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Hepatitis C virus core protein exerts an inhibitory effect on suppressor of cytokine signaling *(SOCS)-1* gene expression

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***Background/Aims*: Suppressor of cytokine signaling (SOCS)-1, a negative feedback regulator of cytokine signaling pathway, also has a tumor suppressor activity, the silencing of its gene by hypermethylation is suggested to contribute to hepatocarcinogenesis. We studied the effect of the core protein of hepatitis C virus (HCV) on the expression of *SOCS-1* gene.**

***Methods*: HCV core gene transgenic mice, which develop hepatocellular carcinoma late in life, HepG2 cells expressing the core protein, and human liver tissues were analyzed.**

***Results*: The expression of *SOCS-1* gene was significantly suppressed in the liver of core gene transgenic mice and HepG2 cells expressing the core protein, while that of *SOCS-3* gene was conserved. *SOCS-1* expression levels also decreased in HCV-positive human liver tissues. The core protein differentially down-regulated the expression of signal transducer and activator of transcription (STAT) target genes, but rather enhanced STAT1 and STAT3 activation after interleukin-6 stimulation in mouse liver tissues and cells.**

***Conclusions*: HCV core protein down-regulates the expression of *SOCS-1* gene. This is a mechanism leading to *SOCS-1* silencing, an alternative to the hypermethylation of the gene; this effect of the core protein may modulate the intracellular signaling pathway, contributing to the pathogenesis in HCV infection including hepatocarcinogenesis.**
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***Keywords*: Tumor suppressor gene; Hepatocellular carcinoma; Transgenic mouse; STAT3**

1. Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis. A substantial proportion of patients with chronic hepatitis C eventually develop hepatocellular carcinoma (HCC), which is one of the leading causes of death worldwide [1,2]. Despite the absence of appropriate in vitro replication systems or practical infectious animal model systems, the mechanism underlying hepatocarcinogenesis in human HCV infection is gradually clarified. Both the direct and indirect effects of HCV on HCC development are demonstrated [3–6]. The accumulation of gene aberrations, such as the inactivation of tumor suppressor genes or the activation of oncogenes, which are induced through the inflammation-mediated continuous death of hepatocytes followed by regeneration, may be one of the mechanisms underlying hepatocarcinogenesis [3]. On the other hand, the viral gene products are suggested to contribute to the development of HCC by their direct effects on hepatocytes [4]. Such direct effects have been demonstrated by the use of model systems including mice

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[7–9]. HCV-infected hepatocytes produce viral structural and nonstructural proteins. Some of these confer certain phenotypes to hepatocytes and may be associated with the pathogenesis of HCV infection including the development of HCC. Among such viral proteins, the core protein of HCV has a variety of biological activities, including oncogenic activity, which substantially affects host cellular functions [7–11].

Suppressor of cytokine signaling (SOCS)-1, also called signal transducer and activator of transcription (STAT)-induced STAT inhibitor-1 or Jak binding protein-1, is a negative feedback regulator of cytokine signaling through the Jak/STAT pathway. SOCS-1 contains the SH2 domain and directly interacts with the kinase domain of Jak to suppress Jak activity. *SOCS-1* gene expression is augmented by various cytokines, such as interferon (IFN)-γ, interleukin (IL)-6 or leukemia inhibitory factor (LIF), resulting in the suppression of the signal transduction downstream pathways of these cytokines [12–14]. Moreover, SOCS-1 has been recently shown to exhibit a tumor suppressor activity. SOCS-1 suppresses the expression of several oncogenes or growth-related genes acting as a negative regulator of cell proliferation: the loss of function of SOCS-1 facilitates tumor progression [15–17]. As a mechanism underlying the loss of function of SOCS-1, a recent study has revealed a frequent silencing of the *SOCS-1* gene by CpG methylation in HCC tissues [18–20]. Alternatively, however, it may be possible that HCV infection, particularly, the proteins that the HCV genome encodes per se, may render the *SOCS-1* gene unable to exhibit its function by gene silencing.

We examined such a possibility using a mouse model for HCV infection that is destined to develop HCC [7,9], as well as cultured cells expressing the HCV core protein [21]. The core protein markedly suppressed the expression of the *SOCS-1* gene in both liver tissues and cultured cells. This silencing of the *SOCS-1* gene may be one of explanations for the pathogenicity of HCV in humans.

2. Materials and methods

2.1. Transgenic mouse and cell lines

HCV core gene transgenic mice have been described previously [7]. These mice develop HCC late in life [7,9]. The mice were cared for according to the institutional guidelines and maintained in a specific pathogen-free state. All the animals received humane care and the study protocol complied with the institution's guidelines for the care and use of experimental animals. HepG2 cell lines expressing the HCV core protein under the control of CAG promoter (Hep39J, Hep396 and Hep397) or a control HepG2 line (Hepswx) carrying the empty vector were described previously [21,22].

2.2. IL-6 Stimulation

For the in vivo experiments, 0.05–0.5 μg/g BW murine IL-6 (Diaclone, Besançon, France) was administered into 8 w.o. male mice i.p., and liver tissues were obtained 60 min later. Cultured cells were treated with human IL-6 (Diaclone) at 10–100 ng/ml or IFN-α at 1.0–10.0 ng/ml and then were harvested 60 min later.

2.3. Reverse transcription (RT)-PCR analysis

Total RNA was extracted from liver tissues or cultured cells before and after the treatment with IL-6 using TRIzol (Invitrogen). RNA was reverse-transcribed using oligo(dT) primers and Superscript II (Invitrogen). Equal amounts of cDNA were then subjected to PCR. The primer pairs used were:

5′-CACTCACTTCCGCACCTTCC-3′ (forward) and
5′-TCCAGCAGCTCGAAAAGGCA-3′ (reverse) for murine *SOCS-1*,
5′-CACGCACTTCCGCACATTCC-3′ (forward) and
5′-TCCAGCAGCTCGAAGAGGCA-3′ (reverse) for human *SOCS-1*,
5′-TCACCCACAGCAAGTTTCCCGC-3′ (forward) and
5′-GTTGACAGTCTTCCGACAAAGATGC-3′ (reverse) for murine *SOCS-3*,
5′-CACGCACTTCCGCACATTCC-3′ (forward), and
5′-GTTGACGGTCTTCCGACAGAGATGC-3′ (reverse) for human *SOCS-3*.

For the RT-PCR analysis, the quantity of cDNA template and the number of amplification cycles were optimized to ensure that the reaction was terminated during the linear phase of product amplification, so that the semiquantitative comparison of mRNA abundance between different samples was possible [23]. The intensities of the bands were determined using a densitometer. RT-PCR was also done using GAPDH primers to adjust the amounts of RNA in each experiment.

2.4. Human liver tissue samples and real-time PCR

Nine patients with HCC who had underlying chronic hepatitis C were studied for *SOCS-1* expression in noncancerous tissues. Additional nine patients, who were found to be negative for both HBsAg and anti-HCV at the time of operation, were also studied. The latter patients underwent liver resection for metastatic liver tumors from colon cancer. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethics Review Committee for Human Experimentation. Informed consent was obtained from each patient. The noncancerous liver tissues obtained from these patients were immediately frozen and stored at −80 °C until further use.

Taqman real-time RT-PCR was performed as described previously [24], using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers and the TaqMan probe for *SOCS-1* were as follows:

Forward primer:5′-CTGGCCCCGGAGCAT-3′
Reverse primer:5′-GTTGTGTGCTACCATCCTACAGA-3′
Probe:5′-FAM-CCGGACGCTATGGCCCA-MGB-3′

Primers and probes for *SOCS-3*, *β-actin*, interferon regulatory factor (*IRF*)-1, *c-myc* and *bcl*-X_L genes were purchased from ABI by Assays-on-Demand system.

2.5. Methylation status

The methylation status of the *SOCS-1* gene was analyzed by methylation-specific PCR as described previously [20].

2.6. Western blotting and immunoprecipitation

Nuclear and cytoplasmic fractions were prepared from HepG2 cells, and Western blotting was performed as described previously [25]. Anti-STAT1 and anti-STAT3 polyclonal antibodies (Cell Signaling Technology, Inc., Beverly, MA), anti-phosphorylated STAT3 (Tyr705) polyclonal antibody (Cell Signaling Technology), anti-phosphorylated STAT3 monoclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY), and anti-protein inhibitor of activated *STAT* (*PIAS*)*1*, *anti-PIAS3* and *anti-SOCS-1* antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were

used. Immunoprecipitation was done as described previously using antibodies followed by protein A-Sepharose [26].

2.7. Immunocytofluorescence

HepG2 cell lines with or without the core gene were grown overnight on chamber slides and treated with 10 ng/ml human IL-6 for 60 min. Cells were fixed with 4% paraformaldehyde plus methanol, and reacted with the anti-STAT3 antibody followed by incubation with a FITC-labeled secondary antibody.

2.8. Statistical analysis

The results are expressed as means ± SD. The significance of the difference in means was determined by Mann–Whitney's *U*-test. $P < 0.05$ was considered significant.

