

The mechanisms of oncogenesis of HBV remain obscure, but several factors have been identified to be associated with a high risk of developing of HCC among CHB patients. In regard to virological factors, hepatitis B e antigen (HBeAg) positivity, high serum HBV DNA levels, HBV genotype (genotype C more than genotype B), pre-core mutations (G1896A), and core promoter mutations (A1762T/G1764A) have been reported [4–11]. In addition, other risk factors such as host factors (male, increasing age) [12] or disease factors (alanine aminotransferase (ALT) levels, cirrhosis) [12, 13] have been mentioned. Among these factors, A1762T/G1764A double mutation has been thought to be the most convincing factor associated with the development of HCC among CHB patients [9, 10, 14–16]. But most of these risk factors were identified by cross-sectional or case-cohort studies with relatively limited numbers of study population, and it has been unclear which factor is the most important in developing HCC. A prospective study for the development of HCC associated with HBV infection is required to solve these problems, but there have been only a few prospective studies about this from Asia [17–19].

In the present report, we describe results of the first large-scale population-based cohort study carried out in Japan to identify the risk factors for the development of HCC among HBV mono-infected Japanese patients.

Methods

Study population and baseline survey

The Japan Public Health Center-based Prospective Study (JPHC Study) was launched in 1993–1994 in the Cohort II among registered Japanese inhabitants aged 40–69 years at the time of the baseline survey in 6 prefectural public health center areas. Details of the study design have been described elsewhere [20, 21]. A part of one public health center area was excluded since its study population was defined differently to the others. Initially, we defined a population-based cohort of 68975 subjects after exclusion of ineligible subjects.

At baseline, a self-administered questionnaire survey on various lifestyle factors was conducted (response rate 82%). Blood was also provided voluntarily by 39% of the respondents during health checkup provided by the respective local government. Clinical laboratory examinations such as ALT and γ -glutamyl transpeptidase (γ -GTP) were carried out on these samples by using commercial assays.

For the present analysis, we restricted subjects to those who responded to the questionnaire and for whom blood samples were available. We further excluded those with a history of HCC and those with missing data on HBV and

hepatitis C virus (HCV) infection status. Finally, a total of 19393 individuals were included in the present analysis.

Subjects were checked for hepatitis B surface antigen (HBsAg) and antibody to hepatitis C virus (anti-HCV). Patients with HBsAg alone were classified into the HBV mono-infected group. All anti-HCV positive subjects were excluded from the study to eliminate the confounding effect of HCV infection on the HCC occurrence.

A baseline self-administered questionnaire survey on several lifestyle factors (body mass index (BMI), alcohol intake, and smoking) was conducted. Alcohol intake exceeding 80 g/day was defined as excessive.

Follow-up and identification of HCC

Enrolled subjects were followed from the baseline survey for average 12.7 years (until 31 December 2006). Residence status and survival were confirmed through the residential registry. Residence and death registration are required in Japan by law and the registries are believed to be complete. Inspection of the resident registry is legally sanctioned by resident registration law.

The occurrence of HCC was determined by notification from the participating hospitals in the study areas and by data linkage with population-based cancer registries. Death certificates were used as a supplementary information source to confirm the cause of death. The site of origin and histological type were coded by using the *International Classification of Diseases for Oncology, 3rd edn.* (ICD-O-3; C22.0) [22]. Through this procedure, a total of 110 newly diagnosed HCC cases were identified among the 19393 subjects during the follow-up period.

Serological analysis

HBsAg, anti-HBs, and anti-HCV in sera were determined by commercial enzyme immunoassay (AxSYM, Abbott Japan, Tokyo, Japan; or Lumipulse forte, Fujirebio, Tokyo, Japan). HBV core-related antigen (HBcAg), which correlates with HBV DNA in serum, was measured in serum, using a chemiluminescence enzyme immunoassay (CLEIA) as described previously (detection limit 1.0 kU/mL) [23]. In brief, HBcAg comprises HBcAg and HBeAg, which share the first 149 amino acids encoded by the core gene. Using the specific enzyme immunoassay within the common region, this assay is able to detect HBcAg and HBeAg even in anti-HBc or anti-HBe antibody-positive specimens [23].

Quantification of serum HBV DNA

HBV DNA parts spanning the S gene [nt 427–606] were amplified by real-time detection polymerase chain reaction (RTD-PCR) using the previously described protocol [24],

with slight modification [25]. The detection limit of this assay was 100 copies/mL.

Sequencing of HBV genome

To investigate HBV genotypes and specific mutations, nucleic acids were extracted from serum samples (100 µL) using the QIAamp DNA extraction kit (Qiagen, Hilden, Germany) and subjected to polymerase chain reaction (PCR) for amplifying genomic areas bearing enhancer II/core promoter/pre-core regions [nt 1628–2364], as described previously [26]. Amplicons were sequenced directly with use of the ABI Prism Big Dye ver. 3.0 kit in the ABI 3100 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were analyzed in both forward and backward directions. HBV genotypes were determined by molecular evolutionary analysis. Reference sequences were retrieved from the DDBJ/EMBL/GenBank database and aligned by CLUSTAL X, and then genetic distances were estimated with the 6-parameter method in the Hepatitis Virus Database (<http://s2as02.genes.nig.ac.jp/>) [27]. Based on obtained distances, phylogenetic trees were constructed by the neighbor-joining (NJ) method with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times.

Statistical analysis

Person-years of follow-up from the date of baseline survey were calculated until the date of diagnosis of HCC, date of death, relocation from the public health center area, or 31 December 2006, whichever occurred first.

Statistical differences were evaluated by Mann–Whitney *U* test, Fisher’s exact probability test, and χ^2 test, where appropriate. Differences were considered to be statistically significant at $P < 0.05$. Multivariate-adjusted Cox hazard model were utilized to sort out independent risk factors for the development of HCC among HBV mono-infected subjects. In addition, Kaplan–Meier method and log-rank test were used to compare the probability of HCC occurrence-free between HBV mono-infected subjects with and without this independent factor. STATA Software ver. 8.0 (Stata-Corp LP, College Station, TX, USA) was employed for all analyses.

Results

Baseline characteristics in HBV mono-infected subjects with and without HCC

Of 19393 subjects, 479 had HBV mono-infection (2.5%), 1051 had HCV mono-infection (5.4%), and 20 (0.1%) had

co-infection of both HBV and HCV. During the 245923 person-years’ follow-up (average follow-up period 12.7 years), a total of 110 cases of newly diagnosed HCC (73 men and 37 women) were documented. Of these, 13 subjects had HBV mono-infection (11 men and 2 women), 78 had HCV mono-infection, 2 had co-infection, and 17 had neither HBV nor HCV. The summary of the population studied is shown in Fig. 1.

The characteristics of these HBV mono-infected subjects with and without HCC are shown in Table 1. The proportion of male was significantly higher in the HCC group (11/13 (85%) vs. 209/466 (45%); $P < 0.005$). As to lifestyles, the proportion of smoking was significantly higher in the HCC group (7/13 (54%) vs. 92/466 (20%); $P < 0.005$), but there were no significant differences in BMI and alcohol intake.

In regard to virological factors, the positivity of HBeAg and HBcrAg was significantly higher in the HCC group (3/13 (23%) vs. 14/466 (3%); $P < 0.001$, and 7/13 (54%) vs. 99/466 (21%), $P < 0.005$, respectively). Furthermore, both serum HBcrAg and HBV DNA levels were significantly higher in the HCC group (Table 1). The proportion of patients with HBV DNA ≥ 5 log copies/mL, which was reported as a risk factor of HCC development, was also significantly higher in the HCC group (6/13 (46%) vs. 39/466 (8%), $P < 0.001$). The proportion of HBV genotype C2/Ce tended to be higher in the HCC group, but there was no significant difference. As to HBV specific mutations, T1753V mutation and A1762T/G1764A double mutation was significantly more frequent in the HCC group (6/13 (46%) vs. 78/421 (19%); $P < 0.05$, and 11/13 (87%) vs. 142/421 (34%), $P < 0.001$, respectively) (Table 1). Figure 2

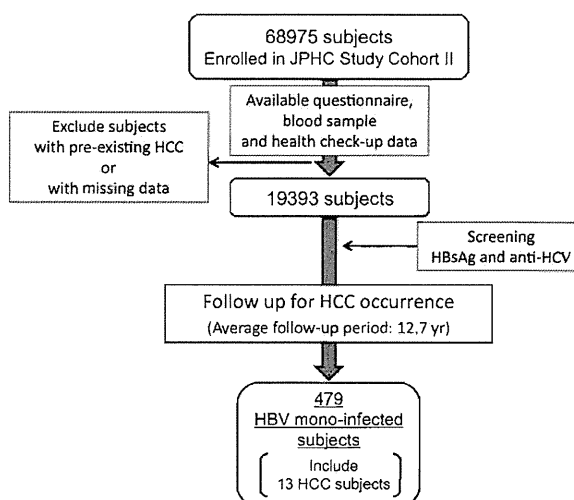
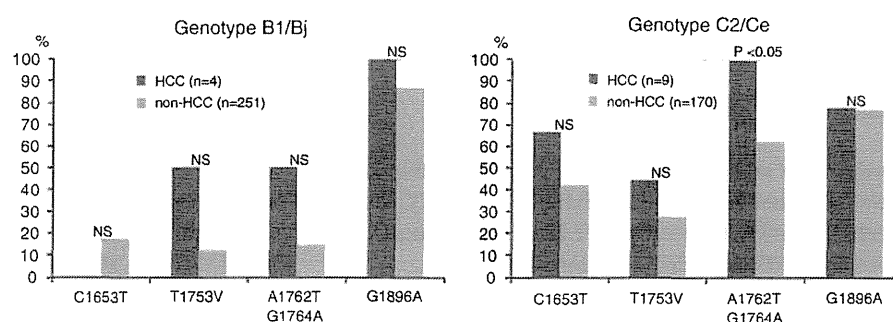


Fig. 1 The summary of the population in this study. Of 479 HBV mono-infected subjects, 13 had HCC

