Specific Polymorphisms in Hepatitis C Virus Genotype 3 Core Protein Associated with Intracellular Lipid Accumulation

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Background. Steatosis is a common histological finding and a poor prognostic indicator in patients with hepatitis C virus (HCV) infection. In HCV genotype 3–infected patients, the etiology of steatosis appears to be closely correlated with unknown viral factors that increase intracellular lipid levels. We hypothesize that specific sequence polymorphisms in HCV genotype 3 core protein may be associated with hepatic intracellular lipid accumulation.

Methods. Using selected serum samples from 8 HCV genotype 3–infected patients with or without steatosis, we sequenced the HCV core gene to identify candidate polymorphisms associated with increased intracellular lipid levels.

Results. Two polymorphisms at positions 182 and 186 of the core protein correlated with the presence (P = .03) and absence (P = .005) of intrahepatic steatosis. Transfected liver cell lines expressing core protein with steatosis-associated polymorphisms had increased intracellular lipid levels compared with non–steatosis-associated core isolates, as measured by oil red O staining (P = .02). Site-specific mutagenesis performed at positions 182 and 186 in steatosis-associated core genes yielded proteins that had decreased intracellular lipid levels in transfected cells (P = .03).

Conclusions. We have identified polymorphisms in HCV core protein genotype 3 that produce increased intracellular lipid levels and thus may play a significant role in lipid metabolism or trafficking, contributing to steatosis.

Steatosis is common in patients infected with hepatitis C virus (HCV) and contributes to the chronic hepatitis and progressive hepatic injury that can lead to end-stage liver disease and hepatocellular carcinoma [1–11]. HCV infection is currently the leading indication for adult liver transplantation in the United States, causes 8000–10,000 deaths per year, and has projected health care costs for 2010–2019 of $20–$50 billion [12–14].

HCV is a single-stranded, plus-sense RNA virus [15]. It is classified within the Flaviviridae family in the Hepacivirus genus [13]. The core protein forms the nucleocapsid of the virus, has 191 aa, and consists of 3 domains [13, 16–18]. Domain 3 (residues 175–191) is the hydrophobic signal-peptide domain that inserts into the endoplasmic reticulum membrane and facilitates (1) its own cleavage at residue 179 and (2) cleavage at the core-E1 junction to allow for subsequent E1 processing [1, 3].

Half of HCV-infected patients have evidence of steatosis on liver biopsy [2–9]. Several studies have shown HCV-related steatosis to be associated with accelerated fibrosis progression, impaired interferon response, and increased risk of hepatocellular carcinoma [1, 9]. Steatosis pathogenesis during HCV infection appears to involve viral and host factors [19–21]. Important host factors identified include alcohol use, obesity, diabetes, insulin resistance, and leptin levels [3, 9, 20, 22–25]. Patients infected with HCV genotype 3 have steatosis that correlates with serum HCV RNA levels, resolves with successful therapy, and is dependent of host factors [9]. Genotype 3–infected patients have steatosis that is more frequent and severe than genotype 1–infected pa-
patients [22]. Despite these findings, not all patients with genotype 3 infection have steatosis. These observations support a 2-pathway model of steatosis formation: one involving viral factor(s) present in most genotype 3 isolates and absent or reduced in other genotypes and another relying on manipulation of susceptible host pathways that is genotype independent [22, 26].

Previous steatosis research has primarily involved in vitro expression of genotype 1 core protein. A recent in vitro study expressed HCV core protein from genotypes 1–5 in HuH7 cells to address the role played by genotype [27]. Genotype 3a core protein showed 3-fold higher levels of triglyceride than genotypes 1b and 3h core, and no accumulation was seen with other genotypes, as detected by oil red O (ORO) staining. No significant sequence variations accounted for the results.

In the present study, we used a well-characterized repository of samples from patients infected with HCV genotype 3a who had either significant or no demonstrable steatosis. We identified polymorphisms within genotype 3a core isolates that correlated with clinical steatosis. Expression of these HCV genotype 3a core protein clones led to differences in intracellular lipid accumulation in liver cell lines. We subsequently manipulated these polymorphisms that differentiated core sequences from individuals with and without steatosis. We demonstrated that core protein residues 182 and 186 may have a significant association with hepatic lipid accumulation in the host.