3. Results

3.1. HCV core protein suppresses SOCS-1 gene expression

To examine the impact of the core protein on *SOCS-1* gene expression, we analyzed mRNA expression levels by semi-quantitative RT-PCR in liver tissues from the HCV core gene transgenic and nontransgenic control mice. *SOCS-1* mRNA expression levels in mouse liver tissues of nontransgenic mice were increased in a dose-dependent manner of IL-6, but were only marginal in the liver tissues from the core gene transgenic mice even in those treated with the maximal dose of IL-6 (0.5 μg/g BW, $n=5$ each) (Fig. 1(A) and (C)). In contrast, the expression levels of *SOCS-3* mRNA in the core gene transgenic mice were comparable to or rather higher than those in nontransgenic mice, before and after stimulation with IL-6 (Fig. 1(A) and (E)) [27,28].

We then examined whether or not this observation in mice is reproducible in HepG2 cell lines that constitutively express the core protein. *SOCS-1* gene expression was suppressed in the core-expressing HepG2 cell lines Hep396, Hep397 and Hep39J, even after stimulation with IL-6, while control bulk HepG2 cells or a control Hepswx cell line expressed *SOCS-1* mRNA at high levels (Fig. 1(B) and (D)). In contrast, the levels of *SOCS-3* gene expression were similar among the core-expressing HepG2 cell lines and control HepG2 cells, and were augmented by stimulation with IL-6 (Fig. 1(B) and (F)). These observations indicate that the core protein selectively suppresses *SOCS-1* gene expression before the translational level. The SOCS-1 protein was not detectable by Western blotting either in the mouse liver or HepG2 cells using currently available anti-SOCS-1antibodies.

These results, obtained in HepG2 cell lines constitutively expressing the core protein, were then evaluated using a transient expression system. In this system, HepG2 cells were infected with baculovirus, expressing the core protein as described previously [29], and *SOCS-1* expression was determined by semiquantitative RT-PCR. The introduction

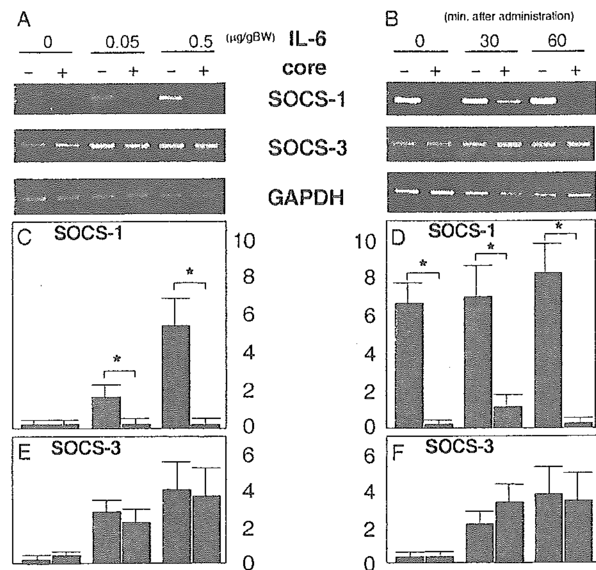

Fig. 1. Suppression of *SOCS-1* gene expression by hepatitis C virus core protein. (A) and (B) RNA from mouse liver tissues (A) or HepG2 cells (B) with or without the core protein was subjected to RT-PCR for the determination of *SOCS-1* and *SOCS-3* gene expression. Liver tissues were taken from mice one hour after inoculation of 0, 0.05 or 0.5 μg/g BW of IL-6. HepG2 cells (Hep396 and Hepswx) were treated with 10 ng/ml of IL-6 for 0, 30 or 60 min before RNA extraction. Bottom panels in (A) and (B) show the expression level of the housekeeping gene GAPDH as an internal control. (C) and (D) Represent means ± SD. of five independent experiments on *SOCS-1* gene expression corresponding to the lanes in (A) and (B), respectively. *, $P < 0.05$. (E) and (F) Represent means ± SD. of five independent experiments on *SOCS-3* gene expression corresponding to the lanes in (A) and (B), respectively.

of the core protein selectively suppressed the expression level of *SOCS-1* mRNA even after stimulation with IL-6 (data not shown).

Modulation of expression by the core protein of STAT-target genes other than *SOCS*s was then examined by determining the mRNA levels in mouse liver tissues. Expression of *IRF-1* gene was suppressed in the presence of the core protein under the stimulation with IL-6, while that of c-*myc* was not affected (Fig. 2(A) and (B)). The expression of *bcl*-X_L gene was rather augmented by the core protein although the difference was not statistically significant (Fig. 2(C)).

The methylation status of *SOCS-1* gene was then explored in liver tissues from the core gene transgenic mice by a method described previously [20], to determine whether or not the *SOCS-1* gene expression may be suppressed by hypermethylation. No hypermethylation was observed in the *SOCS-1* gene of the core gene transgenic mice either at the 5′-noncoding region or the CpG island in the coding region (Fig. 3).

In the analysis of *SOCS-1* expression in noncancerous liver tissues from patients with HCV infection, the *SOCS-1* mRNA expression levels were 0.494 ± 0.352 in HCV-positive patients ($n=9$) and 0.862 ± 0.465 in the control subjects without HCV infection ($n=9$) (in arbitrary units,

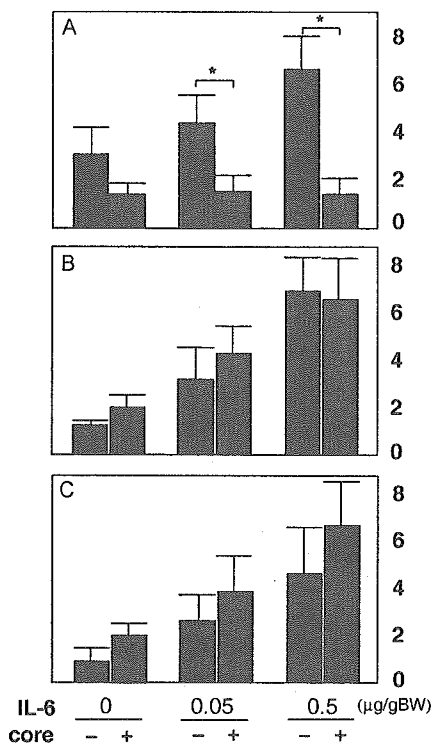

Fig. 2. Effect of hepatitis C virus core protein on the expression of STAT-target genes. RNA from mouse liver tissues with or without the core protein was subjected to RT-PCR for the determination of *IRF1* (A), c-*myc* (B) and *bcl*-X_L (C) gene expressions. Liver tissues were taken from mice one hour after inoculation of 0, 0.05 or 0.5 μg/g BW of IL-6. *, $P<0.05$.

$P=0.0345$). Thus, the SOCS-1 levels in the liver tissues of chronic hepatitis C patients were significantly lower than those of subjects without HCV infection.

3.2. The core protein did not suppress phosphorylation of STAT3 or STAT1

The activation of STAT3 enhances *SOCS-1* expression, thereby forming a negative feedback loop to the STAT3 status [17]. To determine whether or not STAT3 activation is involved in the *SOCS-1* gene suppression in this system, the tyrosine phosphorylation of STAT3 in the mouse liver was examined by Western blotting using an anti-phospho-STAT3 (tyrosine $(Tyr)^{705}$) antibody. At baseline, Tyr^{705} phosphorylation of STAT3 was low in both the core gene transgenic and nontransgenic mice. However, in response to stimulation with IL-6, the levels of Tyr^{705} phosphorylation of STAT3 was higher in the liver tissues from the core gene transgenic mice than that from nontransgenic mice. A representative result is shown in Fig. 4(A). Similarly, the levels of Tyr^{705} phosphorylation of STAT3 were higher in HepG2 cell lines expressing the core protein than those in control cells (Fig. 4(B)). These results observed in HepG2 cells constitutively expressing the core protein was also evaluated in a transient expression system using a recombinant baculovirus, as described above. The Tyr^{705} phosphorylation of STAT3 was enhanced in HepG2 cells infected with baculovirus expressing the core protein compared with mock-infected HepG2 cells (data not shown). The activation of STAT1 was also analyzed using HepG2 cell lines. As shown in Fig. 4(C), the levels of STAT1 phosphorylation was higher in HepG2 cells expressing the core protein than in control cells similar to the result on STAT3.

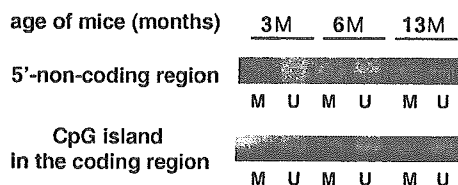

Fig. 3. Methylation status of *SOCS-1* gene in liver from hepatitis C virus core gene transgenic mice. DNA from the liver tissues of core gene transgenic mice at the age of 3 months (3M), 6 months (6M) or 13 months (13M) was subjected to methylation-specific PCR. Only PCR with unmethylation-specific primers yielded bands indicating that the *SOCS-1* gene was unmethylated in the liver tissues of core gene transgenic mice. M, methylation-specific primers; U, unmethylation-specific primers.