Table 1 Characteristics of HBV mono-infected subjects with and without HCC

	HCC group (n = 13)	Non-HCC group (n = 466)	P value
Age (years)	58.8 ± 6.3	55.1 ± 8.5	NS
Male	11 (85%)	209 (45%)	<0.005
BMI	22.3 ± 2.9	23.4 ± 3.0	NS
Alcohol	0	36 (8%)	NS
Smoking	7 (54%)	92 (20%)	<0.005
ALT (IU/L)	44.7 ± 30.0	23.8 ± 20.4	<0.001
γ-GTP (IU/L)	31.7 ± 16.2	23.2 ± 25.2	NS
HBeAg positive	3 (23%)	14 (3%)	<0.001
HBcrAg (kU/mL)	39276 ± 121639	6486 ± 47987	<0.05
HBcrAg positive	7 (54%)	99 (21%)	<0.005
HBV DNA (log copies/mL) ^a	6.1	4.1	<0.001
HBV DNA ≥ 5 log copies/mL	6 (46%)	39 (8%)	<0.001
Genotype B1/Bj	4 (31%)	264 (57%)	NS (0.0637)
Genotype C2/Ce	9 (69%)	202 (43%)	NS (0.0637)
C1653T	6 (46%)	116/421 (28%)	NS
T1753 V	6 (46%)	78/421 (19%)	<0.05
A1762T/G1764A	11 (87%)	142/421 (34%)	<0.001
G1896A	11 (87%)	348/421 (83%)	NS

NS not significant

^a Median value**Fig. 2** The proportion of HBV specific mutations between HCC and non-HCC group when divided into each HBV genotype (B1/Bj or C2/Ce). In the HBV genotype C2/Ce, only A1762T/G1764A double mutation was significantly higher in HCC group ($P < 0.05$)

shows the proportion of HBV specific mutations between HCC and non-HCC group when divided into each HBV genotype (B1/Bj or C2/Ce). Only A1762T/G1764A double mutation was significantly higher in HCC group than non-HCC with genotype C2/Ce ($P < 0.05$).

Factors independently associated with the development of HCC in HBV mono-infected subjects

To identify the independent risk factors associated with the development of HCC, the following factors were evaluated by using multivariate-adjusted Cox hazard model among HBV mono-infected subjects: age, gender, BMI, alcohol intake, smoking, ALT, γ-GTP, HBeAg positivity, HBcrAg positivity, HBV DNA ≥ 5 log copies/mL, genotype (C2/Ce or not), and HBV specific mutations (C1653 V, T1753 V, A1762T/G1764A and G1896A). As a result, only A1762T/G1764A double mutation (hazard ratio 7.05

[95% CI 1.03–48.12, $P = 0.046$]) was an independent risk factor for the development of HCC among HBV mono-infected subjects (Table 2).

Kaplan–Meier method also showed that the probability of HCC occurrence-free was lower in HBV mono-infected subjects with A1762T/G1764A double mutation than without these mutations (log-rank: $P = 0.0001$) (Fig. 3).

Discussion

This is the first large-scale prospective population-based cohort study to identify the risk factors for HCC development among Japanese CHB patients on the basis of the prospective cohort study. To date, several cross-sectional or case-cohort studies identified factors associated with a high risk of developing of HCC among CHB patients; however, few prospective studies were carried out on this

Table 2 Independent risk factors associated with HCC among HBV mono-infected subjects by multivariate-adjusted Cox hazard model

Factors	Hazard ratio	95% confidence interval	P value
Age (years)			
<57 ^a	1		
≥57	1.42	0.38–5.35	0.603
Gender			
Female	1		
Male	2.38	0.38–15.04	0.357
BMI			
<23 ^a	1		
≥23	0.60	0.15–2.31	0.454
Alcohol			
Absent	1		
Present	–	–	
Smoking			
Absent	1		
Present	2.84	0.63–12.76	0.172
ALT (IU/L)			
<18 ^a	1		
≥18	0.36	0.05–2.81	0.332
γ-GTP (IU/L)			
<16 ^a	1		
≥16	4.41	0.71–27.26	0.110
HBeAg			
Negative	1		
Positive	1.15	0.10–13.74	0.913
HBcrAg			
Negative	1		
Positive	3.68	0.48–28.27	0.211
HBV DNA			
<5 log copies/mL	1		
≥5 log copies/mL	3.66	0.56–23.94	0.176
Genotype			
Non-Cc	1		
Cc	0.67	0.10–4.36	0.678
Mutations			
C1653T			
Absent	1		
Present	2.05	0.52–8.09	0.308
T1753V			
Absent	1		
Present	1.18	0.26–5.44	0.830
A1762T/G1764A			
Absent	1		
Present	7.05	1.03–48.12	0.046
G1896A			
Absent	1		
Present	2.69	0.40–18.27	0.312

^a Median value

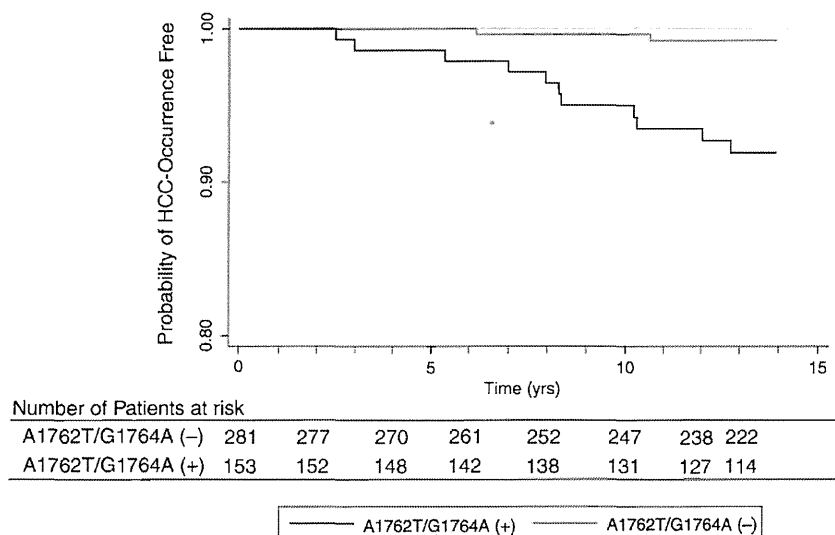
subject [17–19]. Fang et al. first reported that in men aged over 30 years, A1762T/G1764A double mutation was an etiological risk factor of HCC after 36 months of follow-up in a prospective study [17]. No large-scale population based cohort study in Japan was carried out to identify the risk factors for the development of HCC and analyze the clinical impact of HBV mono-infection and/or the other risk factors on the incidence of HCC among CHB patients in Japan. To address this issue, we elucidated the risk factors for HCC development among Japanese patients in this cohort study.

Most of the subjects enrolled in this cohort study were considered as healthy individuals and the proportion of HBV mono-infected subjects among them (2.5%) nearly corresponds to the estimated HBsAg prevalence in middle-aged or older population in Japan (about 1.5%) [28]. During the follow-up period of about 13 years, 2.7% of HBV mono-infected subjects in this cohort developed HCC. In univariate analysis, the following factors were significantly associated with the development of HCC: male, smoking, high ALT, HBeAg positivity, high levels of serum HBcrAg (the positivity of serum HBcrAg), high levels of serum HBV DNA (the proportion of HBV DNA ≥ 5 log copies/mL), T1753V mutation, and A1762T/G1764A double mutation. In multivariate-adjusted Cox hazard model, however, only A1762T/G1764A double mutation remained as an independent risk factor for the development of HCC among HBV mono-infected subjects, and this was also confirmed by further survival time analysis.

However, note that there were some limitations in this study: (1) As a result of the relatively small number of HBV-infected subjects (479), the statistical power might be not so strong. (2) Most of the HBV mono-infected subjects in this study had no medical therapy for HBV infection at entry because they seemed to be healthy HBV carriers, but the use of antiviral therapy was unknown during the follow-up period. (3) The analyses such as lifestyle, clinical data, and HBV specific mutations were based on only one time-point at entry, so the changes of these factors during the follow-up could not be assessed. To confirm our results strictly, further investigations to eliminate these limitations seem to be necessary.

It has been previously reported by a number of studies that A1762T/G1764A double mutation was associated with the development of HCC [4–11]. However, these results were cross-sectional or case-control studies and their observation required confirmation in prospective population-based study. Several mechanisms of hepatocarcinogenesis have been hypothesized in the context of the A1762T/G1764A double mutation: enhancement of HBV virulence by increasing the host immune response [29, 30], increasing viral replication [31–33], or altering the coding

Fig. 3 Kaplan–Meier survival curve for the probability of HCC occurrence in HBV mono-infected subjects with and without A1762T/G1764A double mutation. The probability of HCC occurrence-free was lower in HBV mono-infected subjects with A1762T/G1764A double mutation than without this mutation (log-rank: $P = 0.0001$)



region for the X antigen [9, 34]. In this prospective cohort study, the A1762T/G1764A double mutation was the only independent risk factor of HCC development that is consistent with the results of Fang's prospective study in Guangxi [17], and others [18, 19].

High serum HBV DNA levels ($\geq 10^4$ copies/mL) have been reported to be associated with the development of HCC [18, 35–37]. We also measured serum HBV DNA levels and confirmed that the level of serum HBV DNA was significantly higher in the HCC group. Furthermore, the proportion of serum HBV DNA ≥ 5 log copies/mL was also significantly higher in the HCC group. HBcrAg levels in serum were closely correlated with serum HBV DNA levels in HBV patients without antiviral therapy [23]. However, as the majority of the HBV mono-infected subjects enrolled in this study seemed to be inactive carriers, the overall levels of both serum HBV DNA and serum HBcrAg were low. This might have affected the result of the multivariate analysis.

In several studies, stronger association between HBV genotype C and HCC was reported in comparison with HBV genotype B [38–40]. This might be due to higher frequency of A1762T/G1764A double mutation in the genotype C [41, 42]. In our cohort study there was no association between HBV genotype C2/Ce and the development of HCC in spite of the involvement of A1762T/G1764A double mutation (Table 1; Fig. 2).

