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ORIGINAL ARTICLE

Replication and infectivity of a novel genotype 1b hepatitis C virus clone

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ABSTRACT

Hepatitis C virus infection is a major public health problem because of an estimated 170 million carriers worldwide. Genotype 1b is the major subtype of HCV in many countries and is resistant to interferon therapy. Study of the viral life cycle is important for understanding the mechanisms of interferon resistance of genotype 1b HCV strains. For such studies, genotype 1b HCV strains that can replicate and produce infectious virus particles in cultured cells are required. In the present study, we isolated HCV cDNA, which we named the NC1 strain, from a patient with acute severe hepatitis. Subgenomic replicon experiments revealed that several mutations enhanced the colony-formation efficiency of the NC1 replicon. The full-length NC1 genome with these adaptive mutations could replicate in cultured cells and produce infectious virus particles. The density gradient profile and morphology of the secreted virus particles were similar to those reported for the JFH-1 virus. Further introduction of a combination of mutations of the NS3 and NS5a regions into the NC1 mutants further enhanced secreted core protein levels and infectious virus titers in the culture medium of HCV-RNA-transfected cells. However, the virus infection efficiency was not sufficient for autonomous virus propagation in cultured cells. In conclusion, we established a novel cell culture-adapted genotype 1b HCV strain, termed NC1, which can produce infectious virus when the viral RNA is transfected into cells. This system provides an important opportunity for studying the life cycle of the genotype 1b HCV.

Key words genotype 1b, hepatitis C virus (HCV), replicon, virus culture.

Hepatitis C virus infection leads to chronic liver diseases including cirrhosis and hepatocellular carcinoma, and is a major public health problem because of an estimated 170 million carriers worldwide (1–3). HCV is a plus-strand RNA virus that displays marked genetic heterogeneity and is currently classified into six major genotypes (4). Some HCV genotypes display regional distribution, although genotypes 1 and 2 occur worldwide. Genotype 1b is the major subtype in Japan, whereas genotype 2a is the most common minor subtype (5). Infection with genotype 1b HCV is known to be resistant to interferon therapy, whereas infection with genotype 2a is

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List of Abbreviations: CFU, colony-forming units; DMEM, Dulbecco's modified Eagle's medium; EMCV, encephalomyocarditis virus; ffu, focus-forming units; HCV, hepatitis C virus; IFN, interferon; IRES, internal ribosomal entry site; PI, protease inhibitors; RTD-PCR, real-time detection RT-PCR.

usually sensitive to such intervention (6). Current standard therapy for HCV-related chronic hepatitis is based on a combination of IFN and ribavirin, although virus eradication rates are limited to approximately 50% for genotype 1b HCV infection (7–9). PI have been approved for clinical use against HCV infection in the USA, Europe and Japan, and triple combination therapy that includes PI is expected to improve treatment efficacy. However, the development of other anti-HCV drugs with different modes of action is important to achieve greater efficacy and to avoid the emergence of drug-resistant viruses. To that end, a detailed understanding of the viral replication mechanism is needed to identify novel antiviral targets.

Although HCV belongs to the *Flaviviridae* family and has a genome structure similar to the other flaviviruses, efficient virus propagation in cultured cells has been difficult ever since the discovery of the virus (10). A subgenomic HCV-RNA replicon system was developed using the genotype 1b Con1 strain (11), which enabled the assessment of HCV replication in cultured cells. Subsequently, the genotype 2a JFH-1 strain was cloned and a HCV culture system was established using this strain (12–14). However, such efficient virus production could not be reproduced using genotype 1b HCV strains, even when adaptive mutations were introduced to enhance their replication efficiency in cultured cells (15). Thus, it remains necessary to obtain genotype 1b HCV strains that can replicate and produce infectious virus particles in cultured cells.

In the present study, we isolated HCV cDNA, which we named the NC1 strain, from a patient with acute severe hepatitis. In a subgenomic replicon experiment using the NC1 clone, we found several mutations that enhanced colony-formation efficiency of the NC1 replicon. Interestingly, the full-length NC1 genome with these adaptive mutations could replicate in cultured cells and produce infectious virus particles. However, the viral infection efficiency was not sufficient for autonomous virus propagation in cultured cells even though virus production efficiency could be increased by the introduction of multiple mutations into the virus genome.

MATERIALS AND METHODS

Cell culture system

Huh7 and Huh7.5.1 cells (a generous gift from Dr Francis V. Chisari) were cultured in 5% CO_2 at 37°C in DMEM containing 10% fetal bovine serum (DMEM-10) (13, 16).

HCV clones

The genotype 1b HCV clone NC1 was isolated from a patient with acute severe hepatitis (prothrombin time <40%). The patient was a 48-year-old woman who had no history of blood transfusions. Total RNA was extracted from the serum during the acute phase, and HCV cDNA covering the entire genome was amplified by RT-PCR using HCV-specific primers (S-Table 1). All amplified products were purified and then cloned into pGEM-T Easy^{TM} vectors (Promega, Madison, WI, USA). PCR products and plasmids were sequenced using HCV-specific primer sets (S-Table1), a Big Dye Terminator Mix and an automated DNA sequencer (PE Biosystems, Foster City, CA, USA). Based on the consensus sequence of the NC1 strain, we then assembled pNC1 (DDBJ/EMBL/GenBank accession number: AB691953), which contained the full-length NC1 cDNA downstream of the T7 RNA polymerase promoter. An NC1 subgenomic replicon construct, pSGR-NC1, was also assembled according to previously published methods used for Con1 replicon and pSGR-JFH1 construction (10, 17). The first 213 nucleotides at the NS3 region of pSGR-NC1 are identical with the Con1 sequence because of the restriction site used for plasmid construction. Several mutations were introduced into the pNC1 and pSGR-NC1 constructs as reported previously using specific primer sets (S-Table 2) (17). pNC1/wt contained the wild-type sequence of the NC1 strain. pNC1/SY and pNC1/SG contained S2197Y and S2204G mutations, respectively. pNC1/EGSY contained both E1202G and S2197Y mutations. pNC1/KTSY contained both K1846T and S2197Y mutations. pNC1/EGKTSY contained the three mutations, E1202G, K1846T and S2197Y.

Subgenomic replicon assay

Subgenomic replicon RNA was synthesized as reported previously (17). The synthesized replicon RNA (3 μg) was adjusted to a total RNA amount of 10 μg using cellular RNA isolated from non-transfected Huh7 cells and was then electroporated into naive Huh7 and Huh 7.5.1 cells as reported previously (17). G418 (1 mg/mL) was added to the culture medium for 3 weeks, and drug-resistant colonies were fixed with buffered formalin and stained with crystal violet or were cloned for further analysis. For the cloning analysis of the replicon cells, G418-resistant colonies were isolated by using cloning cylinders (Asahi Techno Glass Co., Tokyo, Japan) and were expanded until they reached 80% to 90% confluency in 10-cm diameter dishes. Total RNA was extracted from the cloned G418-resistant cells by using the ISOGEN reagent (Nippon Gene, Tokyo, Japan), and the replicon RNA was quantified using real-time detection RT-PCR (RTD-PCR) as reported previously (18). The cDNAs of the HCV-RNA replicon were synthesized and then amplified by PCR. The sequence of each replicon was determined (17).

Full-length HCV-RNA transfection

Full-length HCV-RNA was synthesized from pNC1 and its derivatives that contained adaptive mutations as described previously (12, 19, 20). Synthesized HCV-RNA (10 μg) was transfected into Huh7.5.1 cells. The HCV core protein level in the culture supernatant was measured using Lumipulse Ortho HCV Ag (Ortho-Clinical Diagnostics, Tokyo, Japan) (21), and infectivity of the supernatant was determined by measurement of its focus-formation efficiency (13, 19). In some experiments, the transfected cell pellet was harvested and dissolved with RIPA buffer containing 0.1% SDS. HCV core protein levels in cell lysates were also measured using the Lumipulse Ortho HCV Ag.

Density gradient analysis of secreted virus particles

Culture medium (1 mL) derived from transfected cells was harvested for density gradient analysis 2 or 15 days after transfection of full-length NC1/SY HCV RNA. The cleared culture medium was layered onto a stepwise sucrose gradient (60% [wt/vol] to 10%) and centrifuged for 16 hrs in an SW41 rotor (Beckman, Palo Alto, CA, USA) at 200,000 $\times$ *g* at 4°C. After centrifugation, 18 fractions were harvested from the bottom of the tubes. The core protein concentration in each fraction was determined by analysis of 100 μL of each fraction.

Electron microscopy

Culture medium (150 mL) containing NC1/SY virus particles were collected from the cells 15 days after transfected cells. The collected culture medium was ultrafiltrated and concentrated 20-fold using a pellicon-2 filter system (Millipore, Bedford, MA, USA). The sample was layered onto a stepwise sucrose gradient (60% [wt/vol] to 10%) and centrifuged for 4 hrs in an SW28 rotor (Beckman, Palo Alto, CA, USA) at 140,000 $\times$ *g* at 4°C. Thereafter, fractions were harvested from the bottom of the tubes. The core protein concentration in each fraction was determined and the fraction with the highest core protein level was ultrafiltrated for electron microscopic analysis. To visualize HCV particles, we adsorbed the purified virus samples onto carbon-coated grids for 1 min. The grids were then stained with 1% uranyl acetate for 1 min and examined under an H-7650 transmission electron microscope (Hitachi High-Technologies Co., Tokyo, Japan) (22).

