by either IFN-α or IFN-γ in hepatocytes, we tested the effect of stimulation by both IFNs on 2 hepatocyte-derived cell lines, HuH-7 and HepaRG cells. By comparing the ratio of the IFN-α/IFN-γ fold change values in HuH-7 and HepaRG cells 24 hours following stimulation, we identified 14 preferentially IFN-α-responsive genes and 74 preferentially IFN-γ-responsive genes, 3 of which were consistently downregulated by IFN-γ (Table S4). Comparing the in vitro and in vivo results, the gt1-infected liver biopsies had a greater upregulation of 9 of the 14 identified preferentially IFN-α-responsive genes, while gt3-infected liver biopsies had a greater upregulation of 18 of the 22 identified preferentially IFN-γ-responsive genes.
genes (Fig. 2B and C). These results support a role for IFN-γ in driving gene transcription in the context of gt3 infection, while HCV gt1 infection is associated with a type I IFN-like transcriptional signature.

The influence of IFNL4 polymorphisms on ISG expression is evident in gt1 infection but not gt3 infection.

Previous studies have linked polymorphisms rs12979860 and rs8099917 located near IFNL3 to HCV susceptibility (21) and response to IFN-α-based therapy (22, 23). While the biological mechanisms underlying this association are debated, elevated induction of ISGs is observed in individuals carrying the unfavourable polymorphisms (24). More recent genetic analysis has revealed another polymorphism (rs368234815; previously designated ss469415590) in exon 1 of IFNL4, which is in high linkage disequilibrium with rs12979860 and, indeed, is a better marker for HCV clearance in certain ethnic populations (18). The expression of

![Graphs showing ISG mRNA transcription in gt3-infected liver biopsies not influenced by IFNL4 genotype.](image)

**Fig. 3.** ISG mRNA transcription in gt3-infected liver biopsies is not influenced by IFNL4 genotype. Dot plots of Q-PCR data segregated by IFNL4 (rs368234815) genotype (with geometric mean denoted by a horizontal line). Samples with the TT/TT genotype are plotted as circles, while samples with the AG/TT or AG/AG are plotted as squares. Fold changes values are calculated relative to the mean of the control group. For the control group, n = 3 for both the TT/TT and AG/TT or AG/AG genotypes (except for CXCL9 and IDO9 where n = 2 for both genotypes). For the gt1-infected group, n = 2 for the TT/TT genotype and n = 6 for the AG/TT or AG/AG genotypes (except for CXCL9 and IDO9 where n = 1 and n = 5 respectively). For the gt3-infected group, n = 6 for the TT/TT genotype and n = 3 for the AG/TT or AG/AG genotypes.
IFNL4 in tissue culture cells upregulates a number of ISGs. However, the rs368234815 TT variant results in a frameshift which disrupts the expression of IFNL4.

To determine whether the observed differences in gene expression were related to not only viral genotype but also host genetic background, we performed IFNL4 genotyping (rs368234815) on all but one of the samples used in our study (as detailed in Table 1). As shown in Figure 3, the presence of the ΔG variant, which would lead to expression of IFNL4, correlated with higher expression levels of ISGs such as IFIT1, ISG15, RSAD2 and USP18, within the gt1-infected group. Due to the low number of samples with TT/TT genotype, statistical analysis could not be performed within the gt1-infected group; however, our results parallel previous studies of gt1-infected individuals (24). In stark contrast, the expression of genes specifically induced in gt3-infected biopsies, such as CXCL9, IDO1 and IFNG, was not significantly different regardless of IFNL4 genotype (Fig. 3).

**Discussion**

Using high-throughput transcriptome sequencing, we identified distinct hepatic transcriptomic profiles associated with different viral genotypes within HCV-infected liver biopsies. Gt1 infection correlated with elevated type I IFN-like ISG transcription, while gt3 infection was associated with higher transcription of genes involved in cell-mediated immune responses and IFN-γ signalling. While previous studies have documented the induction of type I IFN-inducible ISGs and antiviral cellular pathways during chronic HCV infection, our results highlight that elevated ISG transcription is dependent on viral genotype. Hence, in contrast to gt1, gt3 infection is associated with the enrichment of pathways relating to adaptive immune responses and a weaker induction of ISGs. Despite the clinical association of gt3 infection with liver steatosis, we failed to detect any transcriptional differences in lipid metabolic pathways in these patients with early stages of liver disease. A recent metabolic study has identified a gt3-specific decrease in the serum levels of cholesterol metabolites (25), suggesting that changes associated with gt3 infection and steatosis may only be evident at a post-transcriptional level.