In conclusion, the risk factors associated with the development of HCC were identified among Japanese HBV carriers in this prospective population-based cohort study. Only A1762T/G1764A double mutation in HBV BCP/X region could be associated with HCC development.

Appendix

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Production of Infectious Chimeric Hepatitis C Virus Genotype 2b Harboring Minimal Regions of JFH-1

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To establish a cell culture system for chimeric hepatitis C virus (HCV) genotype 2b, we prepared a chimeric construct harboring the 5' untranslated region (UTR) to the E2 region of the MA strain (genotype 2b) and the region of p7 to the 3' UTR of the JFH-1 strain (genotype 2a). This chimeric RNA (MA/JFH-1.1) replicated and produced infectious virus in Huh7.5.1 cells. Replacement of the 5' UTR of this chimera with that from JFH-1 (MA/JFH-1.2) enhanced virus production, but infectivity remained low. In a long-term follow-up study, we identified a cell culture-adaptive mutation in the core region (R167G) and found that it enhanced virus assembly. We previously reported that the NS3 helicase (N3H) and the region of NS5B to 3' X (N5BX) of JFH-1 enabled replication of the J6CF strain (genotype 2a), which could not replicate in cells. To reduce JFH-1 content in MA/JFH-1.2, we produced a chimeric viral genome for MA harboring the N3H and N5BX regions of JFH-1, combined with a JFH-1 5' UTR replacement and the R167G mutation (MA/N3H+N5BX-JFH1/R167G). This chimeric RNA replicated efficiently, but virus production was low. After the introduction of four additional cell culture-adaptive mutations, MA/N3H+N5BX-JFH1/5am produced infectious virus efficiently. Using this chimeric virus harboring minimal regions of JFH-1, we analyzed interferon sensitivity and found that this chimeric virus was more sensitive to interferon than JFH-1 and another chimeric virus containing more regions from JFH-1 (MA/JFH-1.2/R167G). In conclusion, we established an HCV genotype 2b cell culture system using a chimeric genome harboring minimal regions of JFH-1. This cell culture system may be useful for characterizing genotype 2b viruses and developing antiviral strategies.

Hepatitis C virus (HCV) is a major cause of chronic liver disease (5, 13), but the lack of a robust cell culture system to produce virus particles has hampered the progress of HCV research (2). Although the development of a subgenomic replicon system has enabled research into HCV RNA replication (15), infectious virus particle production has not been possible. Recently, an HCV cell culture system was developed using a genotype 2a strain, JFH-1, cloned from a fulminant hepatitis patient (14, 29, 32), thereby allowing investigation of the entire life cycle of this virus. However, several groups of investigators have reported genotype- and/or strain-dependent effects of some antiviral reagents (6, 17) and neutralizing antibodies (7, 25). Therefore, efficient virus production systems using various genotypes and strains are indispensable for HCV research and the development of antiviral strategies.

The JFH-1 strain is the first HCV strain that can efficiently produce HCV particles in HuH-7 cells (29). Other strains can replicate and produce infectious virus by HCV RNA transfection, but the efficiency is far lower than that of JFH-1 (24, 31). In the case of replication-incompetent strains, chimeric virus containing the JFH-1 nonstructural protein coding region is useful for analyses of viral characteristics (6, 9, 14, 23, 30, 31).

In this study, we developed a genotype 2b chimeric infectious virus production system using the MA strain (accession number AB030907) (19) harboring minimal regions of JFH-1 and cell culture-adaptive mutations that enhance infectious virus production.

MATERIALS AND METHODS

Cell culture. Huh7.5.1 cells (a kind gift from Francis V. Chisari) (32) and Huh7-25 cells (1) were cultured at 37°C in Dulbecco's modified Eagle's

medium containing 10% fetal bovine serum under 5% CO₂ conditions. For follow-up study, RNA-transfected cells were passaged every 2 to 5 days depending on cell status.

Full-length genomic HCV constructs. Plasmids used in the analysis of genomic RNA replication were constructed based on pJFH1 (29) and pMA (19). For convenience, an EcoRI recognition site was introduced upstream of the T7 promoter region of pMA by PCR, and an XbaI recognition site was introduced at the end of the 3' untranslated region (UTR). To construct MA/JFH-1, the EcoRI-BsaBI (nucleotides [nt] 1 to 2570; 5' UTR to E2) fragment of pMA was substituted into pJFH1 (Fig. 1A). Replacement of the 5' UTR was performed by exchanging the EcoRI-AgeI (nt 1 to 159) fragment. A point mutation in the core region (R167G) was introduced into MA chimeric constructs by PCR using the following primers: sense, 5'-TTA TGC AAC GGG GAA TTT ACC CGG TTG CTC T-3'; antisense, 5'-GGT AAA TTC CCC GTT GCA TAA TTT ATC CCG TC-3'. G167R substitution in the JFH-1 construct was performed by PCR using the following primers: sense, 5'-ATT ATG CAA CAA GGA ACC TAC CCG GTT TCC C-3'; antisense, 5'-GGT AGG TTC CTT GTT GCA TAA TTA ACC CCG TC-3'. Point mutations (L814S, R1012G, T1106A, and V1951A) were introduced into MA chimeric constructs by PCR using the following primers: L814S, 5'-GCT TAC GCC TCG GAC GCC GCT GAA CAA GGG G-3' (sense) and 5'-AGC GGC GTC CGA GGC GTA AGC CTG CTG CCG C-3' (antisense); R1012G, 5'-GAG GCT AGG TGG

Received 13 June 2011 Accepted 23 November 2011

Published ahead of print 7 December 2011

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doi:10.1128/JVI.05386-11

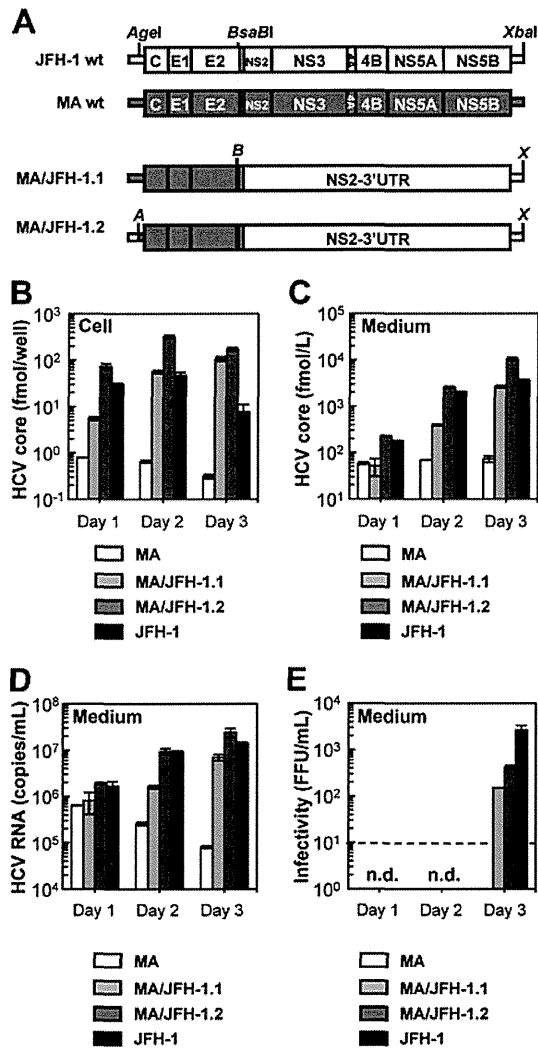


FIG 1 Replication and virus production by MA/JFH-1 chimeras in Huh7.5.1 cells. (A) Schematic structures of JFH-1, MA, and two MA/JFH-1 chimeras (MA/JFH-1.1 and MA/JFH-1.2). The junction of JFH-1 and MA in the 5' UTR is an AgeI site, and the junction of MA and JFH-1 in the NS2 region is a BsaBI site. A, AgeI; B, BsaBI; X, XbaI. (B to E) Chimeric HCV RNA replication in Huh7.5.1 cells. HCV core protein level in cells (B) and culture medium (C) and HCV RNA levels in medium (D) and infectivity of culture medium (E) from HCV RNA-transfected Huh7.5.1 cells are shown. Ten micrograms of HCV RNA was transfected into Huh7.5.1 cells, and cells and culture medium were harvested on days 1, 2, and 3. n.d., not determined. Assays were performed three times independently, and data are presented as means \pm standard deviation. Dashed line indicates detection limit. wt, wild type.

GGA AGT TCT GCT CGG CCC T-3' (sense) and 5'-AGA ACT TCC CCA CCT AGC CTC GCG GAA ACC G-3' (antisense); T1106A, 5'-CAG ATG TAC GCC AGC GCA GAG GGG GAC CTC-3' (sense) and 5'-CTG CGC TGG CGT ACA TCT GGG TGA CTG GTC-3' (antisense); and V1951A, 5'-GTG ACG CAG GCG TTA AGC TCA CTC ACA ATT ACC-3' (sense) and 5'-TGA GCT TAA CGC CTG CGT CAC GCG CAG CGA G-3' (antisense). To construct the MA chimeric virus harboring minimal regions of JFH-1 (MA/N3H+N5BX-JFH1), ClaI (nt 3930), EcoT22I (nt 5294), and BsrGI (nt 7782) recognition sites were introduced into pMA by site-directed mutagenesis. The 5' UTR (EcoRI-AgeI), the region of the NS3 helicase (N3H; ClaI-EcoT22I), and the region of NS5B to 3' X (N5BX;

BsrGI-XbaI) were then replaced with the corresponding regions from JFH-1.

RNA synthesis, transfection, and determination of infectivity. RNA synthesis and transfection were performed as described previously (12, 22). Determination of infectivity was also performed as described previously, with infectivity expressed as the number of focus-forming units per milliliter (FFU/ml) (12, 22). When necessary, culture medium was concentrated 20-fold in Amicon Ultra-15 spin columns (100-kDa molecular-weight-cutoff; Millipore, Bedford, MA) in order to determine infectivity.