3.3. Subcellular localizations of STAT3 and STAT1

STAT activation by tyrosine phosphorylation results in the migration of STAT from the cytoplasm to the nucleus to bind to genomic DNA, modulating of cellular gene expression. We thereby evaluated the subcellular localization of STAT3 and STAT1 by preparing cytoplasmic and nuclear fractions from HepG2 cells followed by Western blotting. The amounts of STAT3 in the nuclei of core-expressing HepG2 cells were similar to or slightly larger

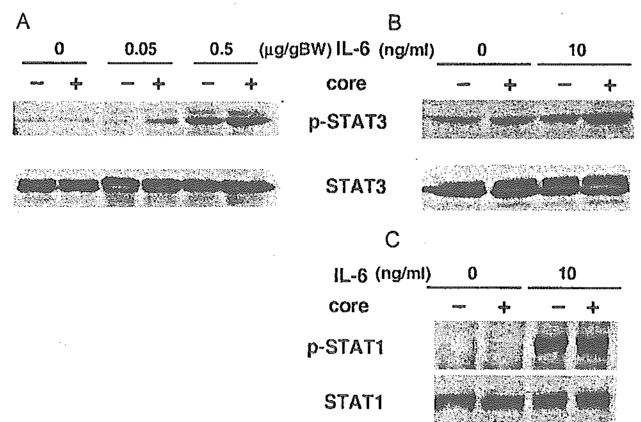

Fig. 4. Increase in the level of tyrosine phosphorylation of STAT3 and STAT1 by hepatitis C virus core protein. Whole cell lysates from mouse liver tissues (A) and HepG2 cells (B) and (C) were subjected to SDS-PAGE followed by Western blotting with anti-STAT3 and anti-*P*-STAT3 (A) and (B) or with anti-STAT1 and anti-*P*-STAT1 (C). Liver tissues were obtained from the mice treated as described in the Fig. 1 legends. HepG2 cells were treated with 10 ng/ml IL-6 or vehicle for 1 h. *P*-STAT3, phosphorylated STAT3; STAT3, total STAT3; p-STAT1, phosphorylated STAT1; STAT1, total STAT1. The experiments were repeated three times.

than those in control HepG2 cells in the presence or absence of IL-6 (Fig. 5(A)). This result was confirmed by an immunofluorescence study (Fig. 5(B)). A similar result was obtained in the analysis of STAT1 subcellular localization (Fig. 5(C)). These observations indicate that the HCV core protein does not inhibit the translocation of STAT3 or STAT1 to the nucleus, and the feedback mechanism is not the cause of *SOCS-1* gene suppression.

Because PIAS3 blocks the nuclear translocation of STAT3 or binding of STAT3 to genomic DNA [30], the expression of PIAS3 was examined by Western blotting. However, there was no significant difference in the levels of PIAS3 between core-expressing HepG2 cells and control HepG2 cells (data not shown). Co-immunoprecipitation analysis was also performed using HepG2 cell lines to know whether or not the core protein affects the association of PIAS1 with STAT1 or PIAS3 with STAT3. However, neither co-immunoprecipitation of STAT1 with anti-PIAS1 antibody nor that of STAT3 with anti-PIAS3 antibody was affected by the presence of the core protein (Fig. 6). We also examined the possibility of the interaction of the core protein with STAT3, which blocks the binding of STAT3 to the promoter of *SOCS-1* gene. For this purpose, a co-immunoprecipitation technique was utilized with whole-cell extracts of core-expressing HepG2 cells. However, no association was observed between these two proteins.

4. Discussion

In the current study, we demonstrated that the core protein of HCV suppresses the expression of *SOCS-1* mRNA in the liver tissues of mice that develop HCC late in their life [4,7]. This observation was reproduced in cultured cells that expressed the core protein. This phenomenon may contribute to the modification of the IFN signaling systems

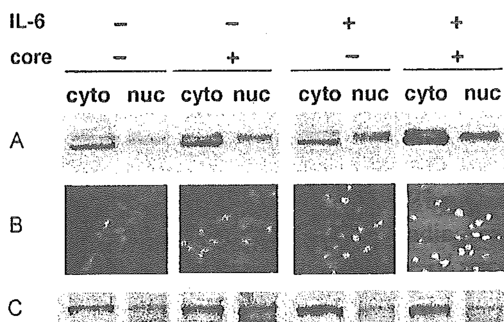

Fig. 5. Hepatitis C virus core protein did not affect subcellular localization of STAT3 or STAT1. Cytoplasmic and nuclear fractions from HepG2 cells with or without the core protein were subjected to Western blotting with the anti-STAT3 antibody (A) or anti-STAT1 antibody (C). HepG2 cells were fixed and an immunocytofluorescence study was performed using the anti-STAT3 antibody (B). Cells were processed before or 60 min after the treatment with 10 ng/ml of IL-6. cyto, cytoplasmic fraction; nuc, nuclear fraction [This figure appears in colour on the web].

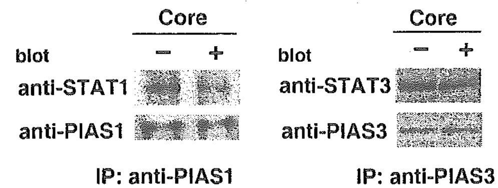

Fig. 6. Effect of the core protein on the interaction of STATs and PIASs. Cell lysates were immunoprecipitated with anti-PIAS1 or anti-PIAS3 antibody, and immunoblotted with anti-STAT1 or STAT3 antibody, respectively. There was no difference in the amounts of STAT1 or STAT3 that were co-immunoprecipitated with anti-PIAS antibodies.

in HCV infection, because SOCS-1 and SOCS-3 play central roles in the Jak/STAT pathway as negative feedback regulators [12–14]. In addition, since SOCS-1 also possesses a tumor suppressor activity [15–17], the down-regulation of *SOCS-1* may contribute to hepatocarcinogenesis in HCV infection. It has been reported that the silencing of the *SOCS-1* gene by hypermethylation is associated with the development of HCC [18–20]. Among patients with HCV infection, a major cause of chronic hepatitis worldwide, HCC develops at a very high incidence [1,2]. Hence, there may be an alternative mechanism of *SOCS-1* silencing to gene methylation in HCV infection. Our current data suggest a possibility of such a mechanism in that HCV per se acts as a negative regulator of SOCS-1, a tumor suppressor. The expression levels of *SOCS-1* mRNA in noncancerous liver tissues in chronic hepatitis C patients were also significantly lower than those in HCV-negative subjects, although the 'shut-off' of the *SOCS-1* gene observed in the experimental systems was not the case. This may be due to the presence of other factors influencing *SOCS-1* gene expression in vivo, including inflammation.

In the exploration of the mechanism underlying the down-regulation of *SOCS-1* expression, we first examined the methylation status of the *SOCS-1* gene in liver tissues from core gene transgenic mice by methylation-specific PCR. Neither the 5′-non-coding region nor the CpG island in the coding region of the *SOCS-1* gene [18–20] was hypermethylated, refuting methylation as a mechanism of SOCS-1 suppression.

We next determined whether or not STAT3, a transcription factor for the *SOCS-1* gene, is involved in the suppression of *SOCS-1* by the core protein: a decreased level or a disturbed phosphorylation of STAT3 may account for the suppression of *SOCS-1*. It was found, however, that STAT3 was rather activated by the core protein, consistent with a previous report [28]. The effect on STAT3 activation by the core protein is yet controversial [31]. Similarly, the activation and nuclear translocation of STAT1 was not disturbed by the presence of the core protein. The core protein differentially affected the expression of STAT-target genes such as *IRF1*, c-*myc* or *bcl*-X_L. The core protein suppressed *IRF1* expression in the mouse liver but did not those of c-*myc* and *bcl*-X_L genes. Regulation of *IRF1*

expression is STAT1-dependent in general, although STAT3 is also involved when stimulated by IL-6 [32]. c-*myc* and *bcl*-X_L inductions by IL-6 are chiefly mediated by STAT3 [33]. Thus, the modulation of expression by the core protein may occur in some other STAT-target genes, suggesting somewhere in Jak/STAT signaling pathway including STAT1 activation is impaired by the core protein. However, no defect was identified in the activation and nuclear translocation of STAT1 and STAT3 in the current study. Thus, although we could not define the precise role of the core protein in *SOCS-1* gene suppression, the direct effect of the core protein on the transcription of the gene is the most likely.

In summary, we found that the HCV core protein selectively suppresses *SOCS-1* gene expression in the liver tissues of animals and cultured cells. These findings may provide a basis for an alternative mechanism of the switch-off of SOCS-1 in the pathogenesis of HCV infection by modulating a tumor suppressor activity or responses to IFNs.

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Murata T, Hijikata M, Shimotohno K.

Enhancement of internal ribosome entry site-mediated translation and replication of hepatitis C virus by PD98059.

Virology. 2005 Sep 15;340(1):105-15.

Translation initiation of hepatitis C virus (HCV) occurs in an internal ribosome entry site (IRES)-dependent manner. We found that HCV IRES-dependent protein synthesis is enhanced by PD98059, an inhibitor of the extracellular signal-regulated kinase (ERK) signaling pathway, while cellular cap-dependent translation was relatively unaffected by the compound. Treatment of cells with PD98059 allowed for robust HCV replication following cellular incubation with HCV-positive serum. Though the molecular mechanism underlying IRES enhancement remains elusive, PD98059 is a potent accelerator of HCV RNA replication.

Murata T, Ohshima T, Yamaji M, Hosaka M, Miyanari Y, Hijikata M, Shimotohno K.