MATERIALS AND METHODS

Patient selection. Patient serum samples were selected from an existing biorepository consisting of all pertinent host and virologic data from >400 patients with chronic HCV infection [3]. The study was approved by the Duke University Institutional Review Board.

Selection criteria included being treatment naive, having HCV genotype 3a infection, and having complete data and available serum samples. Abstinence from alcohol was required for 12 months before therapy. Liver biopsy specimens from a subset of 26 patients were examined, and patients were divided into those with steatosis (grade 3 [≥60% cells with fat]) on liver biopsy and those without steatosis (grade 0 [0%–2% cells with fat]), as judged by an experienced pathologist blinded to patient information [28]. Four samples were selected from each group for this initial study.

Table 1. Custom oligonucleotide primer sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV core genotype 3 forward</td>
<td>ATGCGAATTCGCCACCATGAGCACACTCTCTAAA</td>
</tr>
<tr>
<td>HCV core genotype 3 reverse</td>
<td>AGTCTCTAGATCATCAACCTTGTGCTGGATG</td>
</tr>
<tr>
<td>HCV core mutant V186I reverse</td>
<td>AGTCTCTAGATCATCAACCTTGTGCTGGATGAGATTAAAGCAAGA</td>
</tr>
<tr>
<td>HCV core mutant I186V reverse</td>
<td>AGTCTCTAGATCATCAACCTTGTGCTGGATGAGAAGAAAGCAAGA</td>
</tr>
<tr>
<td>HCV core mutant L182F reverse</td>
<td>AGTCTCTAGATCATCAACCTTGTGCTGGATGAGAAGAAAGCAAGA</td>
</tr>
</tbody>
</table>

NOTE. HCV, hepatitis C virus. Mutated codons are shown in boldface.
Individuals with HCV genotype 3 infection are at increased risk for steatosis; however, not all genotype 3–infected individuals develop steatosis. We speculated that the core protein of HCV genotype 3 has polymorphisms that are associated with steatosis. We hypothesized that expression of these clones would produce different degrees of intracellular lipid accumulation when expressed in vitro.

Sequence analysis. Our patient repository has samples from >400 HCV-infected patients, including >80 with HCV genotype 3a infection. After careful screening of patients with or without a diagnosis of steatosis, 8 patients HCV genotype 3a infection were selected for detailed sequence, molecular, and phenotypic analysis. Half of the patients had severe steatosis, and the other half had HCV infection but no steatosis, serving as a perfect control group. There were no significant differences in demographic variables between the 2 groups (table 2). As expected, the steatosis group had more severe steatosis than the nonsteatosis group (P < .001). We did note that the patients without steatosis had more advanced fibrosis in this small subgroup.

As summarized in figure 1A, our sequence analysis yielded highly related sequences with 96%–100% identity to each other and to a consensus 3a sequence from the HCV sequence database. Several isolates contained nonsynonymous substitutions that occurred at various positions, but most of these substitutions occurred in only 1 isolate from the group. No single substitution segregated the core consensus sequences into their clinical steatosis phenotype, but detailed analysis yielded important differences when residues 182 and 186 were examined in combination. We examined these 2 positions closely because substitutions occurred at these 2 positions in all isolates from patients without steatosis.
with steatosis but in no isolates from patients without steatosis. All core isolates from patients with steatosis had the amino acid pair phenylalanine-valine (FV) or leucine-isoleucine (LI) (figure 1B). All core isolates from patients without steatosis had the pair phenylalanine-isoleucine (FI). Only 1 patient sample (HCV3) yielded discordant sequence results. Statistical analysis showed the polymorphisms to be significantly related to their respective steatosis phenotype (for LI with steatosis, \( P < .028 \); for FI with steatosis, \( P < .005 \); for FV with steatosis, \( P = .3 \); for FV+LI with steatosis, \( P = .07 \)).