Quantification of HCV core protein and HCV RNA. In order to estimate the concentration of HCV core protein in culture medium, we performed a chemiluminescence enzyme immunoassay (Lumipulse II HCV core assay; Fujirebio, Tokyo, Japan) in accordance with the manufacturer's instructions. HCV RNA from harvested cells or culture medium was isolated using an RNeasy Mini RNA kit (Qiagen, Tokyo, Japan) or QiaAmp Viral RNA Minikit (Qiagen), respectively. Copy number of HCV RNA was determined by real-time quantitative reverse transcription-PCR (qRT-PCR), as described previously (28).

HCV sequencing. Total RNA in culture supernatant was extracted with Isogen-LS (Nippon Gene Co., Ltd., Tokyo, Japan). cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). cDNA was subsequently amplified with LA *Taq* DNA polymerase (TaKaRa, Shiga, Japan). Four separate PCR primer sets were used to amplify the fragments of nt 130 to 2909, 2558 to 5142, 4784 to 7279, and 7081 to 9634 covering the entire open reading frame and part of the 5' UTR and 3' UTR of the MA strain. Sequences of amplified fragments were determined directly.

Immunostaining. Infected cells were cultured on Multitest Slides (MP Biomedicals, Aurora, OH) and were fixed in acetone-methanol (1:1, vol/vol) for 15 min at -20°C . After a blocking step, infected cells were visualized with anti-core protein antibody (clone 2H9) (29) and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen), and nuclei were visualized with 4',6'-diamidino-2-phenylindole (DAPI).

Assessment of interferon sensitivity. Two micrograms of *in vitro* transcribed RNA was transfected into 3×10^6 Huh7.5.1 cells. Four hours after transfection, cells were placed in fresh medium or medium containing 0.1, 1, 10, 100, and 1,000 IU/ml of interferon α -2b (Intron A; Schering-Plough Corporation, Osaka, Japan). Culture medium was then harvested on day 3, and HCV core levels in the cells and in the medium were measured.

Statistical analysis. Significant differences were evaluated by Student's *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Transient replication and production of 2b/2a chimeric virus.

We first tested whether the MA strain (genotype 2b) (19) was able to replicate and produce infectious virus in cultured cells. When the *in vitro* transcribed RNA of MA was transfected into Huh7.5.1 cells, a highly HCV-permissive cell line, replication and virus production were not observed (Fig. 1A to C). We then tested whether 2b/2a chimeric RNA harboring the structural region (5' UTR to E2) of the MA strain and the nonstructural region (p7 to 3' UTR) of JFH-1 (Fig. 1A, MA/JFH-1.1) was able to replicate in the cells. After MA/JFH-1.1 RNA transfection, time-dependent accumulation of core protein in the cells (Fig. 1B) and culture medium (Fig. 1C) was observed, indicating that MA/JFH-1.1 RNA was able to replicate in the cells autonomously. HCV RNA levels in the medium were determined by qRT-PCR, and time-dependent increases in HCV RNA level were also observed (Fig. 1D). Infectious virus production was observed on day 3, but infectivity was 17.6-fold lower than that of JFH-1 (Fig. 1E).

In order to improve the level of infectious virus production, we tested another chimeric construct, MA/JFH-1.2, which contained an additional MA-to-JFH-1 replacement of the 5' UTR (Fig. 1A),

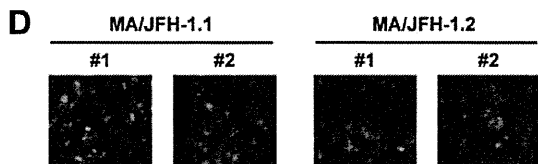
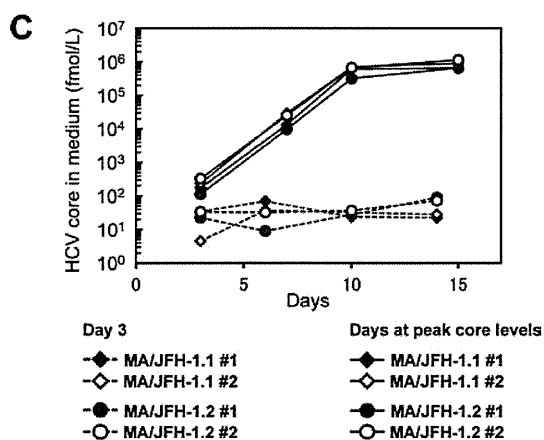
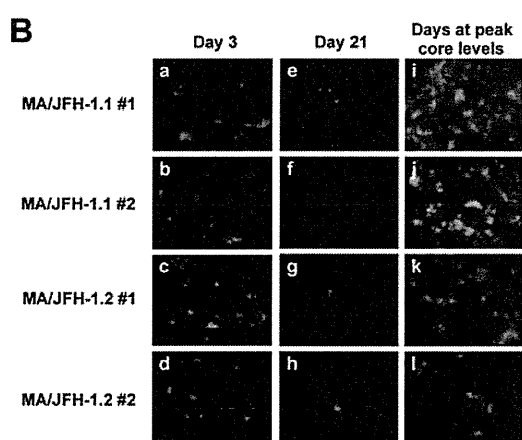
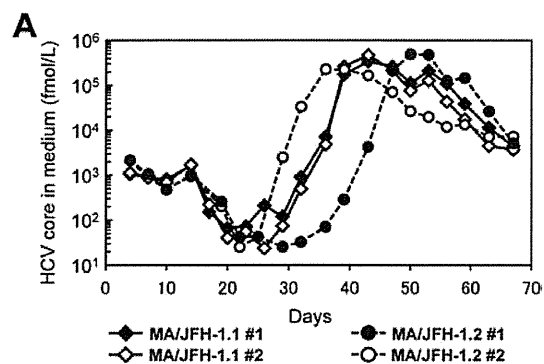


FIG 2 Long-term culture of MA/JFH-1.1 and MA/JFH-1.2 RNA-transfected cells. Ten micrograms of HCV RNA was transfected into Huh7.5.1 cells, and cells were passaged every 2 to 5 days, depending on cell status. Culture medium was collected after every passage, and HCV core protein levels were measured. Transfection was performed twice for each chimeric RNA (1 and 2 for each construct). (A) HCV core protein levels in culture medium from MA/JFH-1.1 and MA/JFH-1.2 RNA-transfected cells. (B) Immunostained cells at 3 days after transfection (a to d), at 21 days after transfection (e to h), and at the time

TABLE 1 HCV core protein levels and infectivity in culture medium immediately after RNA transfection (day 3) and after long-term culture (days 35 to 49)

Sample period and virus	Sample no.	Day no. ^a	HCV core (fmol/liter)	Infectivity (FFU/ml)
After transfection				
MA/JFH-1.1	1	3	1.06×10^3	5.00×10^1
	2	3	1.14×10^3	5.70×10^1
MA/JFH-1.2	1	3	2.14×10^3	7.30×10^1
	2	3	2.15×10^3	9.30×10^1
After long-term culture				
MA/JFH-1.1	1	42	3.38×10^5	1.62×10^5
	2	42	4.70×10^5	3.23×10^5
MA/JFH-1.2	1	35	2.27×10^5	1.61×10^5
	2	49	4.93×10^5	3.27×10^5

^a For the long-term culture, the days are those of peak core protein levels.

as a 5' UTR replacement from J6CF (genotype 2a) to JFH-1 enhanced virus production of chimeric J6CF virus harboring the region of NS2 to 3' X of JFH-1 (J6/JFH-1) (A. Murayama et al., unpublished data). The core protein accumulation levels with MA/JFH-1.2 RNA-transfected cells were higher than those with MA/JFH-1.1 ($P < 0.05$) (Fig. 1B). Similarly, core protein and HCV RNA levels in the medium of MA/JFH-1.2 RNA-transfected cells were higher than those of MA/JFH-1.1 ($P < 0.05$) (Fig. 1C and D). Infectivity on day 3 was also higher than with MA/JFH-1.1 ($P < 0.05$) (Fig. 1E), indicating that the 5' UTR of JFH-1 enhanced virus production. However, infectivity of medium from MA/JFH-1.2 RNA-transfected cells on day 3 remained 6.4-fold lower than that of JFH-1 although HCV RNA levels in the medium were similar to those of JFH-1 (Fig. 1D and E).

These results indicate that 2b/2a chimeric RNA is able to replicate autonomously in Huh7.5.1 cells and produce infectious virus although infectivity remains lower than that of JFH-1.

Assembly-enhancing mutation in core region introduced during long-term culture. Because MA/JFH-1.1 and MA/JFH-1.2 replicated efficiently but produced small amounts of infectious virus, we performed long-term culture of these RNA-transfected cells in order to examine whether these chimeric RNAs would continue replicating and producing infectious virus over the long term. We prepared two RNA-transfected cell lines for each construct (MA/JFH-1.1 and MA/JFH-1.2) as both of these replicated and produced infectious virus at different levels.

Immediately after transfection, core protein levels and infectivity in culture medium were low (1.06×10^3 to 2.15×10^3 fmol/liter and 5.00×10^1 to 9.30×10^1 FFU/ml, respectively) (Fig. 2A and Table 1) although a considerable number of core protein-positive cells were observed by immunostaining (Fig. 2B, frames a to d). Subsequently, core protein levels in the culture medium decreased gradually (Fig. 2A), and core protein-positive cells were rare (Fig. 2B, frames e to h). However, at 30 to 40 days

of peak core levels (days 42 to 49). Infected cells were visualized with anti-core protein antibody (green), and nuclei were visualized with DAPI (blue). (C) Infection of naive cells by culture medium at an MOI of 0.001. (D) Immunostained cells at 15 days after infection with medium at peak core protein levels (Fig. 2A) at an MOI of 0.001. Infected cells were visualized with anti-core antibody (green), and nuclei were visualized with DAPI (blue).

after transfection, core protein levels in the supernatants of all chimeric RNA-transfected cells increased and reached 2.27×10^5 to 4.93×10^5 fmol/liter (Fig. 2A and Table 1). Infectivity in the culture medium also increased (1.61×10^5 to 3.27×10^5 FFU/ml) (Table 1), and at this point, most of the cells were core protein positive (Fig. 2B, frame i to l).