Suppression of hepatitis C virus replicon by TGF-beta.

Virology. 2005 Jan 20;331(2):407-17.

Hepatitis C virus (HCV) is one of the major causative agents of liver diseases, such as liver inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma. Using an efficient HCV subgenomic replicon system, we demonstrate that transforming growth factor-beta (TGF-beta) suppresses viral RNA replication and protein expression from the HCV replicon. We further show that the anti-viral effect of this cytokine is associated with cellular growth arrest in a manner dependent on Smad signaling, not mitogen-activated protein kinase (MAPK) signaling. These results suggest a novel insight into the mechanisms of liver diseases caused by HCV.

Naka K, Takemoto K, Abe K, Dansako H, Ikeda M, Shimotohno K, Kato N.

Interferon resistance of hepatitis C virus replicon-harbouring cells is caused by functional disruption of type I interferon receptors.

J Gen Virol. 2005 Oct;86(Pt 10):2787-92.

Hepatitis C virus (HCV) replicon-harbouring cell lines possessing interferon (IFN)-resistant phenotypes have recently been established. These were divided into two classes: partially IFN resistant and highly IFN resistant. Here, the viral and cellular factors contributing to the IFN resistance of HCV replicon-harbouring cells were evaluated. The results revealed that cellular factors rather than viral factors contributed to a highly IFN-resistant phenotype. The possibility of genetic abnormality of the factors involved in IFN signalling was investigated. As a result, nonsense mutations and deletions in type I IFN receptor genes (IFNAR1 and IFNAR2c) were found in replicon-harbouring cells showing a highly IFN-resistant phenotype, but rarely appeared in cells showing a partially IFN-resistant phenotype. Furthermore, similar genetic alterations were also found in IFN-resistant phenotype, replicon-harbouring cell lines obtained additionally by IFN-beta treatment. Moreover, it was shown that ectopic expression of wild-type IFNAR1 in IFN-resistant phenotype, replicon-harbouring cells possessing the IFNAR1 mutant restored type I IFN signalling.

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Molecular Tracing of the Global Hepatitis C Virus Epidemic Predicts Regional Patterns of Hepatocellular Carcinoma Mortality

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Background & Aims: Molecular evolutionary analysis based on coalescent theory can provide important insights into epidemiologic processes worldwide. This approach was combined with analyses of the hepatitis C virus (HCV) epidemiologic-historical background and HCV-related hepatocellular carcinoma (HCC) in different countries. **_Methods:_** The HCV gene sequences of 131 genotype 1b (HCV-1b) strains from Japan, 38 HCV-1a strains from the United States, 33 HCV-1b strains from Spain, 27 HCV-3a strains from the former Soviet Union (FSU), 47 HCV-4a strains from Egypt, 25 HCV-5a strains from South Africa, and 24 HCV-6a strains from Hong Kong isolated in this study and previous studies were analyzed. **_Results:_** The coalescent analysis indicated that a transition from constant size to rapid exponential growth (spread time) occurred in Japan in the 1920s (HCV-1b), but not until the 1940s for the same genotype in Spain and other European countries. The spread time of HCV-1a in the United States was estimated to be in the 1960s, HCV-3a in the FSU, HCV-5a in South Africa, and HCV-6a in Hong Kong in the 1960s, mid-1950s, and late 1970s, respectively. Three different linear progression curves were determined by analysis of the relationship between HCV seroprevalence and HCC mortality in different geographic regions; a steep ascent indicated the greatest progression to HCC in Japan, a near horizontal line indicated the least progression in the United States and the FSU, and an intermediate slope was observed in Europe. **_Conclusions:_** These findings strongly suggest that the initial spread time of HCV is associated with the progression dynamics of HCC in each area, irrespective of genotype.

Chronic hepatitis C virus (HCV) infection is an endemic disease affecting millions of individuals worldwide.[1,2] HCV infection usually is clinically mild, but the stages of more than 20% of patients can progress during the clinical course, occasionally culminating in hepatocellular carcinoma (HCC) over the course of 2–3 decades, the latter especially in Japan, Spain, and Italy.[3–6] Because the time lag between HCV infection and cancer development is several decades,[4] it is important to estimate the demographic history of HCV infection to predict the future burden of disease.

HCV is classified into 6 major genotypes.[7–9] Within the genotypes there are many subtypes, with varying geographic distributions and modes of transmission.[7] Subtypes 1a (HCV-1a), 1b, 2a, 2b, and 3a are distributed globally and account for the majority of HCV infections worldwide.[10,11] The rapid spread and global dissemination of these subtypes arises from their efficient parenteral transmission via transfusion of contaminated blood products, medical procedures, and illegal injection drug use. Other endemic and epidemic HCV strains are found in restricted geographic areas, including HCV-4a in Egypt, 5a in South Africa, and 6a in southeast Asia (Hong Kong).[7] Because HCV was not identified until 1989, it is difficult to estimate epidemic dynamics associated with the subtypes prevalent before this time.

Abbreviations used in this paper: FSU, former Soviet Union; IDU, injection drug use; *Sj, Schistosoma japonicum*; SRDT, single rate-dated tips.

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Table 1. Characteristics of the Population in Each Country

	United States	Japan *Sj* group	Japan non-*Sj*	Spain	FSU	Egypt	South Africa	Hong Kong
Number	38[a]	64[a]	67[a]	33	27[a]	47[a]	25	24
Mean age	48.2 ± 11.7	69.9 ± 7.7	67.2 ± 8.8	52.9 ± 11.0	24.7 ± 3.6	38.8 ± 9.0	56.8 ± 10.5	49.1 ± 15.4
Sex (M/F)	24/14	34/30	33/34	17/16	22/5	33/14	17/8	12/12
HCV subtypes	1a	1b	1b	1b	3a	4a	5a	6a
Divergence time[b]	1920	1812	1918	1892	1958	1902	1937	1963
Spread time	1965 (1958–1970)	1923 (1890–1937)	1940 (1933–1948)	1942 (1934–1955)	1963 (1958–1974)	1930 (1917–1940)	1955 (1948–1962)	1977 (1968–1982)
Growth rate, y^{-1}	.15298 (.106181–.213943)	.06667 (.044460–.094732)	.12008 (.098260–.149888)	.09715 (.074642–.140650)	.15625 (.121928–.215722)	.09004 (.074114–.115969)	.19762 (.107049–.356198)	.17120 (.090839–.280186)
Risk factors	IDU	PAT	IDU, transfusion, medical	IDU, transfusion, medical	IDU	PAT	Transfusion, medical	IDU

NOTE. 95% confidence intervals shown in parentheses.
PAT, parenteral antischistosomal therapy: transfusion. blood transfusion: medical. medical procedures.
[a]Eight sequences in the United States, 131 in Japan, 20 in the FSU, and 47 in Egypt were obtained from our previous data.[14–17]
[b]Divergence time indicates the most recent common ancestor (MRCA) point of each subtype.

However, using methods based on coalescent theory,[12] the epidemic history of HCV population can be reconstructed from observed genetic diversity of the viral strains.[13] Recently, the molecular clock theory has been applied successfully to estimate the molecular evolutionary rate in long-term serial serum samples obtained from HCV-infected patients in the United States and Japan; a 30-year lag in HCV spread time was shown between these countries. Insofar as a long duration of HCV infection is a critical determinant for the development of HCC, the molecular clock predicted that the incidence of HCC will increase in the United States over the next 2–3 decades[11] and approach the high rates currently observed in Japan.

In a previous study,[13] the spread of HCV-1a, 1b, 4, and 6 infections worldwide was analyzed by the use of HCV sequences obtained from DNA databases; however, corollary clinical and demographic data were limited. In the present study, new sequences from wider geographic regions and with more extensive clinical information are presented. Specifically, HCV-1a strains in the United States, HCV-1b in Japan,[14,15] HCV-1b in Spain, HCV-3a in the former Soviet Union (FSU),[16,17] HCV-4a in Egypt,[18] HCV-5a in South Africa, and HCV-6a in Hong Kong were analyzed by a coalescent-based approach using principles of both population genetics and mathematic epidemiology.[13] Furthermore, the relationship between the estimated spread time and HCC mortality in each country is discussed.

Materials and Methods

HCV Serum Samples From the United States, Spain, FSU, South Africa, and Hong Kong

To elucidate the epidemic history of HCV population in each country, 30 HCV-1a, 33 HCV-1b, 7 HCV-3a, 25 HCV-5a, and 24 HCV-6a samples were obtained from the following blood banks or hospitals, respectively: National Institutes of Health (United States), Hospital Vall d'Hebron (Spain), National Reference Laboratory of Ministry of Health (Uzbekistan), University of the Witwatersrand and National Health Laboratory Services/University of Cape Town (South Africa), and Queen Mary Hospital (Hong Kong). The samples were collected between 2000 and 2003. Along with our previous data,[14–18] the characteristics of the populations studied are shown in Table 1; the mean age of the Japanese population was the oldest and that of the populations from the FSU and Egypt was significantly younger. The study protocol conformed to the 1975 Declaration of Helsinki, and was approved by the Ethic Committees from each institution. Every patient gave written informed consent to participate in the virologic research of HCV at each blood center or hospital. None of the patients had been treated with interferon therapy for HCV infection.