Human hepatocyte chimeric mice experiments

Human hepatocytes were transplanted into uPA$^{+/+}$/SCID$^{+/+}$ mice as described previously (23, 24). These mice were obtained from Phoenix Bio Co., Ltd (Hiroshima, Japan). All mice received hepatocyte transplants from the same donor. Human albumin levels in the sera of the mice were monitored to evaluate the percentage replacement of mouse hepatocytes with human hepatocytes in the mouse liver. These human hepatocyte chimeric mice, in which the mouse liver cells were largely (>90%) replaced with human hepatocytes, were used to reduce the potential influence of mouse-derived mRNA on the results obtained. Two mice were each inoculated with purified NC1/SY HCV particles containing 11.6 fmol core protein. The HCV-RNA titer in inoculated mouse serum was monitored by RTD-PCR each week after inoculation.

RESULTS

Genotype 1b HCV clone isolated from a patient with acute severe hepatitis

HCV cDNA was isolated from a patient with acute severe hepatitis. This patient was a 48-year-old woman without any history of blood transfusions. She developed acute severe hepatitis as diagnosed by acute liver failure associated with stage I encephalopathy and low serum prothrombin time (<40%). In the patient's serum, no marker of cytomegalovirus, Epstein-Barr virus, herpes simplex virus, hepatitis A virus or hepatitis B virus was detected. However, only HCV-RNA was detected using RT-PCR analysis, and the virus RNA titer in her serum at admission was 16,660 KIU/mL. The genotype 1b HCV sequence was detected by sequencing analysis of the PCR fragment. The patient was therefore diagnosed as suffering from acute severe hepatitis caused by genotype 1b HCV infection. She received IFN-α treatment, which cleared the viral infection, and she subsequently recovered. The entire sequence of the amplified HCV genomic cDNA was then determined and this HCV strain was designated as NC1. The sequencing analysis indicated that this NC1 strain shared 91.2% nucleotide and 94.4% amino acid sequence homology with the Con1 strain.

Subgenomic replicon analysis of the NC1 strain

To analyze the replication efficiency of the genotype 1b NC1 strain, we constructed the subgenomic replicon, pSGR-NC1, in the form of a Con1 replicon and pSGR-JFH1 (10, 17). This synthesized NC1 replicon RNA was transfected by electroporation into Huh7 or Huh7.5.1 cells. The transfected cells were then grown for 3 weeks in a selection culture that contained 1 mg/mL G418. Several colonies survived this selection culture, as illustrated by crystal violet staining (Fig. 1). The

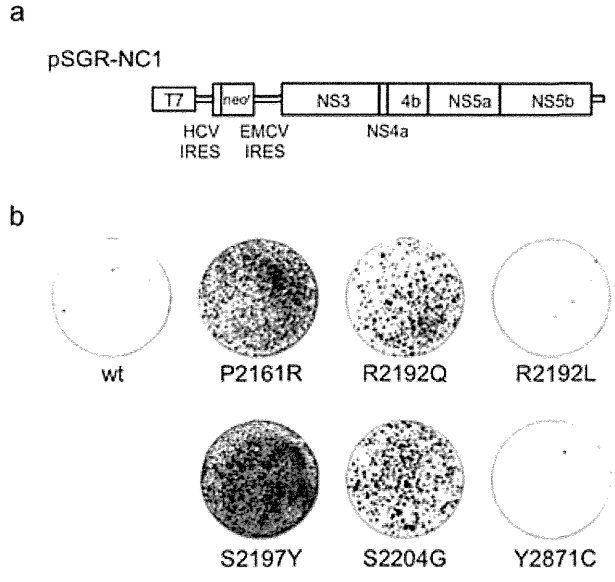


Fig. 1. NC1 subgenomic HCV replicon construct and colony formation of NC1 replicon RNA-transfected Huh7 cells. (a) Organization of the subgenomic replicon construct pSGR-NC1. Open reading frames (wide boxes) are flanked by untranslated regions (narrow boxes). The T7 RNA promoter is located upstream of the 5′ end of the replicon construct. (b) Subgenomic RNAs were synthesized *in vitro* using pSGR-NC1 (wt) and replicon constructs containing one of the mutations P2161R, R2192Q, R2192L, S2197Y, S2204G and Y2871C as templates. Transcribed subgenomic replicon RNAs (3 μg each) were electroporated into Huh7 cells and the cells were cultured with G418 for 3 weeks before staining with crystal violet. Experiments were carried out in triplicate and representative staining examples are shown.

colony-formation efficiency of the NC1 replicons was 1.77 ± 1.54 CFU/μg RNA, which was lower than the colony-formation efficiency of the JFH-1 subgenomic replicon that was determined in our previous study ($5.32 \pm 5.02 \times 10^4$ CFU/μg RNA) (17). Six colonies of the transfected Huh7 cells and 14 colonies of the transfected Huh7.5.1 cells were cloned and expanded for further analysis. Replicon RNA was isolated from each replicon cell clone, and the HCV-RNA titer and sequence of the replicon genome were determined (Table 1). The average HCV-RNA titers in the replicon cell clones derived from Huh7 and Huh7.5.1 cells were determined by RTD-PCR as $4.28 \pm 3.43 \times 10^6$ and $6.72 \pm 7.14 \times 10^6$ copies/μg RNA, respectively.

We next determined the sequence of the replicating replicon genome in each replicon transfected cell clone. All of the clones had at least one non-synonymous mutation and three clones also had a synonymous mutation (Table 1). We found non-synonymous mutations in all of the subgenomic non-structural regions of the replicon genome, and five of the mutations were found in more than one replicon genomic clone. Thus, of the mutations found in the NS5A region, P2161R, R2192L and S2204G mutations were independently detected in two different replicon cell clones, and the S2197Y mutation was detected in three different replicon cell clones. In addition, R2192 was not only mutated to R2192L but was also found as an R2192Q mutation in one replicon. The Y2871C mutation in NS5B was detected in two different replicon cell clones. We therefore selected P2161R, R2192L, R2192Q, S2197Y, S2204G and Y2871C mutations for further analysis to determine their adaptive effects. We inserted P2161R, R2192L, R2192Q, S2197Y, S2204G and Y2871C mutations into the pSGR-NC1 genome and tested

Table 1. Mutations and RNA titer of the NC1 replicon cell clones

Replicon clones†	nt mutation	nt position‡	aa mutation	aa position§	Region	Replicon RNA titer
2	G>A	5302(6916)	R>Q	2192	NS5a	3.58E+05
	T>Y	5490(7104)	S>S,P	2255	NS5a	
5	G>R	4046(5660)	none		NS4b	1.56E+07
	A>G	4088(5702)	I>M	1787	NS4b	
	C>A	5317(6931)	S>Y	2205	NS5a	
	C>S	6579(8193)	Q>Q,E	2618	NS5b	
2–1	T>C	3965(5579)	none		NS4b	3.17E+05
	T>G	5346(6960)	S>A	2207	NS5a	
2–2	C>G	5209(6823)	P>R	2161	NS5a	8.16E+06
2–3	T>C	1721	none		E-I ††	3.97E+06
	C>A	4855(6469)	S>Y	2043	NS5a	
2–5	T>C	3694(5308)	V>C	1656	NS3	1.80E+07
	C>G	5209(6823)	P>R	2161	NS5a	
2–7	G>T	5302(6916)	R>L	2192	NS5a	2.91E+06
2–8	T>C	5067(6681)	C>R	2114	NS5a	1.98E+06
	A>G	7339(8953)	Y>C	2871	NS5b	
2–9	C>A	5317(6931)	S>Y	2197	NS5a	1.58E+07
2–10	A>G	5287(6901)	K>R	2187	NS5a	1.90E+07
	A>G	5337(6951)	S>G	2204	NS5a	
	A>G	7339(8953)	Y>C	2871	NS5b	
2–11	C>A	584	D>E		NEO	2.50E+06
	A>T	3762(5376)	S>C	1679	NS4a	
	T>A	5248(6862)	M>K	2174	NS5a	
2–12	C>T	5291(6905)	none		NS5a	1.02E+06
	G>T	5302(6916)	R>L	2192	NS5a	
2–13	C>A	4850(6464)	N>K	2041	NS5a	3.65E+06
2–15	C>T	5299(6913)	A>V	2191	NS5a	8.07E+05
	A>G	5337(6951)	S>G	2204	NS5a	
	A>G	6014(7628)	none		NS5b	
	A>G	6015(7629)	I>V	2430	NS5b	
H1	C>A	5301(6915)	none		NS5a	1.01E+07
	C>A	5317(6931)	S>Y	2197	NS5a	
H3	G>A	661	G>E		NEO	4.77E+06
	C>A	5323(6937)	A>D	2199	NS5a	
H6	C>A	5265(6879)	H>N	2180	NS5a	1.21E+06
H7	C>A	5308(6922)	S>Y	2194	NS5a	4.53E+05
H10	C>A	5317(6931)	S>Y	2197	NS5a	5.12E+06
H12	A>C	5325(6939)	S>R	2200	NS5a	4.03E+06

†Replicon clones 2~2–15 were derived from Huh7.5.1 cells, and the H1~H12 replicon clones were derived from Huh7 cells. ‡Position within the subgenomic replicon and within full-length NC1 (in parentheses).
§Position within the complete open reading frame of full-length NC1. ¶Copy/μg RNA. ††E-I, EMCV-IRES.

the colony-formation efficiency of the mutant replicons. As shown in Figure 1b, P2161R, R2192Q, S2197Y and S2204G mutations had adaptive effects for colony formation. However, R2192L and Y2871C mutations did not enhance colony-formation efficiency.