As the infectivity of culture supernatant of MA/JFH-1 RNA-transfected cells appeared to increase after long-term culture, we compared viral spread by infection with these supernatants on day 3 (immediately after transfection) and for each peak in core protein levels (after long-term culture). When naïve Huh7.5.1 cells were infected with supernatant on days corresponding to a peak in core protein levels at a multiplicity of infection (MOI) of 0.001, core protein levels in the medium increased rapidly and reached 0.64×10^6 to 1.13×10^6 fmol/liter by day 15 after infection (Fig. 2C). Immunostained images showed that most cells were HCV core protein positive on day 15 (Fig. 2D). When naïve Huh7.5.1 cells were infected with supernatant from day 3 at an MOI of 0.001, core protein levels in the medium did not increase under these conditions (Fig. 2C). These results indicate that both MA/JFH-1 chimeric viruses (MA/JFH-1.1 and MA/JFH-1.2) acquired the ability to spread rapidly after long-term culture.

As the characteristics of the MA/JFH-1 virus changed in long-term culture, we analyzed the possible mutations in the viral genome from the supernatant at each peak in core protein levels (Table 1, days at peak core levels). Nine- to 12-nucleotide mutations were found in the viral genome from each supernatant, and the detected mutations were distributed along the entire genome. Among these mutations, a common nonsynonymous mutation was found in the core region (Arg to Gly at amino acid [aa]167, R167G).

In order to test the effects of R167G on virus production, an R167G substitution was introduced into MA/JFH-1.2 as MA/JFH-1.2/R167G and produced infectious virus more efficiently than MA/JFH-1.1. HCV core protein levels in cells and medium of MA/JFH-1.2 with R167G (MA/JFH-1.2/R167G) were higher than with MA/JFH-1.2 ($P < 0.05$) (Fig. 3A and B). HCV RNA levels in the medium of MA/JFH-1.2/R167G RNA-transfected cells were also higher than with MA/JFH-1.2 ($P < 0.05$) (Fig. 3C). Infectious virus production was also increased by the R167G mutation ($P < 0.05$) (Fig. 3D) and was 8.7-fold higher than that of JFH-1 RNA-transfected cells on day 3 ($P < 0.05$) (Fig. 3D).

We then tested whether R167G was responsible for the rapid spread observed in culture supernatant after long-term culture by monitoring virus spread after infection of naïve Huh7.5.1 with culture medium taken 3 days after RNA transfection of MA/JFH-1.2 and MA/JFH-1.2/R167G at an MOI of 0.005. Core protein levels in medium from MA/JFH-1.2/R167G-infected cells increased with the same kinetics as levels of JFH-1 (Fig. 3E), and the population of core protein-positive cells was almost the same as with JFH-1-infected cells (Fig. 3F), indicating that MA/JFH-1.2/R167G virus spread as rapidly as JFH-1 virus. In contrast, we observed no infectious foci in the MA/JFH-1.2 virus-inoculated cells (Fig. 3F). These data suggest that the R167G mutation in the core region was a cell culture-adaptive mutation and that it enhanced infectious MA/JFH-1.2 virus production.

In order to determine whether R167G enhances RNA replication or other steps in the viral life cycle, we performed a single-cycle virus production assay (11) using Huh7-25 cells, a HuH-7-derived cell line lacking CD81 expression on the cell surface (1).

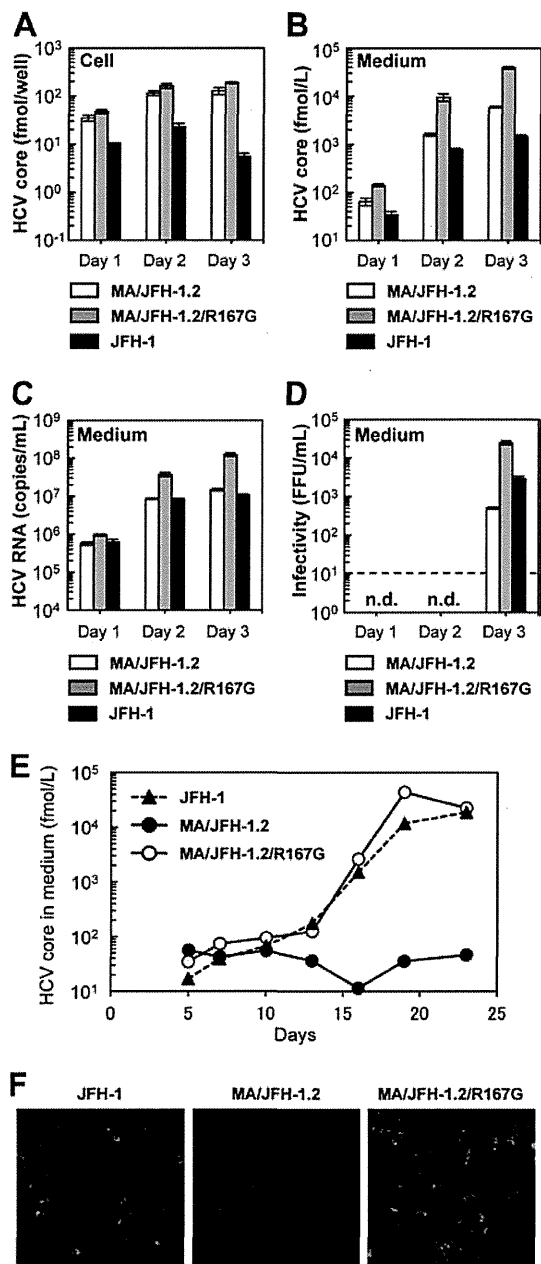


FIG 3 Effects of R167G on replication and virus production of MA/JFH-1.2 in Huh7.5.1 cells. Ten micrograms of HCV RNA was transfected into Huh7.5.1 cells, and cells and medium were harvested on days 1, 2, and 3. HCV core protein levels in the cells (A) and culture medium (B) and HCV RNA levels in the medium (C) and the infectivity of culture medium (D) from HCV RNA-transfected Huh7.5.1 cells are shown. n.d., not determined. Dashed line indicates the detection limit. Assays were performed three times independently, and data are presented as means \pm standard deviation. (E) HCV core protein levels in culture medium from cells infected with medium at 3 days posttransfection at an MOI of 0.005. (F) Immunostained cells at 19 days postinfection. Infected cells were visualized with anti-core antibody (green), and nuclei were visualized with DAPI (blue).

This cell line can support replication and infectious virus production upon transfection of HCV genomic RNA but cannot be reinfectious by progeny virus, thereby allowing observation of a single cycle of infectious virus production without the confounding ef-

fects of reinfection. R167G did not affect HCV core protein levels in the chimeric RNA-transfected Huh7-25 cells (Fig. 4A), demonstrating that R167G did not enhance RNA replication. Nevertheless, R167G increased HCV core protein levels in the medium ($P < 0.05$ on days 2 and 3) and infectivity (Fig. 4B and C). These results suggest that R167G did not affect RNA replication but affected other steps such as virus assembly and/or virus secretion.

Virus particle assembly efficiency was then assessed by determining intracellular-specific infectivity from infectivity and RNA titer in the cells, as reported previously (11). As shown in Fig. 4G, R167G enhanced intracellular-specific infectivity of MA/JFH-1.2 virus 10.2-fold. Virus secretion efficiency was also calculated from the amount of intracellular and extracellular infectious virus, but R167G had no effect (Fig. 4G).

To confirm the effects of Arg167 in other HCV strains, we tested its effects on JFH-1. As aa 167 of JFH-1 is Gly, we replaced it with Arg (G167R). HCV core protein levels in the cells were not affected by G167R (Fig. 4D), and no effects on RNA replication were confirmed. HCV core protein levels in the medium and infectivity decreased after G167R mutation (Fig. 4E and F). As the G167R mutation decreased intracellular infectious virus production of JFH-1 to undetectable levels, we were unable to determine the intracellular-specific infectivity and virus secretion efficiency of JFH-1 G167R (Fig. 4G). These results indicate that Gly is favored over Arg at core position 167 for infectious virus assembly in multiple HCV strains.

MA harboring the R167G mutation, 5' UTR, and N3H (NS3 helicase) and N5BX (NS5B to 3' X) regions of JFH-1 replicated and produced infectious chimeric virus. In order to establish a genotype 2b cell culture system with the MA strain with minimal regions of JFH-1, we attempted to reduce JFH-1 content in MA/JFH-1.2. We previously reported that replacement of the N3H and N5BX regions of JFH-1 allowed efficient replication of the J6CF strain, which normally cannot replicate in cells (21). Thus, we tested whether the N3H and N5BX regions of JFH-1 could also support MA RNA replication.

We prepared two chimeric MA constructs harboring the 5' UTR and N3H and N5BX regions of JFH-1, MA/N3H+N5BX-JFH1 (Fig. 5A) and MA/N3H+N5BX-JFH1/R167G. After *in vitro* transcribed RNA was transfected into Huh7.5.1 cells, intracellular core protein levels of MA/N3H+N5BX-JFH1 and MA/N3H+N5BX-JFH1/R167G RNA-transfected cells increased in a time-dependent manner and reached almost the same levels as with MA/JFH-1.2 RNA-transfected cells on day 5 (Fig. 5B). Extracellular core protein and HCV RNA levels of MA/N3H+N5BX-JFH1 and MA/N3H+N5BX-JFH1/R167G RNA-transfected cells also increased in a time-dependent manner (Fig. 5C and D). However, they were more than 10 times lower than with MA/JFH-1.2 RNA-transfected cells although intracellular core levels were comparable on day 5 (Fig. 5B to D).