HCV Gene Sequences

The HCV subtypes studied in each country are shown in Table 1, including 38 HCV-1a sequences from the Untied States (30 newly determined and 8 previously reported sequences[11]); 131 previously reported HCV-1b sequences including 64 from *Schistosoma japonicum* (*Sj*)-positive sera and 67 from *Sj*-negative (non-Sj) sera in Japan[15]; 33 HCV-1b sequences from Spain; 27 HCV-3a sequences from the FSU (7 newly determined and 20 previously reported sequences[16,17]); 47 previously reported HCV-4a sequences from Egypt[18]; 25 HCV-5a sequences from South Africa; and 24 HCV-6a sequences from Hong Kong. The GenBank/DDBJ accession numbers of the sequences obtained in the present study are AB204592–AB204708. Japanese HCV-1b and Egyptian HCV-4a sequences were obtained from our previous data (AB103424–AB103457, AF271800–AF271812).[15,18] Available related sequences in

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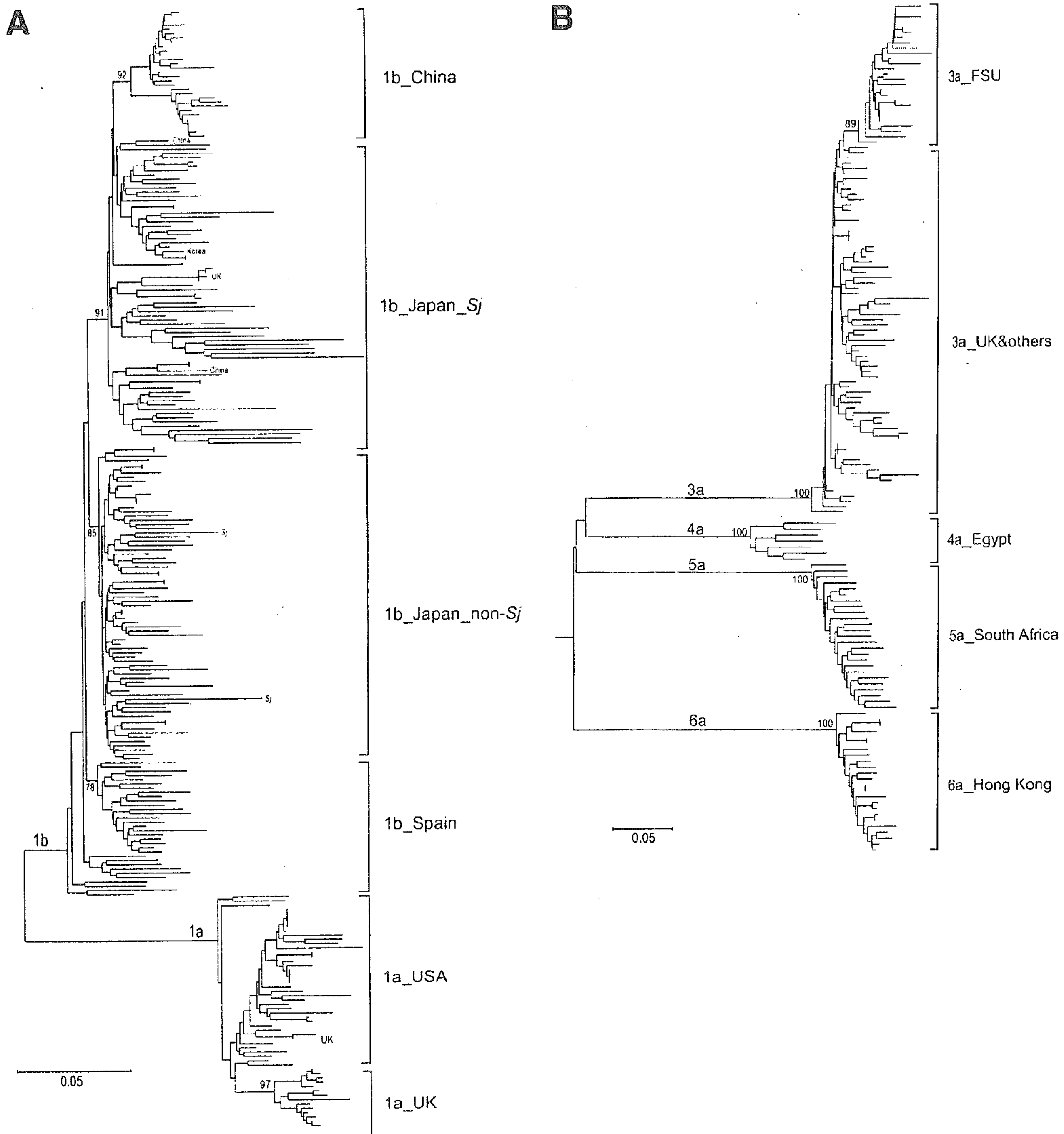

Figure 1. Phylogenetic trees constructed on NS5B sequences of (*A*) HCV-1a and HCV-1b strains and (*B*) HCV-3a, -4a, -5a, and -6a strains. The HCV-1a strains in the United States and HCV-1b strains in Japan (*Sj* and non-*Sj*) and Spain had each significant cluster, and HCV-3a in the FSU, HCV-4a in Egypt, HCV-5a in South Africa, and HCV-6a in Hong Kong. The numbers in the tree indicate bootstrap reliability by the interior branch test. Exceptional strains were labeled according to their country of origin. Significant clusters were subjected to population history analyses.

Figure 1 were recruited from the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp).

Genotyping and Sequencing

Nucleic acids were extracted from the serum samples using a SepaGene RV-R Nucleic acid extracting kit (Sanko Junyaku Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. Viral RNA was reverse-transcribed to complementary DNA using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) as described previously.[19] A sequence spanning 339 nucleotides

in the NS5B region was amplified by polymerase chain reaction with primers described previously.[14] Polymerase chain reaction products were sequenced directly with Prism Big Dye (Applied Biosystems, Foster City, CA) in an ABI 3100 DNA automated sequencer. To reduce the number of artificial substitutions arising in polymerase chain reaction, Platinum Pfx DNA Polymerase (Invirogen Corp.) with a very high fidelity was used. The sequences determined were used to confirm HCV genotypes and to construct phylogenetic trees. To confirm the reliability of the phylogenetic tree, bootstrap reliability was performed by the interior branch test.[20] The overall mean genetic distances in all nucleotide positions and synonymous and nonsynonymous positions were estimated by MEGA version 2.1.[21]

Analysis of Isolation and Migration of HCV Sequences Among Countries

The phylogeny of the HCV-1a, -1b, and -3a sequences from all of the countries under investigation were estimated by the Neighbor–Joining method[22] using the MEGA software (version 3).[21] The analysis of isolation and migration of the sequences was performed by using a method conducted by Nakano et al.[23] The migration histories of the HCV-1a, -1b, and -3a infections were inferred from the phylogeny by the parsimony method whereby the states are the sampling countries of each sequence and the state changes represent migration events. We calculated the expectations and the statistical significances based on the null distribution generated by 1000 times randomization of the sequences with fixing the topology of the phylogeny, where the null hypothesis is that all of the sequences were sampled from a hypothetic panmictic population, using software we developed ourselves.

Estimating Evolutionary Rates and Dating the Origin of HCV

A reconstructed tree was built on the NS5B sequence of 339 nucleotides by a heuristic maximum-likelihood topology search with stepwise addition and the nearest-neighbor–interchange algorithms. Tree likelihood scores were calculated using the HKY85+G method with the molecular clock enforced by PAUP version 4.0b8. By using the estimated topology, all possible root positions were evaluated under a single rate-dated tips (SRDT) model with the computer software TipDate v1.2 and the root that yielded the highest likelihood was adopted.[22] The program provided a maximum-likelihood estimate of the rate and also the associated date of the most recent common ancestor of the sequences, using a model that assumed a constant rate of nucleotide substitution. The molecular clock was tested by a likelihood ratio test between the SRDT model and a general unconstrained branch length model (different rate model). To confirm the reliability of the phylogenetic tree, bootstrap resampling tests also were performed 1000 times.