The core cDNAs isolated from patient samples were subsequently cloned for in vitro expression. Three of the isolates, HCV1, HCV11, and HCV12, were mutated using PCR with custom 3' primers that were designed to change the amino acid at position 182 or 186 depending on the clone: HCV1 V186I, HCV11 I186V, and HCV12 L182F. Plasmids were transfected

Figure 1. Amplification and sequence analysis of hepatitis C virus (HCV) core protein clones. A. Comparison of full predicted amino acid sequence of HCV core isolates in our study against a consensus genotype 3a clone (Con 3a) from the HCV sequence database (Los Alamos National Laboratory), performed using the ClustalW program. Differences in the individual amino acid sequence from the Con 3a clone are underlined. Asterisks at the bottom of a sequence comparison indicate complete identity at that position, and colons indicate a different amino acid that is closely related to the most commonly appearing residue. Sequences from patients with steatosis are underlined. B. Highlight of the comparison in panel A, focusing on domain 3 of HCV core protein, amino acid positions 181–190 (ClustalW). Con 3a is the consensus sequence shown in panel A. The sample nos. correspond to those internally assigned as part of the study. HCV1, HCV12, HCV17, and HCV26 are from patients with steatosis on biopsy. The amino acids at positions 182 and 186 are the phenylalanine-valine or leucine-isoleucine (LI) pair from their samples and are shown in boldface and are underlined. Three of 4 patients with steatosis had the LI pair in those positions. HCV3, HCV11, HCV16, and HCV23 are from patients without steatosis on biopsy. All 4 patients had the amino acid pairs phenylalanine-isoleucine (FI) in their samples at the same positions; these are shown in boldface and are underlined. The correlation between steatosis and the LI amino acid pair was significant (\( P < .005 \)), as was the correlation between no steatosis and the FI pair (\( P = .005 \)).
into Huh7 cells; figure 2A shows Western blot analysis of the 3 core isolates HCV1, HCV11, and HCV12 and corresponding mutants after 72 h. The expression of all clones resulted in size-appropriate protein bands of equivalent abundance.

**IF and ORO staining.** We designed a protocol that combined IF staining for HCV core protein and ORO staining to address (1) whether in vitro expression of steatosis clones resulted in increased intracellular lipid accumulation and (2) whether there were significant differences between expression of steatosis versus nonsteatosis clones. We transfected HepG2 cells with HCV core protein (data not shown) and analyzed samples at 48 h by IF/ORE staining. Although there were many lipid droplets within cells staining positive for HCV core protein, most other cells also had a significant quantity of intracellular lipid. This “background” lipid was seen in both Huh7 and Hep3B cell lines (data not shown), so analysis of subtle differences between clones would not have been possible. Despite transfection of many different liver cell lines, this high-lipid-level background was seen in most liver cell lines. Ultimately, we found the ideal model for our experiments—the HSC5H cell line, a clonally derived rat hepatic cell line with stellate cell characteristics that had previously been used for lipid metabolism experiments [29, 30]. This cell line had little or no lipid present under normal culture conditions. Control experiments showed equivalent levels of protein expression as detected by IF between HCV core clones (data not shown). Figure 2B illustrates 5H cells transfected with HCV core protein and analyzed after 48 h by IF/ORE staining. Cells expressing HCV core protein contain numerous lipid droplets, whereas cells not expressing HCV core protein have minimal detectable intracellular lipid in the field evaluated.

Detecting subtle differences using conventional biochemical techniques would have been difficult with our low transfection efficiency (5%) after optimization. Instead, image analysis with MetaMorph software was used to obtain quantitative measures of lipid accumulation (figure 3). During the core protein expression experiments, regions were set on the IF image and directly transferred to the ORO image, and total red was analyzed and recorded. The advantage was that no subjective bias from an investigator could be introduced at this stage. Figure 4A and 4B show validation experiments using 5H cells incubated in medium with 2%, 10%, and 20% FBS. The increasing fat content with increasing FBS content shown in the images correlated well with the levels of lipid accumulation detected by IF/ORE staining.

The figure is available in its entirety in the online edition of the *Journal of Infectious Diseases.*
with the increasing percentage of ORO staining measured by MetaMorph.

Transfections were performed in 5H cells, using each of the 3 HCV core clones expressed along with its corresponding mutant. Green fluorescent protein (GFP) and empty vector were used as controls. Transfections were performed in duplicate, followed by IF/ORO staining after 48 h. Analysis was performed on twenty ×63 images for each transfected well (figure 5A). GFP resulted in minimal (1%) ORO staining, whereas expression of all of the HCV core clones caused increased lipid accumulation. The lipid droplets were primarily perinuclear except in cells with lipid overload, in which the droplets were distributed throughout the cytoplasm. Expression of the steatosis-associated HCV1 and HCV12 clones resulted in significantly more ORO staining per region compared with the nonsteatosis HCV11 clone (HCV1, 11.4% ± 6.7%; HCV12, 10.8% ± 4.7% vs. 7.8% ± 3.3%; $P = .02$ and $P = .01$, respectively) (figure 5A and 5B). Expression of HCV-N core, which is a genotype 1b isolate, resulted in less intracellular lipid (8.99% ± 4.7%; $P = .14$).