We then tested whether the medium from MA/N3H+N5BX-JFH1 and MA/N3H+N5BX-JFH1/R167G RNA-transfected cells was infectious. Infectivity of the medium from MA/N3H+N5BX-JFH1 RNA-transfected cells was below the detection limit, and that of MA/N3H+N5BX-JFH1/R167G RNA-transfected cells on day 5 was very low ($3.3 \times 10^1 \pm 2.1 \times 10^1$ FFU/ml) (Fig. 5E). To confirm infectivity, the culture media were concentrated, and their infectivity was determined. Infected foci were observed after infection with concentrated medium in MA/N3H+N5BX-JFH1/R167G RNA-transfected cells (Fig. 5F), and infectivity was found

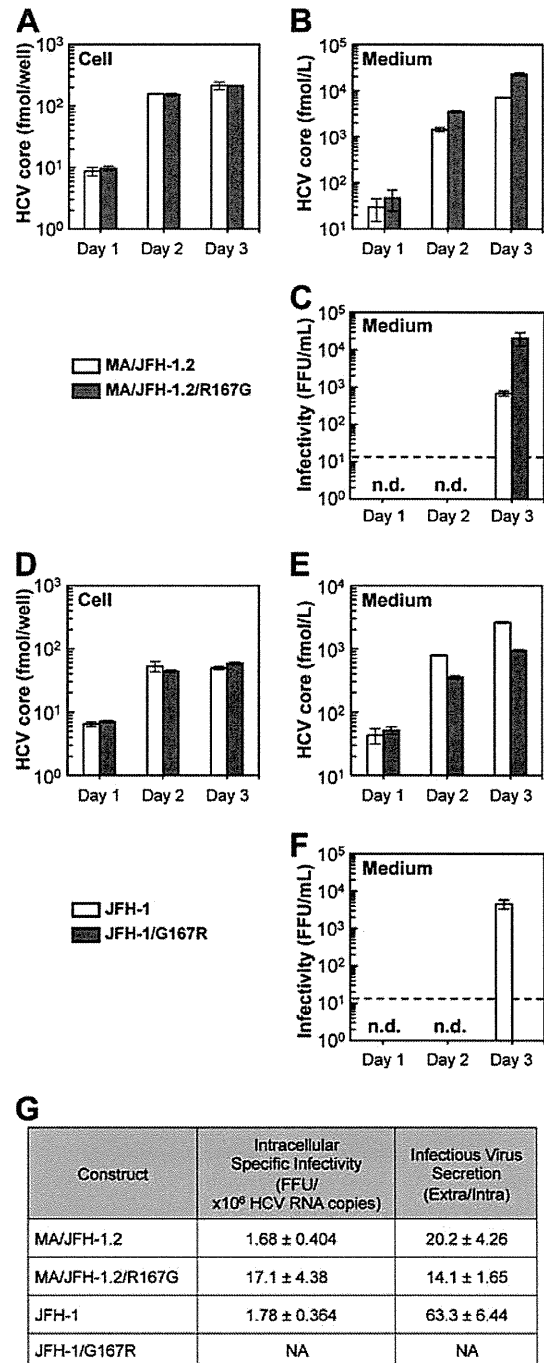


FIG 4 Effects of R167G on replication and virus production of MA/JFH-1.2 and JFH-1 in Huh7-25 cells. Ten micrograms of HCV RNA was transfected into Huh7-25 cells, and cells and medium were harvested on days 1, 2, and 3. HCV core protein levels in cells (A and D) and in medium (B and E) were measured, and infectivity of medium (C and F) was determined. n.d., not determined. Dashed line indicates the detection limit. (G) Intracellular specific infectivity and virus secretion efficiency of chimeric HCV RNA-transfected cells. Intracellular and extracellular infectivity of day 3 samples was determined, and specific infectivity and virus secretion rate were calculated. Assays were performed three times independently, and data are presented as means \pm standard deviation. NA, not available.

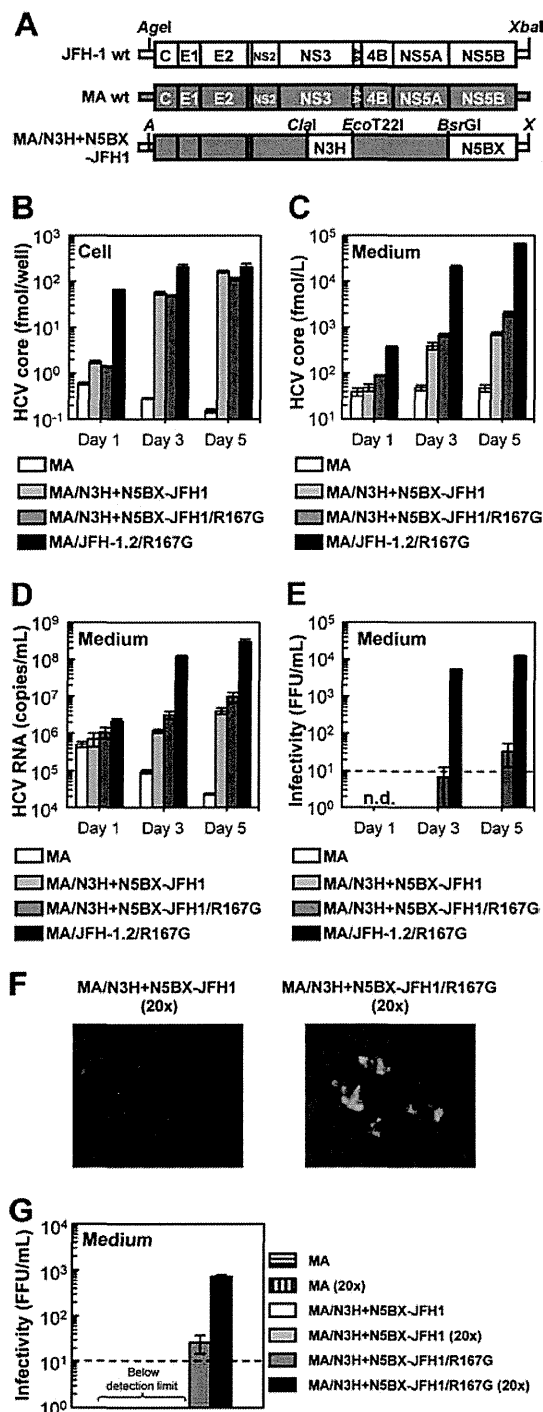


FIG 5 Replication and virus production of MA/N3H+N5BX-JFH1/R167G in Huh7.5.1 cells. (A) Schematic structures of JFH-1, MA, and MA/N3H+N5BX-JFH1. The junction of JFH-1 and MA in the 5' UTR is an AgeI site; the junctions of MA and JFH-1 in the NS3 regions are ClaI and EcoT22I sites, and the junction in the NS5B region is a BsrGI site. A, AgeI; X, XbaI. (B to G) Chimeric HCV RNA replication in Huh7.5.1 cells. Ten micrograms of HCV RNA was transfected into Huh7.5.1 cells, and cells and medium were harvested on days 1, 3, and 5. HCV core protein levels in cells (B) and in medium (C) and HCV RNA levels in medium (D) were measured, and infectivity of medium (E) was determined. Assays were performed three times independently, and data are presented as means \pm standard deviation. n.d., not determined. Dashed line indicates the detection limit. (F) Immunostained cells. Huh7.5.1

to be $7.27 \times 10^2 \pm 7.57 \times 10^1$ FFU/ml (Fig. 5G). No infected foci were observed after infection of MA/N3H+N5BX-JFH1 RNA-transfected cells, even when medium was concentrated (Fig. 5F), although intracellular and extracellular core protein levels were comparable to those with MA/N3H+N5BX-JFH1/R167G RNA-transfected cells (Fig. 5B and C). These results indicate that replacement of the 5' UTR and N3H and N5BX regions in JFH-1 were necessary to rescue autonomous replication in the replication-incompetent MA strain and for secretion of infectious chimeric virus. However, the secretion and infection efficiencies of the virus were low.

Cell culture-adaptive mutations enhanced infectious virus production of MA/N3H+N5BX-JFH1/R167G. Because MA/N3H+N5BX-JFH1/R167G replicated efficiently but produced very small amounts of infectious virus, we performed a long-term culture of the RNA-transfected cells in order to induce cell culture-adaptive mutations that could enhance infectious virus production. We prepared RNA-transfected cells using two constructs, MA/N3H+N5BX-JFH1 and MA/N3H+N5BX-JFH1/R167G; both of these replicated efficiently, and MA/N3H+N5BX-JFH1/R167G produced infectious virus at low levels while MA/N3H+N5BX-JFH1 did not. Immediately after transfection, the HCV core protein levels in the medium of each RNA-transfected cell culture peaked at 3.0×10^3 fmol/liter and declined thereafter. However, the core protein level in the medium with MA/N3H+N5BX-JFH1/R167G RNA-transfected cells continued to increase and reached a peak of 2.7×10^5 fmol/liter 54 days after transfection, at which point most cells were core protein positive (Fig. 6B). The core protein level in the medium with MA/N3H+N5BX-JFH1 RNA-transfected cells did not increase and core-positive cells were scarce on day 54 (Fig. 6B). We analyzed the viral genome in the culture supernatants from day 54 for possible mutations and identified four nonsynonymous mutations in the MA/N3H+N5BX-JFH1/R167G genome: L814S (NS2), R1012G, (NS2), T1106A (NS3), and V1951A (NS4B). In order to test whether these amino acid substitutions enhance infectious virus production, L814S, R1012G, T1106A, and V1951A were introduced into MA/N3H+N5BX-JFH1/R167G, and the product was designated MA/N3H+N5BX-JFH1/5am (where am indicates adaptive mutation). On day 1, although HCV core protein levels in the MA/N3H+N5BX-JFH1/5am RNA-transfected cells were higher than those of MA/N3H+N5BX-JFH1/R167G RNA-transfected cells, they were still lower than those of MA/JFH-1.2/R167G RNA-transfected cells; however, on days 3 and 5, they reached a level comparable to that of MA/JFH-1.2/R167G RNA-transfected cells (Fig. 6C). HCV core protein and HCV RNA levels in the medium of MA/N3H+N5BX-JFH1/5am RNA-transfected cells were higher than those of MA/JFH-1.2/R167G RNA-transfected cells ($P < 0.05$, Fig. 6D and 6E, respectively). MA/N3H+N5BX-JFH1/5am, containing the four additional adaptive mutations, produced infectious virus at the same level as MA/JFH-1.2/R167G on day 5 (Fig. 6F). These results indicate that the

cells were infected with concentrated medium from RNA-transfected cells on day 5. Infected cells were visualized with anti-core antibody (green), and nuclei were visualized with DAPI (blue). (G) Infectivity of concentrated culture medium from HCV RNA-transfected cells. Culture medium was concentrated by 20 times. Infectivities of original and concentrated culture media were determined. Dashed line indicates detection limit.