Demographic Model

As estimates of the demographic history, a nonparametric function, known also as the *skyline plot*, was obtained by transforming coalescent intervals of an observed genealogy into a piecewise plot that represents an effective number of infections through time.[15-23] A parametric maximum-likelihood was estimated by several models with the computer software Genie v3.5 to build a statistical framework for inferring the demographic history of a population on phylogenies reconstructed on sampled DNA sequences.[25] This model assumes a continuous epidemic process in which the viral transmission parameters remain constant through time. Model fitting was evaluated by likelihood-ratio tests of the parametric maximum-likelihood estimates.[24,25]

Results

Analysis of Isolation and Migration of HCV Sequences Among Countries

Preliminarily, all sequences generated in this study were subjected to phylogenetic analyses together with all previously reported sequences available from DDBJ/GeneBank. The most significant phylogenetic clusters containing a total of 325 representatives of the HCV endemic populations from different regions (Table 1) were determined and subjected to further maximum-likelihood phylogenetic analysis with enforced molecular clock, as previously described.[13,14] Figure 1 shows the phylogenies of the HCV-1a and -1b (Figure 1*A*) and HCV-3a, -4a, -5a, and -6a (Figure 1*B*) sequences obtained in the present study along with closely related sequences. As shown in Figure 1*A*, 4 clusters of HCV-1b sequences were found, and some sequences from China, Korea, and the United Kingdom belonged to the Japanese *Sj* group. Also, 2 *Sj*-positive strains clustered with non-*Sj* strains in Japan. To measure country-wise clustering statistically, the isolation and migration of HCV-1b sequences were analyzed by use of a parsimony method. The estimated number of changes in location between groups (ie, migration events) was 7 for HCV-1b, whereas the expected number of location changes for the 1,000 simulated trees created with randomized locations was 40.38 for HCV-1b (Table 2). The observed number of migration events was significantly smaller ($P < .001$) than that expected under the null hypothesis of complete geographic mixing; therefore, this hypothesis can be rejected. This result suggests that there is considerable subdivision by location among the HCV-1b strains sampled. The parsimony analysis also provided clues about the movement of HCV-1b strains among the 6 groups. Table 2 shows the difference between the observed and expected number of changes for each pair of countries. In most cases, the observed number of migration events was

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Table 2. Isolation and Migration of HCV Subtype 1b Among 6 Groups

	Japan (*Sj*)	Korea	China	United Kingdom	Spain	Japan (non-*Sj*)
Number of observed changes in tree (total, 7)						
Japan (*Sj*)	-	1	2	1	0	0
Korea	0	-	0	0	0	0
China	0	0	-	0	0	0
United Kingdom	0	0	0	-	0	0
Spain	0	0	0	0	-	0
Japan (non-*Sj*)	2	0	0	0	0	-
Number of expected changes per tree (total, 40.38)						
Japan (*Sj*)	-	.09	3.30	.48	3.22	8.16
Korea	0	-	0	0	0	0
China	.02	0	-	.01	.63	1.36
United Kingdom	.02	0	.01	-	.01	.03
Spain	1.23	.02	.63	.09	-	1.39
Japan (non-*Sj*)	10.27	.13	4.26	.65	4.31	-
P value (total, $P < .001$)						
Japan (*Sj*)	-	NS	NS	NS	.027	<.001
Korea	NS	-	NS	NS	NS	NS
China	NS	NS	-	NS	NS	NS
United Kingdom	NS	NS	NS	-	NS	NS
Spain	NS	NS	NS	NS	-	NS
Japan (non-*Sj*)	<.001	NS	.012	NS	.011	-

smaller than expected, indicating no significant movement of HCV-1b strains among these groups. For HCV-1a and -3a, the observed number of migration events was significantly smaller than the expected number, again suggesting significantly less overall migration than would be expected by chance.

The Origin of HCV Subtypes in Each Country

Figure 2 shows a comparison of genetic distances estimated on all synonymous and nonsynonymous nucleotide positions between HCV strains in each country. The genetic distances were the greatest among HCV-1b strains in the Japanese *Sj*-positive group, followed by the Egyptian HCV-4a, Spanish HCV-1b, South African HCV-5a, and HCV-1b strains in the Japanese non-*Sj* group. The genetic distances among the US HCV-1a, the FSU HCV-3a, and Hong Kong's HCV-6a were similar and comparatively smaller. These data indicate that the Japanese HCV-1b population is the oldest, and Hong Kong's HCV-6a population is the youngest among the populations studied.

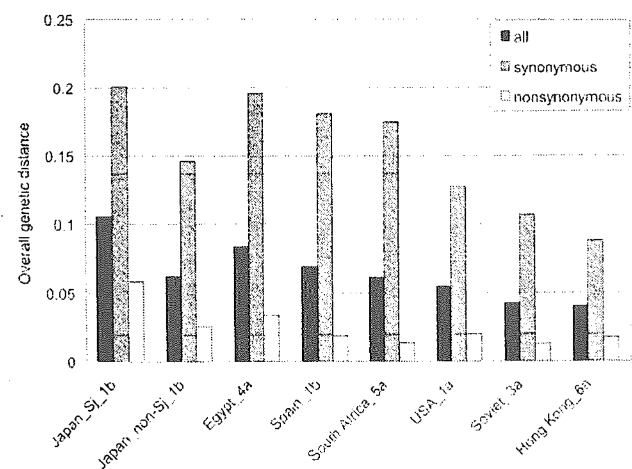

Figure 2. The overall mean genetic distances of all nucleotides positions, synonymous positions, and nonsynonymous positions in each country.

The molecular evolutionary rate was estimated by 2 independent methods. First, our previous linear regression analyses indicated that a molecular evolutionary rate was .58 (range, .53–.61) $\times 10^{-3}$ nucleotide substitutions/site/y.[14] Second, TipDate v1.2 was used to compare the different-rate model with the single rate and SRDT models. The SRDT model provides an adequate fit to most datasets ($P > .05$); the rates were similar (range, .50–.72) $\times 10^{-3}$ nucleotide substitutions/site/y. Although the SRDT model was rejected ($P < .05$) in 1 dataset, simulations have shown that even when the molecular clock is rejected, the confidence limits of the substitution rate sometimes may include the true rate.[26] Hence, we used the substitution rate estimated previously: .58 $\times 10^{-3}$ substitutions/site/y for the NS5B region.

Based on TipDate,[22] to investigate the origin of HCV subtypes we converted the genetic distance in the phylogenetic tree into a timescale by using the molecular clock. According to the timescale, the most recent com-

mon ancestor for HCV subtypes in each country was established (Table 1); the divergence time of the most recent common ancestor for HCV-1b in the Japanese *Sj*-group was estimated before 1850, followed by that of Spanish HCV-1b strains (in 1892). The divergence time in the other groups was estimated to be in the 20th century. Notably, HCV-3a and HCV-6a strains have been introduced relatively recently into the FSU (in 1958) and Hong Kong (in 1963), respectively (Table 1).

Historical Analyses of the HCV Population by Using the Coalescent Theory

The level of population subdivision shown in the parsimony analysis described earlier suggests that much of the transmission of the sampled HCV strains occurred within the sampled groups. Therefore, the epidemic history of HCV strains in each group was estimated from separate trees.

Based on the phylogenetic analysis, the effective number of HCV infections through time, $N(t)$, was analyzed using a skyline plot for the HCV strains. The parameters for several models in Genie v3.5 were examined. Time (t) then was transformed to year using the same rate, assuming the collecting time to be the present. Figure 3 shows the skyline plots and HCV population growth in each country according to a piecewise expansion growth model that was evaluated by likelihood-ratio testing (data not shown).[24,25] An expansion growth model gave the best fit for only the *Sj*-positive HCV-1b population, but because this likelihood ratio was almost the same as that of the piecewise expansion growth model, all populations were applied to the same piecewise expansion growth model. Our estimates of the effective numbers of HCV infections showed a transition from constant size to rapid exponential growth in the 1920s among the Japanese *Sj*-positive HCV-1b population, as we have reported earlier.[15] This indicates the oldest outbreak among all studied populations, whereas the exponential growth among the Japanese *Sj*-negative HCV-1b population was dated in the 1940s,[15] which is close in time to the HCV-1b populations in Spain (Figure 3*A*) and other European countries,[27,28] and the HCV-4a population in Egypt.[18] The exponential growth of the HCV-5a population in South Africa occurred in the 1950s (Figure 3*B*), and comparatively recent HCV endemics were dated in the 1960s for both HCV-1a in the United States (Figure 3*C*) and HCV-3a in the FSU (Figure 3*D*), and in the late 1970s for HCV-6a in Hong Kong (Figure 3*E*).

The exponential growth rates also varied among the subtype populations (Table 1). The estimated rates for HCV-5a in South Africa, HCV-6a in Hong Kong, HCV-3a in the FSU, and HCV-1a in the United States were higher than those for HCV-1b in Japan, HCV-1b in Spain, and HCV-4a in Egypt. Hence, our findings indicate that the particular epidemics worldwide, associated with the corresponding HCV subtype, had different patterns in terms of divergence time, exponential spread time, and the dynamic growth rate. The different ages of the studied viral subpopulations, best assessed by synonymous genetic distance values, are shown in Figure 2.

Relative Population Size of HCV Subtypes in Each Country

Current estimates of the HCV subtype distribution in each sampled country were used to transform the epidemic histories shown in Figure 3 and previous data[14,15,18] to reflect the relative historical levels of HCV subtype infection in each country (Figure 4). As shown in Figure 4, 3 different growth patterns were found; one is the oldest historical pattern of HCV-1b in the Japanese *Sj* group, and the second group consists of HCV-1b in the Japanese non-*Sj* group, HCV-1b in Spain, and HCV-4a in Egypt. The last group, with the latest exponential growth, includes 4 different subtypes in independent countries: HCV-1a in the United States, HCV-3a in the FSU, HCV-5a in South Africa, and HCV-6a in Hong Kong.

Discussion

The world map of HCC occurrence still contains many wide gaps owing to difficulties collecting exact clinical and epidemiologic data from many countries. The positive correlation between HCV seroprevalence and HCC mortality was documented in a recent European report.[29] However, most of the HCV seroprevalence data worldwide are derived from studies of blood donors who represent a selective relatively low-risk, younger population. Therefore this approach underestimates the absolute burden of infection[30] and complicates a comparative analysis of the results obtained in different countries, especially for the age-specific HCV seroprevalence.