Full-length HCV replication

As shown above, some of the mutations detected in the NC1 subgenomic replicon that was transfected into cells exhibited adaptive effects that increased colony-formation efficiency. We next determined whether full-length NC1 HCV clones with these mutations were able to replicate in cultured cells and to produce infectious virus. We therefore introduced the same six mutations that we tested in the subgenomic replicon assay into the full-length NC1 HCV cDNA (Fig. 2a). Full-length viral RNA was synthesized from linearized pNC1 and its mutant derivatives and was electroporated into Huh7.5.1 cells. All of the transfected cells were serially passaged, and HCV core protein levels in the culture supernatant were monitored over time (Fig. 2b). Interestingly, only cells transfected with the NC1 HCV-RNA with an S2197Y or S2204G mutation secreted a significant amount of HCV core protein into the culture medium. Secreted HCV core protein levels reached a maximum at 5–10 days post- transfection and subsequently continuously decreased in the culture medium of the cells transfected with the viral RNA containing S2197Y or S2204G mutation until 32 days after transfection. This reduction in HCV core protein levels after a certain number of cell passages was reproducible and was confirmed in repeated experiments.

Characterization of the NC1 virus particles secreted from NC1 RNA-transfected cells

Of the six NC1 constructs with mutations that were transfected into cells, transfection of NC1/SY RNA, which had the S2197Y mutation, resulted in secretion of the highest level of HCV core protein into the culture medium (Fig. 2b). We therefore further analyzed the HCV particles produced from NC1/SY RNA-transfected cells. Synthesized NC1/SY RNA was transfected again into Huh7.5.1 cells, the transfected cells were passaged and the culture medium was harvested at cell passaging. HCV core protein levels were relatively high in the culture medium from the NC1/SY RNA-transfected cells on days 2 and 15 post-transfection (Table 2). Infectivity of the culture medium was also detected at both time points, although at very low levels (Table 2). We also analyzed HCV core protein in the culture media obtained at these two time points using a density gradient assay. Both media exhibited a high, narrow peak of HCV core protein in fraction 9, which had a density of 1.15 g/mL, as well as a broader

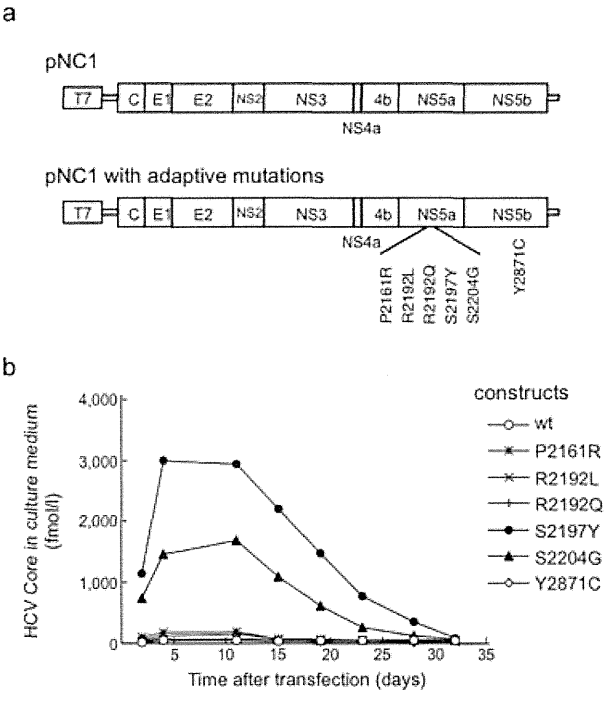


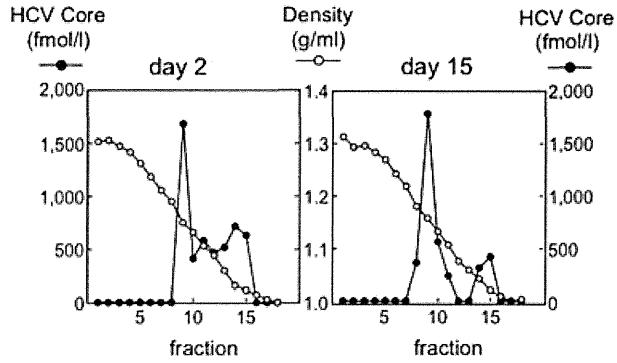
Fig. 2. Analysis of the HCV core protein released into the cell culture medium after transfection of NC1 full-length HCV-RNA. (a) Organization of the full-length NC1construct, pNC1 (upper). Open reading frames (wide boxes) are flanked by untranslated regions (narrow boxes). A T7 RNA promoter is located upstream of the 5′ end of the HCV cDNA construct. Six of the mutations identified in the replicon cell clones were independently introduced into the pNC1 construct (lower). (b) Huh7.5.1 cells were transfected with RNA transcribed from pNC1 with the wild-type HCV sequence (wt) or with RNA from one of the six constructs containing one of the mutations P2161R, R2192Q, R2192L, S2197Y, S2204G or Y2871C. Two independently transfected cell lines were passaged for each construct. At each time point after transfection, the culture medium was harvested and analyzed for the presence of HCV core protein. Average data of duplicate transfections are indicated.

Table 2. HCV core protein and infectivity levels in the culture medium of NC1/SY RNA-transfected cells

Days post-transfection	HCV Core (fmol/L)	Infectivity (ffu/mL)
2	1721	3
15	1009	16

minor peak which had a density of 1.02 to 1.04 g/mL (Fig. 3a). Interestingly, these density gradient profiles were quite similar to the previously described profile of JFH-1 (12). We further identified viral particles in the peak fraction of NC1/SY virus particles obtained following density gradient centrifugation by electron microscopic analysis (Fig. 3b). The observed particles exhibited a spherical shape with diameters of approximately 50–55 nm.

a



b

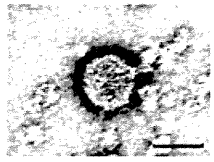


Fig. 3. Characterization of secreted HCV particles from NC1/SY RNA-transfected cells. (a) Sucrose density gradient analysis of the culture supernatant from NC1/SY RNA-transfected Huh7.5.1 cells. One ml of the culture supernatant of transfected cells that was collected on day 2 and day 15 post-transfection (Table 2) was cleared by centrifugation and filtration. Each supernatant was overlaid on a stepwise sucrose density gradient (0, 10, 20, 30, 40, 50 and 60% sucrose) and centrifuged for 16 hrs at 200,000 × *g* at 4°C. Eighteen fractions were collected from the bottom of the tubes and the concentration of HCV core protein in each fraction was determined. Closed and open circles indicate HCV core protein levels (fmol/L) and the sucrose density (g/mL) of the fractions, respectively. (b) Negative-stained HCV particles were observed by electron microscopy. NC1/SY virus particles were purified from the culture medium at 15 days post-transfection and observed by electron microscopy using negative staining. Scale bar, 50 nm.

Infectivity of the NC1/SY virus in human hepatocyte-transplanted uPA/SCID mice

We next determined the *in vivo* infectivity of the NC1/SY virus using human hepatocyte-transplanted uPA/SCID mice, which were reported to be permissive for HCV infection (23). We harvested cell culture media containing NC1/SY virus at 6 days after RNA transfection and was concentrated approximately 20-fold. The concentrated NC1/SY virus was inoculated into two mice designated as PXB 21–39 and PXB 21–40 (Table 3). Human albumin levels in the sera of the inoculated mice were higher than 3 mg/mL during the experiment, which supported the fact that there was a high level of replacement of mouse hepatocytes with human hepatocytes in the mouse liver. Both mice were negative for HCV-RNA at 1 week after inoculation (Table 3). The PXB 21–39 mouse became transiently HCV-RNA positive only at 2 weeks post-inoculation but thereafter remained negative from 3 to 5 weeks post-inoculation. The PXB 21–40 mouse remained HCV-RNA negative until 5 weeks post-inoculation. Thus, the cell culture-adapted NC1/SY virus in the inoculum may have possessed *in vivo* infectivity although this infectivity was at a low level.

Table 3. Serum HCV-RNA titer in human hepatocyte chimeric mice inoculated with the NC1/SY virus produced in cultured cells

Days post-infection	Mouse ID† PXB 21–39	PXB 21–40
7	–	–
14	+	–
21	–	–
28	–	–
35	–	–

†Two mice were independently inoculated with 300 μL NC1/SY (38,600 fmol/L).
+, PCR positive (<2.1×10^4 copies/mL); –, PCR negative.