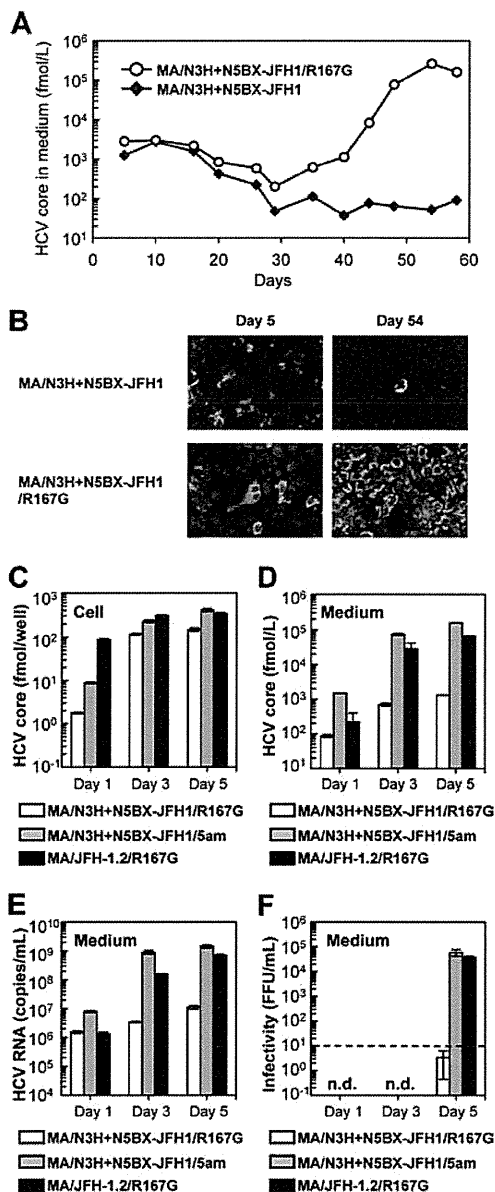


FIG 6 Cell culture-adaptive mutations enhanced infectious virus production of MA/N3H+N5BX-JFH1/R167G. (A) Long-term culture of MA/N3H+N5BX-JFH1 and MA/N3H+N5BX-JFH1/R167G RNA-transfected cells. Ten micrograms of HCV RNA was transfected into Huh7.5.1 cells, and cells were passaged every 2 to 5 days, depending on cell status. Culture medium was collected after every passage, and HCV core protein levels were measured. HCV core protein levels in culture medium from MA/N3H+N5BX-JFH1 and MA/N3H+N5BX-JFH1/R167G RNA-transfected cells are presented. (B) Immunostained cells on days 5 and 54 after transfection. Infected cells were visualized with anti-core antibody (green), and nuclei were visualized with DAPI (blue). (C to F) Effect of four additional cell culture-adaptive mutations on virus production. Ten micrograms of HCV RNA was transfected into Huh7.5.1 cells, and cells and medium were harvested on days 1, 3, and 5. HCV core levels in cells (C) and in medium (D) and HCV RNA levels in medium (E) were measured, and infectivity of medium (F) was determined. Assays were performed three times independently, and data are presented as means \pm standard deviation. n.d., not determined. Dashed line indicates the detection limit.

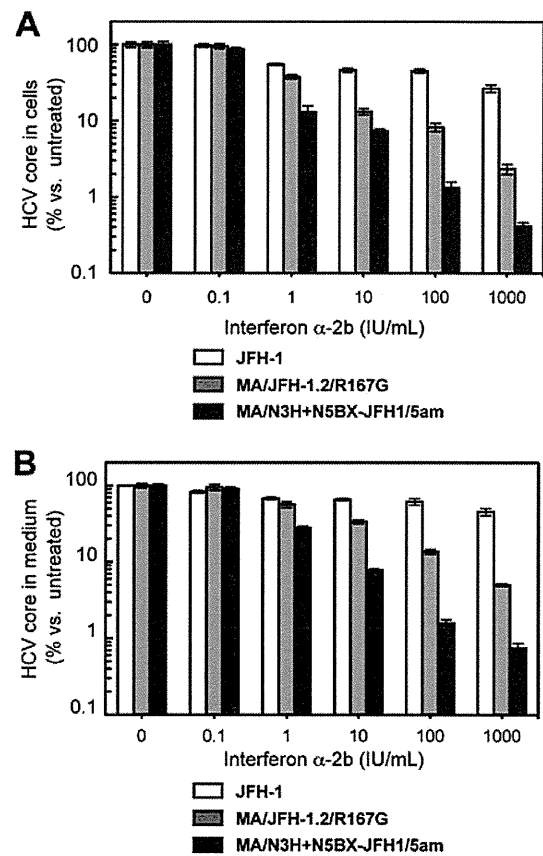


FIG 7 Comparisons of interferon sensitivity between JFH-1, MA/JFH-1.2/R167G and MA/N3H+N5BX-JFH1/5am. Two micrograms of HCV RNA was transfected into Huh7.5.1 cells, and interferon was added at the indicated concentrations at 4 h after transfection. HCV core protein levels in cells (A) and in medium (B) on day 3 were measured, and data are expressed as percent versus untreated cells (0 IU/ml). Assays were performed three times independently, and data are presented as means \pm standard deviation.

four additional adaptive mutations enhance infectious virus production and that MA/N3H+N5BX-JFH1/5am RNA-transfected cells replicate and produce infectious virus as efficiently as MA/JFH-1.2/R167G RNA-transfected cells.

Comparison of interferon sensitivity between JFH-1, MA/JFH-1.2/R167G, and MA/N3H+N5BX-JFH1/R167G. Using the newly established genotype 2b infectious chimeric virus, we compared interferon sensitivity between the JFH-1, MA/JFH-1.2/R167G, and MA/N3H+N5BX-JFH1/5am viruses. JFH-1 or MA chimeric viral RNA-transfected Huh7.5.1 cells were treated with 0.1, 1, 10, 100, or 1,000 IU/ml interferon α -2b, and HCV core protein levels in the cells and in culture media were compared. Interferon decreased HCV core protein levels in the JFH-1 RNA-transfected cells and in the medium in a dose-dependent manner, and production was inhibited to 26.8% \pm 3.0% and 45.6% \pm 4.7%, respectively, of control levels (Fig. 7A and B, respectively). In contrast, HCV core protein levels in cells and medium of MA/JFH-1.2/R167G and MA/N3H+N5BX-JFH1/5am RNA-transfected cells decreased more pronouncedly in a dose-dependent manner (Fig. 7A and B, respectively). HCV core protein levels in cells and medium from MA/N3H+N5BX-JFH1/5am RNA-transfected cells were lower than those from MA/JFH-1.2/

R167G RNA-transfected cells (Fig. 7A and B, respectively) ($P < 0.05$ at 1, 10, 100, and 1,000 IU/ml), indicating that the MA/N3H+N5BX-JFH1/5am virus was more sensitive to interferon than the MA/JFH1.2/R167G virus, which contained more regions from JFH-1.

DISCUSSION

In this study, we developed a novel infectious HCV production system using a genotype 2b chimeric virus. To improve infectious virus production, we introduced two modifications into the chimeric genome.

First, we replaced the 5' UTR from MA with that of JFH-1. Similarly to J6/JFH-1, replacement of the 5' UTR increased core protein accumulation in both the cells and medium when these RNAs were transfected into Huh7.5.1 cells (Fig. 1). The same trend was observed when these RNAs were transfected into Huh7-25 cells (data not shown), indicating that the 5' UTR of JFH-1 enhanced RNA replication. There are two genetic variations in J6CF and seven in MA in the region we replaced (nt 1 to 154 for J6CF and nt 1 to 155 for MA), and some of these mutations may affect RNA replication by changing the RNA secondary structure, RNA-RNA interactions, or binding of host or viral proteins.

Second, we introduced a cell culture-adaptive mutation (R167G) in the core region. This mutation was induced by long-term culture of MA/JFH-1 RNA-transfected cells (Fig. 2). MA/JFH-1 chimeric RNA (MA/JFH-1.1 and MA/JFH-1.2) replicated when synthesized RNA was transfected into the cells. However, infectious virus production was low, and virus infection did not spread over the short term. In early stages of long-term culture, the number of core protein-positive cells gradually decreased, and core protein-positive cells were scarcely detectable. Subsequently, the population of core protein-positive cells increased, reaching almost 100%. At this time point, we identified a common mutation in the core region (R167G) of the viral genome as a cell culture-adaptive mutation and found that it enhanced infectious virus production (Fig. 3). Several nonsynonymous mutations other than R167G were identified in the viral genome from each supernatant, and these mutations may enhance infectious virus production. However, there was a discrepancy between RNA levels and the infectivity of the culture media of MA/JFH-1.2 and MA/JFH-1.2/R167G RNA-transfected cells (Fig. 3C and D). The MA/JFH-1.2/R167G mutant had a 2-log increase in viral infectivity compared to that of MA/JFH-1.2 but only a 1-log increase in secreted RNA. The replication efficiency of MA/JFH-1.2 RNA-transfected cells was comparable to that of MA/JFH-1.2/R167G RNA-transfected cells, but the efficiency of infectious virus assembly within the cells was low, indicating that mainly noninfectious virus may be produced.