Because HCC is associated directly with the duration of HCV infection in a given carrier, the time of exposure to HCV infection is another critical determinant of HCC incidence at the population level. To investigate further the relative role played by the duration of HCV infection, we analyzed current and previously published data from populations throughout the world for the general and age-related HCV seroprevalence, the estimated time of HCV exponential spread, the association with primary risk factors for virus transmission, and HCC mortality.

The past population dynamics of a virus can be inferred from viral gene sequence data using a population

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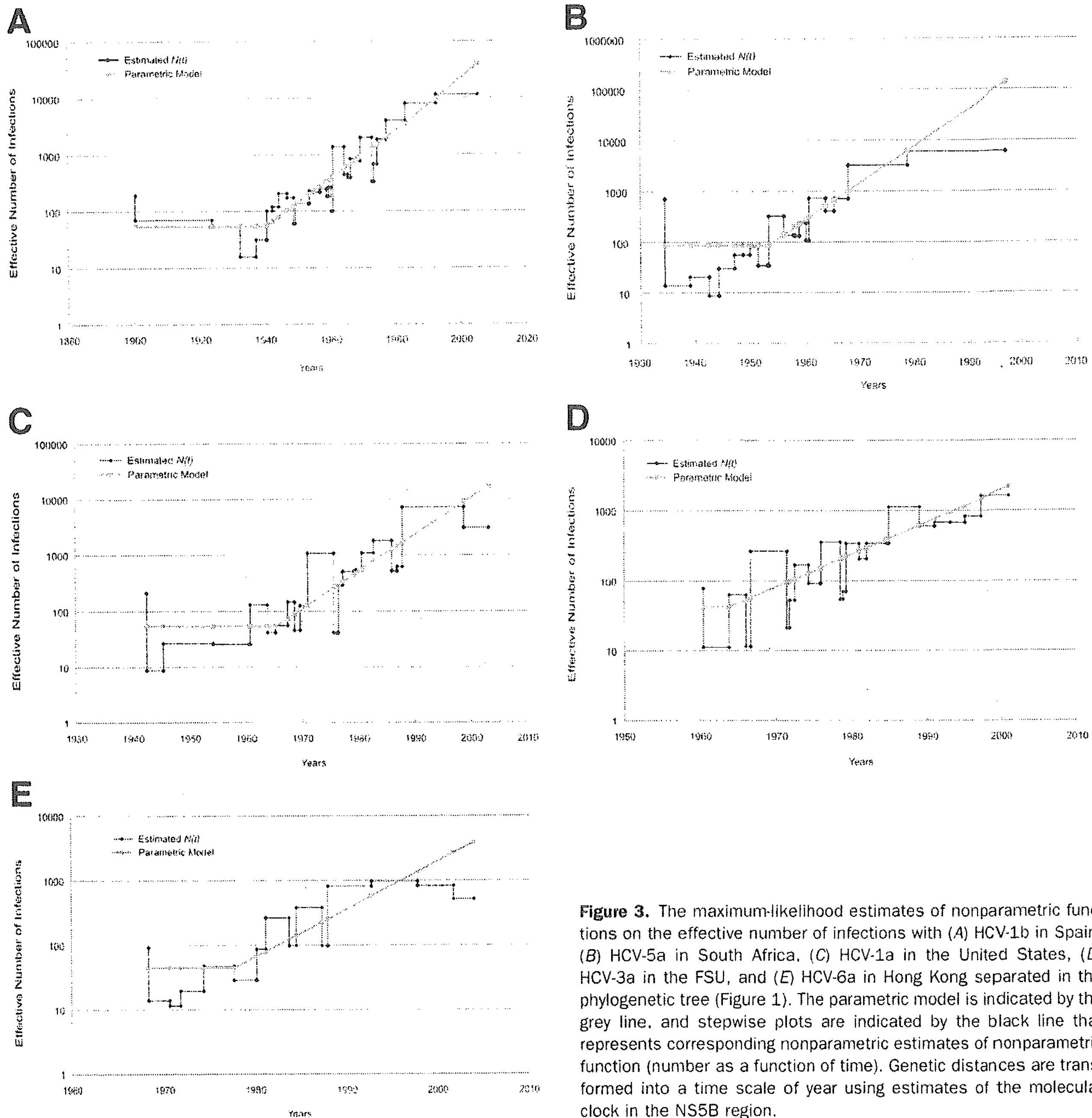

Figure 3. The maximum-likelihood estimates of nonparametric functions on the effective number of infections with (*A*) HCV-1b in Spain, (*B*) HCV-5a in South Africa, (*C*) HCV-1a in the United States, (*D*) HCV-3a in the FSU, and (*E*) HCV-6a in Hong Kong separated in the phylogenetic tree (Figure 1). The parametric model is indicated by the grey line, and stepwise plots are indicated by the black line that represents corresponding nonparametric estimates of nonparametric function (number as a function of time). Genetic distances are transformed into a time scale of year using estimates of the molecular clock in the NS5B region.

genetic model called *the coalescent theory*.[31] The coalescent framework requires a demographic model, denoted $N(t)$, that describes the effective population size through time. A demographic model based on neutral theory, which infers that a constant-size population in the past changes to grow exponentially starting at a specific point in time,[13,24,25] was applied to investigate the HCV population history worldwide. Various HCV genotypes and subtypes circulating in the geographic regions were studied. However, in each country we selected a single subtype representative of the subpopulation conforming to the largest indigenous phylogenetic cluster; this cluster would play an important role in the particular epidemic network (see later), and at the same time fulfill the requirements for calculations by the methods used.[13,14]

In Japan, HCC incidence is exceedingly high and comparatively well studied. Chronic HCV infection is responsible for the majority of HCC cases in Japan even though the overall HCV seroprevalence is relatively low at 1.4%. Notably, however, the highest incidence of HCC occurs in persons older than age 70 (>150 per 100,000 men), in whom HCV prevalence is correspond-

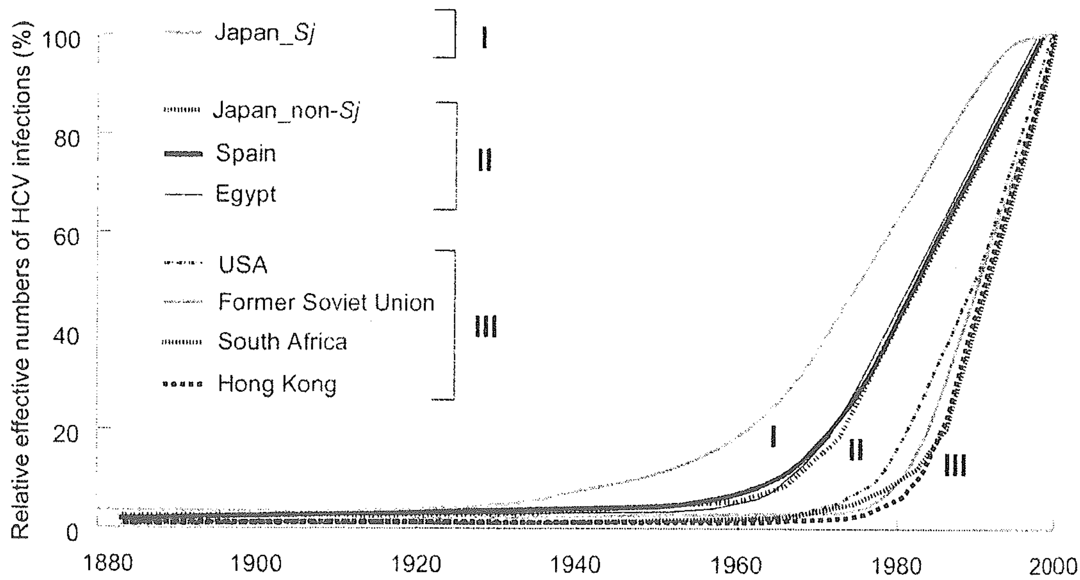

Figure 4. Relative effective numbers of HCV infections in each country. Three different growth patterns were defined as I, II, and III.

ingly high at approximately 7%.[32] HCV-1b is the predominant genotype in Japan and 2 relatively distinctive waves of its spread were described recently in detail.[15] Briefly, the first wave was associated with treatments for *Sj* beginning in 1921,[33] and the second wave coincided with World War II (1940s) when war-associated injection drug use (IDU), blood transfusions, and medical procedures intensified and contributed to HCV transmission.[15,32,34]

In Europe, HCC mortality rates are highly variable in different countries,[35] and a positive correlation with HCV seroprevalence was documented recently (summarized in Figure 5).[29] Detailed data were obtained in France using a model based on epidemiologic analyses of HCV-infected patients and mortality data from national statistics surveys that allowed tracing of the HCV epidemic back to the 1940s.[27] These data were similar to our results estimated for the epidemic in Spain, where exponential growth of the virus population began after 1940. These data are consistent with the likelihood of HCV transmission during and after the Spanish Civil War in the late 1930s, and with the widespread use of shared needles for penicillin treatments in the early 1940s. In Italy, as in Japan, the prevalence of anti-HCV was the highest among elderly people (age, 75–79 y) suggesting a cohort effect dating to exposure during World War II.[28]

Indeed, in the United States, prevalent subtypes in the general population are HCV-1a (57%) and 1b (17%)[36]; an association of the HCV-1a epidemic with IDU has been reported.[37] Our estimates of the epidemic history of HCV-1a in the United States are consistent with the onset of injection opiate use between 1950 and 1960 and more widespread use in the late 1960s and 1970s.[38] The relative importance of IDU and blood transfusion associated with HCV transmission in the United States has changed over time,[39] and IDU has been the predominant mode since the 1970s.[40]

HCV-1a and 3a are the most prevalent among patients with a history of IDU and appear to be increasing in prevalence worldwide.[10,16,41] In the FSU, HCV-1b was predominant in all population groups until the late 1970s, and was associated primarily with medical procedures and unscreened blood products during and after World War II. After blood screening started in the 1990s, an HCV genotypic shift occurred first in the drug-addicted population and, more recently, has been observed in the general population (Musabaev EI, personal communication). HCV-3a represents a comparatively recent and growing epidemic associated with IDU in the FSU.[16,41] Our estimates traced that HCV-3a spread from the 1960s, coinciding with the start of mass vaccination campaigns against measles when there were no disposable syringes in the FSU. However, a rapid increase of the estimated viral population size started in the late 1970s, probably associated with the expanded IDU network that was stimulated dramatically by the Afghan War (Ruzibakiev R, personal communication, web information-analytical recourses: http://druglibrary.org/ and http://www.irinnews.org/).