Combinatory effects of adaptive mutations in different regions

The above experiment showed that NC1/SY RNA-transfected cells could produce infectious virus particles but at very low efficiency. To increase the replication and virus production efficiency of the NC1/SY virus, we tested the effect of introducing additional adaptive mutations into NC1. Thus, the E1202G mutation in NS3 (25) and/or the K1846T mutation in NS4B (26) were introduced into pNC1/SY (Fig. 4a) yielding pNC1/EGSY, pNC1/KTSY and pNC1/EGKTSY. RNAs transcribed from these synthesized constructs were transiently transfected into Huh7.5.1 cells. In this transient transfection experiment, NC1/EGSY and NC1/EGKTSY-RNA-transfected cells expressed higher levels of HCV core protein both in the culture medium and in the cell lysate from 48 to 96 hrs after transfection than cells transfected with RNA of the other NC1 mutants (Fig. 4b and c). However, the NC1/KTSY RNA-transfected cells expressed a similar level of HCV core protein as that expressed by NC1/SY. Interestingly, the E1202G mutation not only increased the level of the HCV core protein but also the infectivity of the culture medium. At 3 days post-transfection, HCV core protein levels reached a concentration of 8193 fmol/L in the medium of NC1/EGSY RNA-transfected cells, and, at the same time point, the infectivity titer in the culture medium of these cells reached 792 ffu/mL (Fig. 4b and Table 4). However, by 25 days post-transfection, the

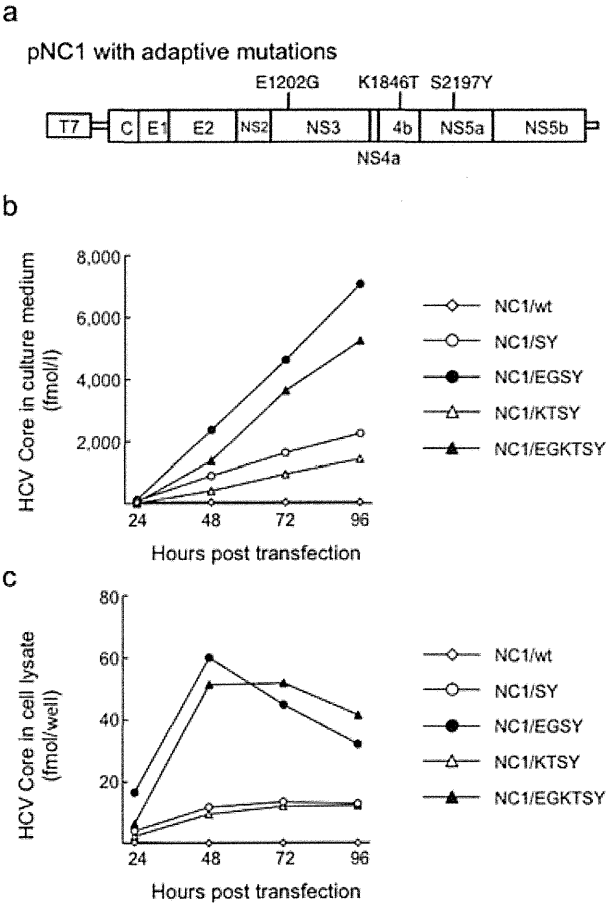


Fig. 4. Kinetics of HCV core protein release into the culture medium and cellular HCV protein expression. (a) Position of the E1202G and K1846T mutations, which were introduced alone or in combination into the full-length pNC1 containing the S2197Y mutation, is shown. Transcribed full-length HCV-RNAs from pNC1 and its mutated derivatives were transfected into Huh7 cells. At each time point, the culture medium and an aliquot of transfected cells were harvested. HCV core protein levels in the culture medium (b) and the cell lysate (c) were determined as described in Materials and Methods. Data indicate the average core protein concentration from two independent transfections. wt, wild type; EG, E1202G; KT, K1846T; SY, S2197Y.

infectivity titer in the culture medium of the NC1/EGSY RNA-transfected cells had decreased to 23.3 ffu/mL. Furthermore, reinfection of the secreted virus did not result in productive infection.

DISCUSSION

In previous studies, we isolated a cell culture infectious genotype 2a HCV strain, the JFH-1 strain, from a patient with fulminant hepatitis (12, 27). In the present study, we isolated a novel genotype 1b HCV cDNA, the NC1 strain. We tested NC1 strain replication efficiency in cultured cells using a drug-selectable subgenomic replicon

Table 4. HCV core protein levels in, and infectivity of, the culture medium of NC1 RNA-transfected cells

Days post-transfection	HCV construct	HCV core (fmol/L)	Infectivity (ffu/mL)
3	NC1/SY	2468	23.3
	NC1/EGSY	8193	792.0
	NC1/KTSY	1079	3.3
	NC1/EGKTSY	5064	467.0
25	NC1/SY	963	3.3
	NC1/EGSY	515	23.3
	NC1/KTSY	319	0.0
	NC1/EGKTSY	995	6.7

assay. Although the colony-forming efficiency of the NC1 replicon was lower than that of JFH-1, we still isolated multiple NC1 replicon cell clones. This result suggested that adaptive mutations in the replicon genome were necessary for efficient replication and that clones that expressed neomycin-resistant gene products were selected. Indeed, some mutations were identified in the NC1 replicon that enhanced colony-formation efficiency (Fig. 1). Notably, the S2197Y and S2204G mutations also increased core protein secretion from cells transfected with full-length NC1 HCV-RNA (Fig. 2). The culture medium of the NC1/SY RNA-transfected cells showed marginal infectivity for naive Huh7.5.1 cells. The *in vivo* infectivity of the NC1/SY virus was confirmed by its inoculation into human hepatocyte-transplanted uPA/SCID mice. Although the *in vivo* NC1/SY virus infectivity was very weak and transient, it was detected in one of the two inoculated mice. By introducing additional adaptive mutations into the NC1/SY virus, we found that a combination of E1202G and S2197Y mutations further enhanced HCV replication and virus production in RNA-transfected cells.

HCV was discovered as a causative agent of non-A, non-B hepatitis in 1989 (1, 2). Since then, efforts to understand the viral life cycle of HCV and to identify effective antiviral agents have been hampered by the lack of an efficient cell culture system for this virus. There have already been many attempts to develop a system for HCV infection and replication in cell culture (reviewed in ref 10). However, the viral replication efficiencies reported in these studies were modest, requiring detection by RT-PCR.

We previously isolated an HCV clone, JFH-1, from a fulminant hepatitis patient with HCV (27). A JFH-1-derived subgenomic replicon proved capable of highly efficient replication in a variety of cell lines (17), and produced infectious HCV particles in Huh7 cells (12–14). The development of an HCV infection system using the JFH-1 strain has contributed to our understanding of this important virus. A genotype 1a strain, H77S, which contained five adaptive mutations, was reported to produce infectious

virus following transfection of its synthesized RNA into Huh7 cells (28). It is thus clear that JFH-1 is not the only HCV strain that can be propagated in cultured cells. However, it is also important to understand why JFH-1 is the only strain that replicates efficiently in cultured cells in the absence of adaptive mutations. In order to understand this characteristic of JFH-1, we analyzed the mechanisms that underlie the efficient replication of the JFH-1 strain (29, 30) and identified important sequence differences in the JFH-1 strain compared to the J6CF strain. However, introduction of mutations that would abrogate such sequence differences did not enable efficient replication of genotype 1b HCV strains. These data suggested that it is necessary to identify different mutations that would increase the virus production efficiency of genotype 1b HCV. Further analysis of JFH-1 genomic replication mechanisms may help to determine other differences between the replication of JFH-1 and other strains.

Previous reports have indicated that adaptive mutations enhance viral RNA replication at the expense of virus particle formation efficiency (31). A highly cell culture-adapted Con1 strain can replicate in cultured cells, but it cannot produce infectious virus particles. In the present study, we also attempted to establish a replication-competent genotype 1b HCV strain from a patient with acute severe hepatitis. We identified several adaptive mutations in colony-forming experiments using a subgenomic replicon of this NC1 strain. Notably, S2197Y and S2204G mutations exhibited an adaptive effect for both virus replication and virus particle secretion. The adaptive mutations at amino acid positions 2197 and 2204 were previously reported in genotype 1a and 1b replicon studies (reviewed in ref 32). Although the S2197Y mutation enabled the NC1 strain to produce infectious virus, the infection efficiency of this virus was very weak. We therefore hypothesized that it may be necessary to increase replication efficiency by introducing additional mutations that would facilitate efficient virus production. For this purpose, we introduced and tested the effect of introduction of the previously reported mutations, E1202G in NS3 (25) and K1846T in NS4B (26), into the pNC1/SY construct. Interestingly, virus core protein secretion of NC1/SY was enhanced by introduction of the E1202G mutation but not by introduction of the K2864T mutation. This result suggested that the selection and combination of adaptive mutations is very important for establishment of an infectious genotype 1b HCV strain. Although NC1/EGSY RNA-transfected cells produced high levels of HCV core protein into the culture medium with infectivity, not all of the cells were infected with the virus and viral infection did not continue as the cells were passaged. After several cell passages, only a small number of the transfected cells remained infected (data not shown). This result may be due to a low degree of infection efficiency that was not sufficient for productive infection or may be due to some other unknown mechanisms.