The pathways identified as regulated by HCV in previous studies vary considerably. Such variability undoubtedly emerges from differences in the physiological state of liver samples at time of collection, the extent of liver disease and distinct host genetic backgrounds. We have tried to minimise such variability by excluding liver samples with evidence of cirrhosis and using both normal liver samples, as well as samples from uninfected patients with conditions associated with hepatic disease, as controls. This approach has the impact of lowering the number of genes identified due to generic pathological processes within the liver, enabling a greater likelihood of determining host genes that are specifically regulated by HCV. Consequently, we believe that the resultant RNA-Seq data provide greater certainty that the up- and downregulation of cellular genes is virus mediated.

A central question arising from our study is why gt1 and gt3 mediate such different hepatic transcriptional responses. While we observed a general induction of ISGs by both genotypes compared to controls, the panel of induced ISGs was not identical. Whereas there is persistent induction of ISGs in gt1 infection, gt3 promotes differential expression of a larger set of genes, including factors stimulated by IFN-γ. We did not observe alterations in either type I or type III IFN transcription in the infected or uninfected biopsies. However, IFN-γ mRNA was expressed at significantly higher levels in gt3 infection and is associated with elevated expression of type II IFN-regulated genes. Elevated expression of type I IFNs is known to regulate responses to type II IFNs (26, 27), via downregulation of the IFN-γ receptor (28). These exaggerated type I IFN responses can drive immunopathology and antagonise protective IFN-γ responses during bacterial infections (29). In the context of HCV infection, exaggerated type I IFN-like responses may subvert the development of protective cell-mediated immune responses and suppress the expression of antiviral mediators that are driven by IFN-γ signalling (20). The reduced expression of HLA genes in gt1 infection compared to gt3 potentially impacts on antigen presentation within the liver and may lead to the development of defective cellular immune responses during gt1 infection.

From the perspective of treatment, high levels of ISGs are associated with poor response to therapy (15, 24, 30). Basal activation of the type I IFN signalling pathway induces a state of refractoriness to further stimulation (31), most likely through the action of ISGs that act as negative regulators of IFN-α signalling, such as the SOCS proteins and USP18 (32–34). Elevated ISG induction in the liver is associated with IFNL3 polymorphisms (24), although this association is not apparent within peripheral blood (35). The apparent discrepancy between peripheral blood and liver suggests that the effect of IFN-λ polymorphisms is strongest at the local site of HCV infection within the liver. These findings highlight that both viral and host factors influence ISG induction and the relative influence varies between local sites of infection and the periphery.

While the mechanisms underlying the relationship between IFN-λ polymorphism, liver ISG induction and treatment non-response are still unclear, recent studies have suggested that the unfavourable polymorphisms are associated with increased IFN-λ receptor 1 expression (36) and enhanced AU-rich element-mediated decay of IFNL3 mRNA (37). No differences were detected in the expression levels of any of the IFN-λ mRNAs, nor in the mRNA expression of the unique IFNLR1 receptor subunit in our samples. However, the induction of ISGs in gt1-infected liver biopsies did
correlate with the presence of the ΔG allele in IFNL4. By contrast, there was no evidence for a link between IFNL4 genotype and changes in gene expression in gt3 infection. These data provide a rationale for the reduced efficacy of IFNL SNPs in predicting gt3 treatment outcomes (38).

Our data demonstrate that gt1 infection drives a highly skewed type I IFN-like response, which is apparently influenced by host IFNL4 genotype. Conversely, gt3 induces upregulation of IFN-γ and IFN-γ-responsive ISGs, independent of host IFNL4 genotype. These transcriptional differences highlight fundamental differences in host–pathogen interactions that exist between different HCV genotypes and provide a platform for further research into the impact of viral genotype on host responses. Future studies that focus on changes in liver gene expression elicited by other genotypes will be important to understand how the natural history of infection is influenced by viral variation. Such knowledge will undoubtedly be vital to develop strategies for predicting outcomes of therapy and combating the different HCV genotypes circulating within human populations.

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Conflict of interest: All authors declare no conflict of interests.

References

HCV genotype-specific gene transcription

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Supporting information

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

Table S1. Gene selection for Q-PCR assays.
Table S2. Liver biopsy RNA-Seq total reads and % mapped to human genes.
Table S3. Top 10 canonical pathways in gt1- and gt3-infected liver biopsies versus controls.
Table S4. Genes preferentially responsive to either IFN-α or IFN-γ in HepaRG and HuH-7 cell lines.
Fig. S1. Filtered RNA-Seq sequencing library quality scores.
Fig. S2. Viral genotype-specific transcriptional changes within the liver.
Data S1. RNA-Seq Significant Genes.
Data S2. Methods.