**Analysis of core mutant clones.** Mutants for HCV1 and HCV12 were designed to test the significance of polymorphisms at positions 182 and 186 in predicting intracellular lipid accumulation. Mutants were directly compared with the parent sequence. The HCV1 mutant, with the V186I mutation intended to reverse its steatosis phenotype, had 27% less ORO staining per region than did the parent clone (11.4% ± 6.7% vs. 8.3% ± 4.8%; $P = .03$) (figure 6A and 6B). The same was true for another steatosis clone, HCV12, compared with its mutant L182F clone; there was 37% less intracellular lipid with the mutant clone ($P = .01$) (data not shown).

**DISCUSSION**

We have identified amino acid substitutions at positions 182 and 186 of the HCV genotype 3a core protein that cause increased intracellular lipid accumulation in hepatic cells and may contribute to steatosis development. These sequence differences segregated a carefully selected group of patients with or without

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**Figure 4.** Validation of MetaMorph analysis of oil red O (ORO) staining.

**Figure 5.** Graphic representation of the MetaMorph analysis results. Twenty ×68 high-power fields were compared for each of the clones represented. As described for figure 3, results are expressed as the percentage of oil red O (ORO) staining, measured as the area within the region that met or surpassed the red threshold applied. A, Statistical analysis of comparisons between the groups. For 5H cells expressing the green fluorescent protein (GFP) control vector, 1.1% of the area stained with ORO, on average. For cells expressing the hepatitis C virus (HCV) clones HCV1 and HCV12 (steatosis), the respective averages were 11.4% and 10.8%, compared with only 7.8% for cells expressing the HCV11 clone (nonsteatosis). These differences were significant ($P = .02$ and $P = .01$, respectively). For HCV-N, a genotype 1b clone, the average ORO staining was 8.9% (for HCV1 and HCV 12 vs. HCV-N, $P = .14$). B, Images from each group that represent the average percentages of ORO staining in the MetaMorph analysis. Immunofluorescence (IF) and ORO images are shown for GFP, HCV1, and HCV11.
steatosis in all cases except 1 in this pilot study. In vitro expression of these clones led to increased intracellular fat levels, as detected by ORO staining, and we found significant differences of intracellular lipid levels when clones from patients with steatosis were expressed compared with those from patients without steatosis. Mutations at positions 182 and 186 in steatosis clones were successful in decreasing the amount of intracellular lipid. These findings suggest that this domain of core protein plays an important role in regulating cellular lipid metabolism or trafficking.

Our results are consistent with findings of prior studies examining the role played by the HCV genotype 3 core protein in steatosis. The genotype 3a core sequence used in the study of Abid et al. [27] had the FV amino acid pair at positions 182 and 186, consistent with our findings. Abid et al. also found moderate lipid accumulation with genotype 1 core protein, but it was 3-fold less than that with genotype 3a. These experiments indicated that in vitro expression of most HCV core protein isolates led to some intracellular lipid accumulation, which would explain why our nonsteatosis clones still led to increased lipid levels. Our findings are novel because we show distinct differences between genotype 3a isolates, which may explain why certain genotype 3a isolates produce no clinical steatosis. Previous studies used consensus genotype clones, which did not enable any analysis of differences within a genotype. Considering the results of Abid et al. along with our own, we hypothesize that lipid accumulation occurs along a continuum, with genotype 3 steatosis clones at the accumulation end, our genotype 3 nonsteatosis clones and genotypes 1 and 2 toward the middle, and the rest of the genotypes at the nonaccumulation end of this spectrum. The lipid accumulation observed with a genotype 1b clone in our experiments is consistent with this hypothesis. We speculate that domain 3 plays a significant role in determining intracellular lipid accumulation along this spectrum.