The *in vivo* infectivity of the NC1/SY virus was tested using the human hepatocyte-transplanted uPA/SCID mouse system. The NC1/SY virus showed only transient viremia in one out of two inoculated mice. Interestingly, a highly adapted Con1 strain was previously shown not to be infectious for chimpanzees, whereas a moderately adapted Con1 was infectious. However, the virus recovered from the infected animal was wild-type Con1 virus (33). This result clearly suggests that HCV strains with low replication efficiency are favorable for *in vivo* infection. A wild-type virus with low replication capacity is likely to evade the host immune surveillance system and thereby survive in an *in vivo* situation. However, the JFH-1 virus was infectious not only for cultured cells but also for chimpanzees and human liver-transplanted mice (12, 34, 35). It is therefore important to analyze how HCV can evade host defense mechanisms to understand the mechanism of persistent viral infection. Further detailed studies are necessary for such analysis.

In the present study, we established the cell culture-adapted genotype 1b HCV strain, NC1. Infectious virus was produced from RNA-transfected cells. However, virus infection could not be continuously passaged in Huh7.5.1 cells. Novel antiviral drugs targeting genotype 1b HCV are under development and some of these drugs will be used in a clinical setting. It is likely that such new therapy will be accompanied by the appearance of significant adverse effects and by the emergence of drug-resistant virus. It is therefore important to further develop improved genotype 1b infectious HCV culture systems for future studies so that these problems can be circumvented.

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DISCLOSURE

None of the authors have any conflicts of interest associated with this study.

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SUPPORTING INFORMATION

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

S-Table 1: Primers used to amplify and sequence the NC1 HCV strain.

S-Table 2: Primers used to introduce mutations into pNC1 and pSGR-NC1.

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Serum Ferritin Levels Are Associated With a Distinct Phenotype of Chronic Hepatitis C Poorly Responding to Pegylated Interferon-Alpha and Ribavirin Therapy

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Elevated serum ferritin levels may reflect a systemic inflammatory state as well as increased iron storage, both of which may contribute to an unfavorable outcome of chronic hepatitis C (CHC). We therefore performed a comprehensive analysis of the role of serum ferritin and its genetic determinants in the pathogenesis and treatment of CHC. To this end, serum ferritin levels at baseline of therapy with pegylated interferon-alpha and ribavirin or before biopsy were correlated with clinical and histological features of chronic hepatitis C virus (HCV) infection, including necroinflammatory activity (N = 970), fibrosis (N = 980), steatosis (N = 886), and response to treatment (N = 876). The association between high serum ferritin levels (>median) and the endpoints was assessed by logistic regression. Moreover, a candidate gene as well as a genome-wide association study of serum ferritin were performed. We found that serum ferritin ≥ the sex-specific median was one of the strongest pretreatment predictors of treatment failure (univariate $P < 0.0001$, odds ratio [OR] = 0.45, 95% confidence interval [CI] = 0.34-0.60). This association remained highly significant in a multivariate analysis ($P = 0.0002$, OR = 0.35, 95% CI = 0.20-0.61), with an OR comparable to that of interleukin (*IL*)*28B* genotype. When patients with the unfavorable *IL28B* genotypes were stratified according to high versus low ferritin levels, SVR rates differed by >30% in both HCV genotype 1- and genotype 3–infected patients ($P < 0.001$). Serum ferritin levels were also independently associated with severe liver fibrosis ($P < 0.0001$, OR = 2.67, 95% CI = 1.68-4.25) and steatosis ($P = 0.002$, OR = 2.29, 95% CI = 1.35-3.91), but not with necroinflammatory activity ($P = 0.3$). Genetic variations had only a limited impact on serum ferritin levels. *Conclusion:* In patients with CHC, elevated serum ferritin levels are independently associated with advanced liver fibrosis, hepatic steatosis, and poor response to interferon-alpha-based therapy. (HEPATOLOGY 2012;55:1038-1047)

Chronic hepatitis C (CHC) is one of the most significant infectious diseases by being a leading cause of liver-related morbidity and liver transplantation worldwide.[1,2] Triple therapy comprising the hepatitis C virus (HCV) nonstructural protein 3-4A protease inhibitors, telaprevir or boceprevir, in combination with pegylated interferon-alpha (PEG-IFN-α) and ribavirin is becoming the new standard treatment for patients infected with the difficult-to-cure HCV genotype 1.[3] Recently completed phase III

Abbreviations: ALT, alanine aminotransferase; BMI, body mass index; CHC, chronic hepatitis C; CI, confidence interval; GWAS, genome-wide association study; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IL, interleukin; OR, odds ratio; PEG-IFN-α, pegylated interferon-alpha; RVR, rapid virologic response; SCCS, Swiss Hepatitis C Cohort Study; SVR, sustained virologic response; SNP, single-nucleotide polymorphism.

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clinical trials have demonstrated superior efficacy of such triple-therapy regimens in treatment-naïve and in treatment-experienced HCV genotype 1 patients, compared to PEG-IFN-α and ribavirin alone.[4-8] However, such triple-therapy regimens are burdened with additional adverse effects and significant cost. In addition, the success of triple-therapy regimens still depends on the IFN sensitivity of a given patient, as, for example, the efficacy of telaprevir in previous null responders (<2 $\log_{10}$ reduction in HCV RNA after 12 weeks of PEG-IFN-α and ribavirin) remains limited (boceprevir was not evaluated in null responders), and the risk of resistance development decreases with an extended rapid virologic response (RVR) (i.e., undetectable HCV RNA at weeks 4 and 12 of triple therapy).[6,9] Therefore, despite enormous progress, there is still a need to establish algorithms (including, among others, on-treatment viral kinetics and interleukin *[IL]28B* genotype) to predict treatment outcome of IFN-α-based therapy (e.g., to identify patients who do not necessarily need or who may require only short durations of triple therapy).[10-13]

HCV interferes with the host's iron metabolism, and hepatic iron measures were correlated with the grade and stage, as well as with the treatment outcome, of CHC.[14-18] Infection with HCV leads to iron accumulation in the liver and increased serum ferritin levels, which can be, at least partially, explained by down-regulation of hepcidin, a key regulator of iron homeostasis.[17,19] However, serum ferritin is also frequently elevated in inflammatory conditions. Excess iron in the liver promotes liver inflammation, oxidative stress, and mitochondrial dysfunction.[20] Accordingly, repeated phlebotomy in patients with CHC has been shown to reduce necroinflammatory activity, and a recent study has reported a reduced risk of hepatocellular carcinoma development in the phlebotomy group, compared to the control group.[21-23] However, the role of serum ferritin as an independent predictor of sustained virologic response (SVR) to IFN-α-based therapy remains controversial.[18,24-28]

In the present study, we performed a comprehensive analysis of the relationship of serum ferritin levels and the natural course and treatment outcome of CHC in patients enrolled in the large, well characterized Swiss Hepatitis C Cohort Study (SCCS), including a genome-wide association study (GWAS) of determinants of serum ferritin levels in HCV-infected individuals.

Patients and Methods

Patients. Patients were followed within the framework of the SCCS, a multicenter study performed at eight major Swiss hospitals and their local affiliated centers, including a total number of 3,648 patients with chronic or resolved HCV infection.[29,30] SCCS patients were selected for the present retrospective analysis if they met the following inclusion criteria: proven CHC, defined as anti-HCV seropositivity (using enzyme-linked immunosorbent assay and confirmed by recombinant immunoblotting assay) and HCV RNA detectable by a qualitative or quantitative assay; availability of serum ferritin measurements either at baseline before antiviral therapy or before liver biopsy (≤3 months); written informed consent for genetic testing; and availability of genomic DNA for genotyping. Patients with hemochromatosis were excluded from the present study. Assessment of treatment response was restricted to treatment-naïve patients who were treated during clinical practice conditions with either PEG-IFN-α2a or PEG-IFN-α2b in combination with weight-based ribavirin, with standard treatment durations (48 weeks for HCV genotypes 1 and 4 and 24 weeks for HCV genotypes 2 and 3), and who received ≥80% of the recommended dose of both agents during the first 12 weeks of therapy. SVR was defined as HCV RNA below the limit of detection in a sensitive assay ≥24 weeks after treatment completion, and all patients who failed to achieve SVR were classified as no SVR. Data on rapid, early, and end-of-treatment virologic response were not available. Demographic and clinical characteristics, including age, sex, HCV genotype, human immunodeficiency virus (HIV) coinfection, baseline HCV viral load, liver histology, serum ferritin levels, serum alanine aminotransferase (ALT) levels, HCV treatment, alcohol

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Potential conflict of interest: Dr. Negro is a consultant for and received grants from Roche. He advises MSD.

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

consumption, body mass index (BMI), and presence or absence of diabetes, were extracted from clinical databases. Serum ferritin levels were determined at local laboratories. Heavy alcohol intake was defined as consumption >40 g per day over a period of ≥5 years. Liver biopsies were evaluated by experienced local pathologists. Liver fibrosis was classified according to the METAVIR score. Necroinflammatory activity was stratified into two groups: absent to mild activity (METAVIR A0-A1) versus moderate to high activity (METAVIR A2-3). Steatosis was considered present when detected ≥5% of all hepatocytes. Quantitative intrahepatic iron determination was not systematically performed in the SCCS. The study was approved by local ethical committees.