Similar data were obtained from Hong Kong where 2 relatively distinct HCV epidemics were observed: HCV-1b was predominant among older patients with chronic hepatitis whereas HCV-6 was detected predominantly in

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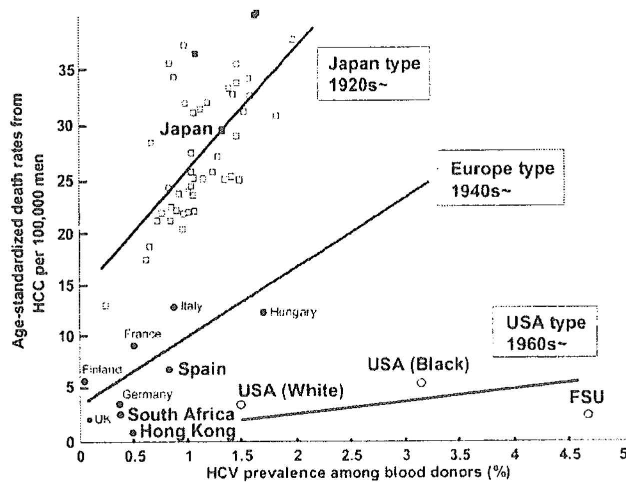

Figure 5. Linear relationship between HCV seroprevalence and HCC annual mortality rates constructed using available data from different countries. Three different patterns were observed: Japan type, European type, and the USA type. Data from European countries were obtained from a previous report.[29] Japan: HCV seroprevalence was approximately 1.4% in the general population from 1988 to 1992. The age-adjusted mortality rate of HCC among Japanese men was approximately 30 per 100,000 during 1990 (■),[32] which is close to the mean age-adjusted death rates of HCC among the 48 prefectures (27.3 per 100,000 persons in 2001). □ and ■, (*S*)-endemic areas) show the relationship between the age-adjusted annual mortality rates of primary liver cancer and anti-HCV among the general population older than 40 years of age in 2002 and a positive significant correlation was found.[50] Spain: data were obtained mainly from a previous European study,[29] which was consistent with another recent study performed in Valencia (Spain) (2,172,796 inhabitants in 1998: 1,060,156 males and 1,112,640 females) in 2000[51]; the estimated incidence of HCC was 8.2 cases per 100,000 inhabitants. United States: HCV seroprevalence was 3.2% among non-Hispanic blacks and 1.5% among non-Hispanic whites from 1988 to 1994.[36] The age-adjusted mortality rate of HCC among black men was 6.0 per 100,000 in 1991–1995, and 3.4 per 100,000 among white men.[52] FSU: HCV seroprevalence was approximately 5% in blood donors, and the HCV-related HCC mortality rate was estimated at approximately 2.5 per 100,000 men in 1990 (Ruzibakiev R and Musabaev M, personal communication). South Africa: The prevalence of HCV infection among blood donors was .41% during 1992 and 1994.[53] The HCV prevalence was .75% among blacks and .16% among non-Hispanic whites.[54] The age-adjusted incidence of HCC was approximately 30 per 100,000.[47] HCV-related HCC was approximately 10%, indicating 3 per 100,000 from HCV-related HCC. Hong Kong: the prevalence of HCV was .5% in the general population, and the estimated HCV-related HCC mortality was much rarer than HBV-related HCC mortality.[42,47]

younger patients with a history of IDU and in young patients with thalassemia major.[12] Age-related prevalence and results of our estimation suggested that HCV-6 infection represents a recent growing epidemic that will have an increasing influence on HCC incidence in the future.

In South Africa (sub-Saharan Africa) and Hong Kong, hepatitis B virus (HBV) infection is much more prevalent than HCV infection and the epidemiology of HCV infection is less well characterized. Our results indicate that both genetic diversity and the growth rate of the HCV-infected population in South Africa are higher than would be expected. This may be explained by the fact that most studied samples were obtained from elder patients in this region, including 8 of 24 patients with HCC (all male blacks; mean age, 65.3 ± 3.3 y). The major risk factors for HCV transmission in South Africa include contaminated blood transfusions and medical procedures performed with inadequately sterilized shared instruments.

In analyzing data on the association between HCC mortality and HCV seroprevalence, 3 general patterns were observed (Figure 5). The first pattern was observed in Japan (Japan type), where the data presented from all geographic parts of the country indicated that HCC mortality was the highest in the world, whereas HCV seroprevalence was comparatively low. The second pattern was observed in European countries (Europe type), where the HCC incidence had a more direct association with HCV prevalence as previously reported,[29] although recent reports in some European countries such as Spain indicated that HCC mortality in men older than 60 years overlapped with the high mortality rate observed in Japan.[13,14] The third pattern was observed in the United States (≃5 million HCV-infected) and the FSU (USA type) with HCV seroprevalence comparable with Japan, but low HCC mortality rates. The greatest progression to HCC in Japan, indicated by the steep ascent in Figure 5, is consistent with the previously reported high annual incidence of HCC (7.9%) among patients with stage F4 fibrosis.[45] Because the HCV epidemic in Japan began early, resulting in a large cohort with a very long duration of infection, more patients in Japan have reached the stage of advanced fibrosis that increases their likelihood of developing HCC. Thus, the slope of the curve is influenced strongly by the age-specific prevalence and the duration of HCV infection. This would predict that as age and the duration of infection increases in other populations, including the United States, the slope of the HCC mortality curve will increase steeply. The patterns observed in South Africa and Hong Kong, characterized by comparatively low HCV prevalence and low HCV-related HCC mortality, were intermediate between that in Europe and the United States. However, precise data regarding the association of HCV with HCC incidence are not available in these regions, and most cases of HCC in Hong Kong and South Africa probably were associated with HBV infection, which is highly prevalent in these countries.[46,47] A total of 80.3% and 62.7%–70.5% were positive for hepatitis B surface antigen in Hong Kong and South Africa, respectively, whereas a relatively low prevalence of HBV-related HCC (≃20%) was reported in the United States, Europe, and Japan.[48] The relative

estimated viral population growth dynamics obtained in different countries are shown in Figure 4. All estimated data were separated into 3 groups according to the time virus exponential spread began: the first rapid spread was associated with schistosomiasis treatments in Japan (*Sj* group) in the 1920s; the second wave occurred in Japan (non-*Sj* group), Spain, and Egypt in the 1940s; and the third wave occurred in the 1960s involving the United States, the FSU, South Africa, and Hong Kong. When we combine these data (Figures 4 and 5), a putative picture of the global HCV epidemic emerges that potentially would allow predictions of HCC dynamics in any region of the world. However, no data on HCC mortality were available from Egypt, where the high general HCV prevalence and the estimated spread time suggest that Egypt might have a very high HCC mortality rate, comparable with that in Japan. A recent report from Egypt showing a high HCC incidence among chronic liver disease patients (4.7%)[19] is consistent with our hypothesis.

It could be argued that our results may not represent the community distribution of HCV strains because of the vast predominance of tertiary institution referral. However, we found no significant difference in the sequences of HCV isolated from blood donors and patients with chronic liver diseases, indicating that little bias would be expected to occur in our molecular evolutionary analyses. Hence, an advantage of the coalescent approach to molecular epidemiology used here is that the entire history of a transmission cluster can be investigated using a relatively small sample of gene sequences. In addition, this approach allows a more complete analysis of global HCC dynamics and a prediction of HCC occurrence rates over time. The implications are that Japan has set the model for HCV-related HCC and that the high HCC incidence in Japan may be replicated by the rest of the world as their HCV-infected population ages and the duration of HCV infection approaches that currently observed in Japan. Clearly, there is a need not only to prevent new HCV infections, but also to eradicate chronic infections with appropriate treatment strategies. Unfortunately, the current costs of antiviral therapy are prohibitive for many of the regions where HCC is likely to escalate in the future. High priority must be given to global prevention of HCV-related cirrhosis, the almost universal predecessor to HCV-induced HCC.

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