Domain 3 is the E1 signal peptide region that facilitates cleavage to mature core protein and proper cleavage at the Core-E1 junction. Its fate after both cleavage events is unknown. Given our findings, we speculate (1) that domain 3 interacts with host proteins within the endoplasmic reticulum membrane that mediate lipid metabolism and trafficking and (2) that this interaction may differ between genotypes. This domain is predicted to form a helix, and different amino acids may act as “helix benders” in this setting [17, 31]. Compared with the LANL HCV sequence database, our analysis identified the 3 combinations that exist within genotype 3a at residues 182 and 186: FI, LI, and FV. Virtually all genotype 1 isolates, including the HCV-N used

Figure 6. Analysis of specific mutations of steatosis-associated clones. A, After the hepatitis C virus (HCV) clone HCV1 had its amino acid at position 186 changed from valine to isoleucine, which should change its phenotype from steatosis to nonsteatosis, cells expressing this HCV1 mutant clone had an average of only 8.3% staining with oil red 0 (ORO). When this clone was compared with the parent HCV1 clone, the difference was significant (P = .03). B, Images from each group that represent the average percentages of ORO staining in the MetaMorph analysis. Immunofluorescence (IF) and ORO images are shown for GFP, HCV1, and HCV1 V186I mutant. GFP, green fluorescent protein.
in our experiment, have leucine and threonine at positions 182 and 186, respectively. We speculate that differences in the amino acid pairs between our clones may alter the helix structure enough to change the nature of the interactions with host proteins that mediate lipid accumulation.

Previous research into the pathogenesis of steatosis has primarily evaluated the expression of genotype 1 core protein. These findings have indicated that transgenic mice expressing core protein inconsistently develop age-related steatosis, that core protein and NS5A colocalize to lipid droplets, that core protein inhibits microsomal triglyceride transfer protein activity and the synthesis of very-low-density lipoprotein, and that core protein causes mitochondrial toxicity and reactive oxygen species production [32–37]. Deletion analysis of a genotype 1a core isolate demonstrated that specific helix elements within domain 2 are required for lipid droplet association [16, 17, 38]. These domain 2 regions are common to many HCV genotypes [17, 31], and the sequences of all of our clones are almost identical to the genotype 1 clone used in the lipid droplet study by Boulant et al. [38]. Given this identity, differences in lipid droplet binding could not account for the differences we found. Our data support the idea that intracellular lipid accumulation is not related to the ability of HCV core protein to bind to lipid droplets.

Recent studies examining a possible mechanism of steatosis formation in genotype 3a isolates have focused on the phenylalanine at position 164 up-regulating fatty acid synthase activity [39, 40]. Comparisons with genotype 1 isolates that have tyrosine at this position and mutational analysis have supported these findings. Fatty acid synthase up-regulation by this residue could not account for the changes observed between our isolates, because all of them had phenylalanine at this position and were identical throughout this region of the protein.

We used a rat-derived liver cell line. Our work with more traditional liver cell lines used with HCV (Huh7, HepG2, and Hep3B) revealed far too much intracellular lipid in standard culture conditions for us to detect any meaningful differences between the clones studied. This problem of “background” steatosis has been seen by other investigators (S. Weinman, personal communication). Many meaningful studies of basic metabolic pathways within the liver have been performed in rodent cells [41–44].

The studies presented here provide novel insights into how the highly related 3a core proteins may dramatically alter lipid accumulation through 2 polymorphisms. These polymorphisms may be significant in determining the degree of lipid accumulation in vivo and thus the degree of subsequent steatosis. Because our study involved a small number of genotype 3a core sequences expressed outside the context of the other HCV proteins, we are currently performing more comprehensive studies comparing various genotype core sequences to further correlate the polymorphisms at positions 182 and 186 with intracellular lipid accumulation and possible clinical progression toward steatosis. Our future efforts to elucidate the cellular and biochemical consequences of domain 3 polymorphisms with their intracellular function will provide a broader view of the viral contributions to HCV-associated steatosis and hepatic fibrosis.

In the present study, we discovered specific sequence polymorphisms in HCV genotype 3 isolates that segregate patients with clinical steatosis from those without steatosis. Expression of the steatosis clones led to significantly more intracellular lipid accumulation than observed with nonsteatosis clones. Mutation of these residues in steatosis clones reduced intracellular lipid accumulation. These results provide us with a model to study HCV core-associated changes in lipid metabolism. In our future work, we will attempt to elucidate the mechanisms involved and explore how steatosis may offer the virus a survival advantage.

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References