GWAS and Genotyping of IL28B Single-Nucleotide Polymorphisms. A GWAS of determinants of serum ferritin levels in CHC patients was performed. Patients were included in this analysis if they met the above-indicated inclusion criteria and if DNA samples were genotyped previously within the framework of a GWAS of spontaneous and treatment-induced clearance from HCV infection.[30] Genotyping and genotype data analyses were described previously.[30] The present GWAS focused on serum ferritin levels measured before treatment initiation and/or liver biopsy, as described above. The following phenotypes were defined: serum ferritin as continuous variable; serum ferritin < versus ≥ the sex-specific median; serum ferritin < versus ≥ the 90% percentile. Each of the more than 500,000 single-nucleotide polymorphisms (SNPs) was tested for associations among each phenotype by linear or logistic regression models, as appropriate. The covariates, age and sex, were included in the regression analyses. Bonferroni correction was used to adjust for multiple testing, applying a significance threshold of 5×10^{-8}. *IL28B* rs8099917 genotyping was performed previously or performed as previously described.[30,31]

Statistical Analysis. Associations between serum ferritin < versus ≥ the sex-specific median with continuous (i.e., HCV viral load and serum ALT levels) and dichotomic variables (e.g., SVR versus no SVR, necroinflammatory activity [none-mild versus moderate-high], stage of liver fibrosis [METAVIR F0-F1 versus F2-F4], and presence or absence of steatosis) were assessed in linear and logistic regression models, respectively. After univariate analyses, multivariate analyses were performed for significant associations. Multivariate analyses were obtained by using backward selection, using a P value ≥0.15 for removal from the model. Only patients with complete data for the remaining covariates were included in multivariate analyses. Sex, age, and serum ferritin levels were forced into the model. Group differences (e.g., serum ferritin levels according to *IL28B* rs8099917 TT versus GT/GG) were assessed by means of χ^2 contingency tables or Wilcoxon-Mann-Whitney U tests, as appropriate. P values <0.05 were considered to be significant.

Results

Patient Characteristics. A total of 876 patients were included in the response to treatment study, 980 in the liver histology study, and 707 Caucasian patients in the GWAS of ferritin serum levels in chronic HCV infection. In the response to treatment group, 606 (69%) and 270 (31%) patients were treated with PEG-IFN-α2a and PEG-IFN-α2b, respectively, in combination with weight-based ribavirin. The overall SVR rates in patients infected with HCV genotypes 1, 2, 3, and 4 were 43%, 86%, 78%, and 47%, respectively. Demographic and clinical characteristics of patients are shown in Table 1. The sex-specific median of serum ferritin levels in the whole cohort was 85 μg/L in females and 203 μg/L in males. Ferritin levels above these values were considered as elevated in the following analyses.

Serum Ferritin Levels and Response to Treatment of CHC. Elevated serum ferritin (ferritin >sex-specific median) was among the strongest pretreatment predictors of failure to achieve SVR ($P < 0.0001$, odds ratio [OR] favoring SVR = 0.45, 95% confidence interval [CI] = 0.34-0.60). These results were comparable when serum ferritin was analyzed as a continuous variable ($P < 0.0001$), or when selecting a different cutoff for high serum ferritin ($P < 0.0001$ for ferritin cutoff >200 and >250 μg/L). The association between elevated ferritin and response to treatment remained highly significant in multivariate analyses (P = 0.0002, OR = 0.35, 95% CI = 0.20-0.61) after adjustment for known predictors of SVR, including the *IL28B* genotype, baseline viral load, HCV genotype, presence of diabetes, and liver fibrosis stage (Table 2). When free serum iron and transferrin saturation were entered into the model, the association between ferritin and treatment failure remained significant (OR = 0.42 [0.23-0.78], $P = 0.006$).

The number of HCV genotype 1– and genotype 3–infected patients was sufficient for stratified analyses. In both genotypes, serum ferritin was a strong, independent predictor of failure to achieve SVR (Supporting Table 1). In HCV genotype 3–infected patients, ferritin was an even stronger predictor than *IL28B* rs8099917 genotype ($P = 0.0005$, OR = 0.38, 95% CI = 0.22-0.66 for ferritin versus $P = 0.10$, OR = 0.58, 95%

Table 1. Baseline and Demographic Characteristics

Characteristics	Treatment Response (N = 876)	Liver Histology (N = 980)	GWAS (N = 707)
Male sex, n (%)	569 (65)	638 (65)	450 (0.64)
Caucasian ethnicity	815 (93)	917 (94)	707 (100)
Age (years), mean (range)*	45 (19-75)	44 (17-76)	45 (21-75)
Alcohol (40 g/day ≥5 years), n (%)*,†	157 (18)	182 (19)	104 (16)
Diabetes, n (%)*,‡	69 (8)	66 (7)	51 (7)
BMI (kg/m^2), mean (range)	26 (14-60)	25 (14-42)	24 (16-42)
ALT (U/L), mean (range)*,§	102 (6-693)	103 (4-2,082)	104 (8-720)
Ferritin (μg/L), mean (range)*	237 (3-2,183)	240 (3-4,525)	250 (3-3,358)
Steatosis (presence), n (%)‖	417 (74)	588 (66)	373 (70)
Fibrosis stage, n (%)¶			
0-1	180 (32)	707 (72)	212 (49)
2-4	382 (68)	273 (28)	336 (61)
Activity#			
None to mild	421 (75)	780 (80)	437 (81)
Moderate to severe	139 (25)	190 (20)	105 (19)
HCV genotype, n (%)			
1	383 (44)	528 (54)	343 (49)
2	98 (11)	82 (8)	83 (12)
3	322 (37)	269 (27)	222 (31)
4	73 (8)	101 (10)	59 (8)
HCV RNA (log_{10}), mean (range)*	5.96 (1-8)	5.88 (1-8)	6.00 (1-8)
HIV coinfection, n (%)*,**	35 (5)	61 (6)	40 (7)

A number of patients overlap between the treatment response, liver histology, and GWAS groups.
*Detection before treatment initiation in the treatment response group and before first liver biopsy in the liver histology group.
†Alcohol consumption data were missing in 16 treatment and 21 biopsy patients.
‡Diabetes data were missing in 11 treatment and 77 biopsy patients.
§ALT data were missing in 278 treatment and 1 biopsy patients.
‖Steatosis data were missing in 309 treatment and 94 biopsy patients.
¶Fibrosis data were missing in 314 treatment patients.
#Activity data were missing in 316 treatment and 10 biopsy patients.
**HIV serostatus was missing in 155 treatment and 116 biopsy patients.

CI = 0.31-1.10 for rs8099917). In the multivariate model, ferritin remained a strong, independent predictor of SVR in HCV genotype 3 patients (OR = 25 [0.08-0.78], $P = 0.02$), though its effect was slightly smaller than that of *IL28B* rs8099917 (OR = 20 [0.06-0.65], $P = 0.007$). Detailed test characteristics of

Table 2. Uni- and Multivariate Analyses of Variables Associated With Treatment Response in Patients With CHC

	Univariate Analysis		Multivariate Analysis	
Variables	OR (95% CI)	P Value	OR (95% CI)	P Value
Age (years, continuous)	0.96 (0.95-0.98)	<0.0001	0.98 (0.95-1.01)	0.13
Male sex	0.81 (0.60-1.07)	0.14	0.68 (0.39-1.18)	0.17
BMI (kg/m^2, continuous)	0.95 (0.92-0.99)	0.02		
Diabetes, presence	0.49 (0.30-0.80)	0.005	0.40 (0.14-1.14)	0.09
Alcohol (40 g/day ≥5 years)	0.76 (0.51-1.14)	0.2		
ALT (U/L, continuous)	1.00 (1.00-1.00)	0.6		
Activity, none to mild versus moderate to high	0.93 (0.63-1.37)	0.7		
Fibrosis, F3-F4 versus F0-F2	0.52 (0.36-0.74)	<0.0001	0.47 (0.26-0.85)	0.01
Steatosis, presence	0.62 (0.42-0.93)	0.02		
IL28B rs8099917, TT versus TG/GG	0.35 (0.25-0.48)	<0.0001	0.20 (0.11-0.34)	<0.0001
Iron (ug/dL, continuous)*	0.98 (0.97-1.00)	0.01		
Transferrin saturation (%, continuous)	1.00 (0.99-1.00)	0.5		
Ferritin ≥ median†	0.45 (0.34-0.60)	<0.0001	0.35 (0.20-0.61)	0.0002
HCV genotype 2/3 versus 1/4	5.12 (3.78-6.93)	<0.0001	3.34 (1.95-5.74)	<0.0001
HCV RNA (log10 RNA, continuous)	0.38 (0.31-0.46)	<0.0001	0.30 (0.21-0.44)	<0.0001
HIV coinfection	0.59 (0.24-1.47)	0.3		

*Iron was excluded from the above-shown multivariate analysis, but the association with ferritin remained significant when iron was entered into the model (OR = 0.42 [0.23-0.78], $P = 0.006$).
†A sex-specific median was applied (80 μg/L in women, 200 μg/L in men). Results of this multivariate analysis were comparable when ferritin serum concentrations were included as continuous variables or with fixed cutoffs ≥200/250 μg/L. Overall, 376 patients with complete datasets were included in multivariate analysis.

Table 3. SVR Rates of HCV Genotype 1 and 3 Patients Stratified According to *IL28B* Genotype and Serum Ferritin

HCV Genotype	*rs8099917* Genotypes	Ferritin	SVR Yes	SVR No	SVR Rate (%)	*P* Value
1 (N = 283)	TT	Low	54	27	66.7	0.033
		High	36	38	48.7	
	TG/GG	Low	25	31	44.6	0.0001
		High	10	62	13.9	
3 (N = 218)	TT	Low	69	13	84.2	NS
		High	41	14	74.6	
	TG/GG	Low	40	6	87.0	0.0002
		High	17	18	48.6	

Low and high ferritin indicate a value below or above the sex-specific median. *P* values were calculated using the Fisher's exact test.
Abbreviation: NS, not significant.

serum ferritin versus *IL28B* rs8099917 genotype as predictors of SVR are shown in Supporting Table 2.

To analyze the contribution of both ferritin levels and *IL28B* polymorphisms to response to treatment, patients were stratified according the *IL28B* rs8099917 genotype (i.e., unfavorable TG or GG versus favorable TT) and the ferritin level (i.e., high versus Low, if above or below the sex-specific median; Table 3). In both HCV genotype 1– and genotype 3–infected patients with unfavorable *IL28B* genotype, SVR rates differed by >30% after stratification for low versus high serum ferritin ($P = 0.0001$ for HCV genotype 1, $P = 0.0002$ for HCV genotype 3), whereas the association between serum ferritin and SVR was less significant in patients with favorable *IL28B* genotype ($P = 0.033$ for HCV genotype 1, $P = 0.2$ for HCV genotype 3; Table 3). Therefore, serum ferritin appears to add to the value of *IL28B* genotype in predicting treatment outcome, especially in patients with an unfavorable *IL28B* genotype.

Serum Ferritin Levels and Liver Fibrosis Stage As Well As Necroinflammatory Activity in Patients With CHC. Elevated ferritin (>sex-specific median) was associated with severe liver fibrosis (F3-4, $P < 0.0001$, OR = 3.27, 95% CI = 2.44-4.38; Table 4). This association was still significant in a multivariate model ($P < 0.0001$, OR = 2.67, 95% CI = 1.68-4.25) after adjustment for other known predictors of fibrosis, including male sex, diabetes, steatosis, high necroinflammatory activity, and heavy alcohol intake. Elevated serum ferritin was also associated with moderate to high necroinflammatory activity (univariate $P < 0.0001$, OR = 2.08, 95% CI = 1.50-2.87), but this association lost significance after adjustment for relevant covariates ($P = 0.3$; Table 4).

Serum Ferritin Levels and Liver Steatosis in Patients With CHC. Serum ferritin ≥ the sex-specific median was strongly associated with the presence of steatosis in >5% of hepatocytes (univariate $P < 0.0001$, OR = 2.65, 95% CI = 1.97-3.56). Other variables associated with steatosis in >5% of hepatocytes were high ALT serum levels, high BMI, *IL28B* genotype, male sex, infection with HCV genotype 3, presence of diabetes, high necroinflammatory activity, advanced liver fibrosis, and heavy alcohol intake (Table 5). In a multivariate analysis, high ferritin levels remained a strong, independent predictor of steatosis ($P = 0.002$, OR = 2.29, 95% CI = 1.35-3.91), together with BMI, infection with HCV genotype 3, high necroinflammatory activity, and severe liver fibrosis (Table 5). These findings were comparable when using another steatosis cutoff (i.e., present or absent).

Genetic Study of Determinants of Serum Ferritin Levels in CHC Patients. First, we analyzed the associations of nine SNPs from four genes that were previously associated with elevated serum ferritin at the genome level in healthy subjects (Table 6).[32-36] These SNPs included two major genetic variations in the *HFE* gene associated with hemochromatosis (rs1800562, resulting in C282Y, and rs1799945, resulting in H63D).[37] In our cohort, only candidate SNPs in the *HFE* gene (rs1800562; $P = 0.03$) and in *LRRC16A* (rs2274089; $P = 0.02$), a gene involved in the organization of the cytoskeleton, were weakly associated with serum ferritin levels, although these associations would be no more significant after correction for multiple testing (Table 6). Importantly, patients with proven or suspected hemochromatosis were excluded from the present study. None of these SNPs were significantly associated with SVR, though some *P* values reached close to the level of statistical trends, suggesting that a larger sample size may reveal significant associations (Supporting Table 3).

We next performed a GWAS to test whether genetic variations other than those observed in the general population influence serum ferritin levels in CHC patients. However, no SNP reached genome-wide significance for an association with serum ferritin levels as a continuous variable (Table 7) or as a categorical variable (< versus ≥ the sex-specific median or ≤ versus ≥ the 90% percentile; not shown). To our knowledge, no clear

Table 4. Uni- and Multivariate Analyses of Variables Associated With Liver Fibrosis Stage and Necroinflammatory Activity

Variables	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
Fibrosis stage, F0-2 versus F3-4				
Age at infection (years, continuous)	1.02 (1.01-1.04)	0.002	1.01 (0.99-1.04)	0.3
Male sex	1.35 (1.00-1.83)	0.05	1.57 (0.93-2.65)	0.09
BMI (kg/m^2, continuous)	1.05 (1.02-1.09)	0.003		
Diabetes	4.81 (2.86-8.09)	<0.0001	6.68 (2.63-17.0)	0.0001
Alcohol (40 g/day ≥5 years)	2.76 (1.98-3.86)	<0.0001	2.62 (1.57-4.39)	0.0002
ALT (U/L, continuous)	1.00 (1.00-1.00)	0.03		
Activity (none-mild versus moderate to high)	5.95 (4.24-8.35)	<0.0001	4.46 (2.48-8.02)	<0.0001
Steatosis	3.43 (2.37-4.97)	<0.0001	2.02 (1.17-3.49)	0.01
IL28B rs8099917 (TT versus TG/GG)	0.97 (0.68-1.39)	0.9		
Ferritin, ≥median*	3.27 (2.44-4.38)	<0.0001	2.67 (1.68-4.25)	<0.0001
HCV RNA (log10 RNA, continuous)	0.95 (0.84-1.09)	0.5		
HIV coinfection	1.50 (0.86-2.63)	0.16		
Necroinflammatory activity (none to mild versus Moderate to high)				
Age at infection (years, continuous)	1.04 (1.02-1.06)	<0.0001	1.03 (1.00-1.05)	0.04
Male sex	1.12 (0.80-1.57)	0.5	1.36 (0.73-2.52)	0.3
BMI (kg/m^2, continuous)	1.04 (1.01-1.08)	0.02		
Diabetes	2.54 (1.49-4.31)	0.0006		
Alcohol (40 g/day ≥5 years)	1.38 (0.94-2.03)	0.1		
ALT (U/L, continuous)	1.00 (1.00-1.00)	0.007		
Steatosis	3.15 (2.05-4.86)	<0.0001	3.68 (1.56-8.67)	0.003
Fibrosis (F0-F2 versus ≥F3-F4)	5.95 (4.24-8.35)	<0.0001	4.71 (2.67-8.29)	<0.0001
IL28B rs8099917 (TT versus TG/GG)	0.74 (0.51-1.07)	0.11		
Ferritin, ≥median*	2.08 (1.50-2.87)	<0.0001	1.38 (0.77-2.48)	0.3
HCV RNA (log10 RNA, continuous)	0.82 (0.71-0.94)	0.005		
HIV coinfection	0.58 (0.26-1.31)	0.2		

*A sex-specific median was applied (80 μg/L in women, 200 μg/L in men). Results of this multivariate analysis were comparable when ferritin serum concentrations were included as continuous variables or with fixed cutoffs ≥200/250 μg/L and when a cutoff for fibrosis stage of F0-F1 versus F2-F4 was applied. Overall, 312 and 519 patients with complete datasets were included in multivariate analyses of fibrosis and activity, respectively.

biological evidence for a role in iron metabolism is known for each of the top SNPs of this GWAS (Table 7).

Discussion

In the present study, we show that serum ferritin is a strong predictor of failure to achieve SVR after treatment of CHC with PEG-IFN-α and ribavirin independently of other predictors of virologic response, including the *IL28B* genotype. In addition, serum ferritin was independently associated with advanced liver fibrosis and the presence of steatosis, but not with necroinflammatory activity. In a candidate gene approach and in a GWAS, we did not identify significant genetic

Table 5. Uni- and Multivariate Analyses of Variables Associated With Liver Steatosis (≤5% Versus >5% of Hepatocytes)

Variables	Univariate Analysis		Multivariate Analysis	
	OR (95% CI)	*P* Values	OR (95% CI)	*P* Values
Age at infection (years, continuous)	1.01 (1.00-1.03)	0.14	1.01 (0.98-1.04)	0.4
Male sex	1.69 (1.26-2.27)	0.0005	1.65 (0.98-2.79)	0.1
BMI (kg/m^2, continuous)	1.13 (1.09-1.18)	<0.0001	1.15 (1.07-1.22)	<0.0001
Diabetes	5.02 (2.34-10.75)	<0.0001		
Alcohol (40 g/day ≥5 years)	1.56 (1.08-2.26)	0.02		
ALT (U/L, continuous)	1.00 (1.00-1.01)	0.0004		
Fibrosis, F0-F2 versus ≥F3-F4	3.13 (2.19-4.48)	<0.0001	1.79 (0.96-3.35)	0.07
Activity, none to mild versus moderate to high	2.60 (1.73-3.92)	<0.0001	2.56 (1.14-5.77)	0.02
IL28B rs8099917, TT versus TG/GG	1.44 (1.00-2.05)	0.05		
Ferritin, ≥median*	2.65 (1.97-3.56)	<0.0001	2.29 (1.35-3.91)	0.002
HCV genotype 3 versus non-3	2.40 (1.71-3.36)	<0.0001	4.08 (2.33-7.14)	<0.0001
HCV RNA (log10 RNA, continuous)	1.07 (0.90-1.28)	0.4		
HIV coinfection	0.96 (0.54-1.70)	0.9		

*A sex-specific median was applied (80 μg/L in women, 200 μg/L in men). Results of this multivariate analysis were comparable when steatosis was analyzed as present versus absent. Overall, 392 patients with complete datasets were included in multivariate analysis.

Table 6. Associations Between Candidate SNPs and Serum Ferritin Levels in Patients With CHC

Gene	SNP rs#	Chr	Noneffect Allele	Effect Allele	r2_hat	Beta	SE	P Value
TMPRSS6	rs855791	22	A	G	0.99	0.072	0.051	0.16
HFE	rs1800562	6	A	G	0.99	−0.255	0.12	0.033
	rs1799945	6	C	G	1.00	0.065	0.068	0.34
	rs17342717	6	C	T	0.99	0.099	0.090	0.27
	rs1408272	6	G	T	0.99	−0.265	0.13	0.032
Transferrin	rs3811647	3	A	G	1.00	−0.042	0.053	0.435
	rs1799852	3	C	T	0.99	−0.095	0.079	0.26
	rs2280673	3	A	C	1.00	0.011	0.054	0.85
LRRC16A	rs2274089	6	C	T	1.00	0.237	0.103	0.022

Candidate SNPs included in this analysis were identified by previous GWAS to be associated with serum ferritin concentrations in healthy populations.
Abbreviations: Chr, chromosome; r2_hat, imputation quality score; SE, standard error.

determinants of elevated serum ferritin levels in patients with CHC.

Importantly, serum ferritin was one of the strongest predictors of treatment response in our cohort, with an OR for treatment failure comparable to the *IL28B* genotype. In this regard, the effect of serum ferritin on treatment response, in comparison to the *IL28B* genotype, appeared to be somewhat less pronounced in HCV genotype 1 patients than in patients infected with HCV genotype 3. However, in both HCV genotype 1 and 3 patients, serum ferritin appeared to provide significant additional information to *IL28B* genotyping in patients with a poor-response *IL28B* genotype, in whom SVR rates differed by >30% after stratification by serum ferritin levels. An association between serum ferritin levels and outcome of IFN-α-based therapy has been reported previously in smaller studies, although it remained controversial as to whether serum ferritin is an independent predictor of treatment response or whether it is merely a surrogate marker for advanced stages of liver fibrosis.[18,24-27] The present, to our knowledge, largest, most comprehensive study, which was restricted to adherent patients who had received the recommended dose of PEG-IFN-α and ribavirin during the first 12 weeks of therapy, had the power to clearly demonstrate that serum ferritin is an independent predictor of treatment failure, even though serum ferritin was also independently associated with advanced liver fibrosis and steatosis. This observation indicates that serum ferritin is a promising candidate to be included in the panel of predictors of response to IFN-α-based therapy.[12,26,38-42] This may be especially relevant in patients infected with HCV genotype 3, in whom the utility of *IL28B* genotyping is limited, even though a substantial number of patients cannot be cured with PEG-IFN-α and ribavirin alone.[43,44] In addition, the development of directly acting antivirals for HCV genotype 3 patients is less advanced, compared to HCV genotype 1.[3] In this context, it may also be worthwhile to assess the relationship of serum ferritin and response to triple therapy, including telaprevir, in treatment-experienced patients with previous null response—a group of patients with limited chances to achieve cure, even by triple therapy.[6]

For our statistical analyses, we have chosen a sex-specific median as the cutoff between high and low serum ferritin levels, but such cohort-specific values are not suitable for clinical decision making. Nevertheless, the sex-specific median for both women (85 ug/L) and men (203 ug/L) in our cohort of HCV-infected patients was relatively close to the upper limit of normal of the general population (e.g., 150 ug/L in

Table 7. Genome-Wide Association Study of Determinants of Serum Ferritin Levels in Patients With CHC (Top 10 SNPs Are Shown)

Gene	SNP rs#	Chr	Position	Dist (kb)	Aa	Ab	r2_hat	Beta (Ab)	SE	P Value
KIAA0494	rs10789491	1	46951897	0	A	G	0.99	0.312	0.064	$9.57\ 10^{-07}$
ARHGEF10L	rs2254135	1	17707329	31,587	C	T	0.97	0.269	0.058	$3.41\ 10^{-06}$
DNASE2B	rs12144715	1	84637818	0	A	T	0.91	−0.636	0.14	$6.55\ 10^{-06}$
SCG2	rs16864968	2	224167927	1,978	A	G	0.99	−0.826	0.177	$3.28\ 10^{-06}$
CWH43	rs11725957	4	48757649	0	A	G	1.00	0.727	0.163	$7.74\ 10^{-06}$
SLC22A3	rs2504916	6	160744018	0	A	T	0.99	0.306	0.065	$2.01\ 10^{-06}$
RP11-157J24.1	rs11242704	6	1480997	20,474	A	G	0.98	−0.257	0.056	$5.47\ 10^{-06}$
EPHA7	rs12527818	6	93967840	40,023	C	T	0.98	0.274	0.061	$7.29\ 10^{-06}$
LAMA4	rs10782172	6	112687675	5,133	C	T	0.96	0.33	0.075	$9.73\ 10^{-06}$
OLFM1	rs10776934	9	137169522	16,671	G	T	0.95	−0.281	0.058	$1.05\ 10^{-06}$

Aa, allele a; Ab, allele b; chr, chromosome; dist, distance from gene; kb, kilobase; SE, standard error.

women and 300 ug/L in men, with some variations according to the assay used). Therefore, a threshold of serum ferritin at the upper limit of normal, or even slightly below, might be used to predict treatment failure, though additional studies are required to validate these suggestions.

In our study, serum ferritin levels were also strongly associated with advanced liver fibrosis, as well with the presence of steatosis. These associations remained highly significant in multivariate models, and serum ferritin did not appear to be a simple surrogate of either steatosis or advanced fibrosis. In our study, we could confirm that infection with HCV genotype 3 is an independent risk factor of liver steatosis (Table 5), as described previously.[45-47] However, according to subanalyses, the association between serum ferritin and steatosis remained highly significant in uni- and multivariate models in patients infected with HCV genotype 1, but only in the univariate analysis in HCV genotype 3 patients (Supporting Table 4). Altogether, serum ferritin appears to provide additional information in describing a distinct phenotype of CHC characterized by advanced liver fibrosis and steatosis as well as a poor response to IFN-α-based therapy.

We also aimed to investigate potential genetic determinants of serum ferritin levels in patients with CHC. No significant associations between SNPs and serum ferritin levels were identified in a GWAS including more than 700 patients with CHC, suggesting that at least common genetic variants do not substantially influence ferritin levels in this context. Among the top hits of this GWAS was rs16864968 very close to *SCG2*, encoding secretogranin II, a protein with immune-modulating properties.[48] However, for none of the top hits was an obvious link to iron metabolism described thus far. The known association between serum ferritin and rs1800562 and rs1408272 in *HFE* and rs2274089 in *LRRC16A* could be confirmed by a candidate gene approach, although the association appeared to be weak, compared to individuals not infected with HCV.

Our study had some limitations, because stainable hepatic iron deposition was not quantitatively assessed in the SCCS, and it therefore was not possible to assess the relationship between serum ferritin and intrahepatic iron load. Thus, our study does not provide any evidence on a causal role of serum ferritin in treatment response. In addition, we were unable to take potential diurnal and nonfasting variability of serum ferritin and serum iron levels into account, and because of the lack of on-treatment viral load data, we could not assess associations between serum ferritin and rapid or early virologic response. Moreover, it remains unclear whether our findings could be extrapolated to patients of other ethnicities, because almost all patients included in our study were of Caucasian origin.

In principle, increased serum ferritin levels in the context of HCV infection could reflect, at least partially, an increased hepatic iron load, which might be explained by dysregulated hepcidin expression.[17] However, serum ferritin is also frequently increased as a consequence of inflammatory responses during acute and chronic infectious diseases, autoimmune diseases, or cancer, a finding that may substantially contribute to increased serum ferritin levels in patients with CHC. Both increased hepatic iron and an ongoing systemic inflammatory response can contribute to an unfavorable course of CHC, of which serum ferritin appears to be a surrogate. In this regard, it was shown recently that a strong host response against translocated gut microbial products correlates with an unfavorable outcome of chronic hepatitis B and C as well as with elevated serum ferritin levels.[49] Our finding, that free serum iron, but not transferrin saturation, is associated with treatment failure may point to such a role of serum ferritin levels as a surrogate for a systemic inflammatory response in CHC. Nevertheless, it has been shown that iron reduction by phlebotomy results in a significant reduction of serum ferritin levels in patients with CHC, and iron reduction may improve liver histology and treatment response in CHC patients.[15,21,22,50] This may be especially relevant in patients who are unable to receive or who did not respond to triple therapy.

In conclusion, we show that serum ferritin is strongly and independently associated with failure to achieve HCV eradication by IFN-α-based therapy as well as with advanced liver fibrosis and steatosis. Serum ferritin may be included in clinical decision making in patients with CHC, especially in patients with a poor *IL28B* genotype.

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