Infection of MA/JFH-1.2/R167G virus spreads rapidly, similarly to that of the JFH-1 virus, when it is inoculated into naïve Huh7.5.1 cells. On a single-cycle virus production assay, we found that the R167G mutation did not affect RNA replication or virus secretion but enhanced infectious virus assembly within the cells (Fig. 4). Efficient infectious virus assembly within the cells was mainly responsible for the rapid spread and high virus production of MA/JFH-1.2/R167G.

The amino acid at 167 (aa 167) is located in domain 2 of the core region, which is important for localization of the core

protein (3, 8). Lipid droplet localization of the core protein and/or NS5A is important for infectious virus production (4, 18, 26). The interaction between the core protein and NS5A is also important for infectious virus production (16). Thus, aa 167 affects infectious virus production possibly by altering subcellular localization of the core protein or interaction between the core protein and NS5A. We examined the amino acid sequence of the core protein in 2,078 strains in the Hepatitis Virus Database (<http://s2as02.genes.nig.ac.jp/>) and found that aa 167 is Gly in all other strains. These data strongly suggest that Gly at aa 167 is important for the HCV life cycle. As the MA strain was cloned from the serum of a patient with chronic hepatitis C, the low virus production by this Gly at aa 167 may be important for persistent infection.

We then attempted to reduce the contents of JFH-1 from MA/JFH-1.2/R167G. We previously reported that the N3H and N5BX regions of JFH-1 were sufficient for replication of the J6CF strain (21). We also reported that this effect was observed only in genotype 2a strains (J6CF, JCH-1, and JCH-4). In this study, we tested whether the N3H and N5BX regions of JFH-1 could also support replication of a genotype 2b strain, MA. We constructed an MA chimeric virus harboring the N3H and N5BX regions of JFH-1 and combined this with the 5' UTR of JFH-1 and the R167G mutation (MA/N3H+N5BX-JFH1/R167G). This chimeric RNA was able to replicate in the cells and produce infectious chimeric virus in culture medium although infectious virus production levels were low (Fig. 5).

We showed in this paper that the N3H and N5BX regions of JFH-1 were able to support RNA replication by both genotype 2a clones and genotype 2b clones, but the nucleotide sequence similarity between JFH-1 and MA was lower than that between JFH-1 and J6CF (77% versus 89%, respectively). Compared to MA/JFH-1.2/R167G, MA/N3H+N5BX-JFH1/R167G RNA showed the same levels of RNA replication and low levels of infectious virus production. To clarify whether there were any differences in the characteristics of the secreted virus, we performed density gradient ultracentrifugation with the MA/JFH-1.2/R167G and MA/N3H+N5BX-JFH1/R167G viruses. The distributions of the HCV core protein and infectivity showed similar profiles (data not shown).

The differences between MA/JFH-1.2/R167G and MA/N3H+N5BX-JFH1/R167G are the NS2, NS3 protease domain (N3P), and NS4A to NS5A regions. Nucleotide variation(s) other than aa 167 in these regions of the MA strain may be associated with reduced virus assembly. We identified four additional cell culture-adaptive mutations, L814S (NS2), R1012G (NS2), T1106A (NS3), and V1951A (NS4B), which resulted from long-term culture of MA/N3H+N5BX-JFH1/R167G RNA-transfected cells. Consequently, cells transfected with MA/N3H+N5BX-JFH1/5am constructed by insertion of these four adaptive mutations into MA/N3H+N5BX-JFH1/R167G replicated and produced infectious virus as efficiently as MA/JFH-1.2/R167G RNA-transfected cells (Fig. 6).

This system is able to contribute to studies into the development of antiviral strategies. It has been reported that HCV genotype 2a was more sensitive to interferon therapy than HCV genotype 2b in a clinical study (20). To assess the interferon resistance of genotype 2b, a cell culture system with multiple genotype 2b strains is necessary. The previously reported replicable genotype 2b chimeric virus harbored only structural

regions of 2b strains (6, 27). The 2b/JFH-1 chimeric virus containing the region of the core protein to NS2 from the J8 strain (genotype 2b) and the region of NS3 to 3' X of JFH-1 was able to replicate and showed that there were no differences in interferon sensitivity among the JFH-1 chimeric viruses of other genotypes (6, 27). Another 2b/JFH-1 chimeric virus containing the regions of the core protein to NS2 (nt 342 to 2867) of a genotype 2b strain and of NS2 to 3' UTR (nt 2868) of JFH-1 has been reported (6, 27). The authors reported that their 2b/JFH-1 chimeric virus was more sensitive to interferon than JFH-1 (6, 27). We developed the genotype 2b HCV cell culture system with another HCV genotype 2b strain (MA). We identified a virus assembly-enhancing mutation in the core region, the minimal JFH-1 regions necessary for replication, and four additional adaptive mutations that enhance infectious virus production and demonstrated that MA harboring the five adaptive mutations and the 5' UTR and N3H and N5BX regions of JFH-1 (MA/N3H+N5BX-JFH1/5am) could replicate and produce infectious virus efficiently.

Using these novel genotype 2b chimeric viruses, we assessed interferon sensitivity. We found that MA/JFH-1.2/R167G chimeric virus and MA/N3H+N5BX-JFH1/5am virus were more sensitive to interferon than the JFH-1 virus (Fig. 7). Furthermore, we found that MA/N3H+N5BX-JFH1/5am was more sensitive to interferon than MA/JFH-1.2/R167G, indicating that the genetic variation(s) in the NS2, N3P, and NS4A to NS5A regions affect interferon sensitivity. Although genotype 2a viruses are more sensitive to interferon than genotype 2b viruses in clinical studies, JFH-1 displayed interferon resistance in our study.

These results suggest that the JFH-1 regions in the 2b/JFH-1 virus affect the interferon sensitivity of the chimeric virus. Moreover, it was reported that amino acid variations in E2, p7, NS2, and NS5A were associated with the response to peginterferon and ribavirin therapy in genotype 2b HCV infection (10). Therefore, our MA/JFH-1 chimeric virus harboring minimal regions from JFH-1 (MA/N3H+N5BX-JFH1/5am) is more suitable for assessing the characteristics of the MA strain than the MA/JFH-1 chimeric virus, which includes a nonstructural region from JFH-1 (MA/JFH-1.2/R167G). We showed here that replacement of the 5' UTR and N3H and N5BX regions in MA with those from JFH-1 is able to convert MA into a replicable virus. Using the same strategy, numerous HCV cell culture systems with various genotype 2b strains, as well as genotype 2a strains, may be available.

In conclusion, we established a novel HCV genotype 2b cell culture system using a chimeric genome in MA harboring minimal regions from JFH-1. This cell culture system using the chimeric genotype 2b virus will be useful for characterization of genotype 2b viruses and the development of antiviral strategies.

ACKNOWLEDGMENTS

We are grateful to Tetsuro Suzuki of Hamamatsu University School of Medicine for helpful comments and suggestions. Huh7.5.1 cells were kindly provided by Francis V. Chisari.

A.M. is partially supported by the Japan Health Sciences Foundation and Viral Hepatitis Research Foundation of Japan. This work was partially supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labor and

Welfare of Japan, from the Ministry of Education, Culture, Sports, Science and Technology, from the National Institute of Biomedical Innovation, and by Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation.

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ORIGINAL ARTICLE

Hepatitis B virus strains of subgenotype A2 with an identical sequence spreading rapidly from the capital region to all over Japan in patients with acute hepatitis B

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Revised 3 October 2011
Accepted 5 October 2011

ABSTRACT

Objective To examine recent trends of acute infection with hepatitis B virus (HBV) in Japan by nationwide surveillance and phylogenetic analyses.

Methods During 1991 through 2009, a sentinel surveillance was conducted in 28 national hospitals in a prospective cohort study. Genotypes of HBV were determined in 547 patients with acute hepatitis B. Nucleotide sequences in the preS1/S2/S gene of genotype A and B isolates were determined for phylogenetic analyses.

Results HBV genotype A was detected in 137 (25% (accompanied by genotype G in one)) patients, B in 48 (9%), C in 359 (66%), and other genotypes in the remaining three (0.5%). HBV persisted in five with genotype A including the one accompanied by genotype G; another was co-infected with HIV type 1. The genotype was A in 4.8% of patients during 1991–1996, 29.3% during 1997–2002, and 50.0% during 2003–2008 in the capital region, as against 6.5%, 8.5% and 33.1%, respectively, in other regions. Of the 114 genotype A isolates, 13 (11.4%) were subgenotype A1, and 101 (88.6%) were A2, whereas of the 43 genotype B isolates, 10 (23.3%) were subgenotype B1, 28 (65.1%) were B2, two (4.7%) were B3, and three (7.0%) were B4. Sequences of 65 (64%) isolates of A2 were identical, as were three (23%) of A1, and five (18%) of B2, but none of the B1, B3 and B4 isolates shared a sequence.

Conclusions Acute infection with HBV of genotype A, subgenotype A2 in particular, appear to be increasing, mainly through sexual contact, and spreading from the capital region to other regions in Japan nationwide. Infection persisted in 4% of the patients with genotype A, and HBV strains with an identical sequence prevailed in subgenotype A2 infections. This study indicates the need for universal vaccination of young people to prevent increases in HBV infection in Japan.

Significance of this study

What is already known about this subject?

- ▶ In Japan, a national prevention programme was started in 1986 with selective vaccination of babies born to mothers who carry hepatitis B virus (HBV). Since then, the prevalence of hepatitis B surface antigen among younger generations has decreased sharply.
- ▶ However, retrospective studies indicate that the frequency of HBV genotype A is increasing among patients with acute hepatitis B (AHB) within the capital region of Japan.
- ▶ Infection with genotype A more often persists than infection with other genotypes.
- ▶ Because there is no reliable and comprehensive surveillance system for AHB in Japan, the incidence of AHB and factors responsible for changes over many years are not known.

What are the new findings?

- ▶ This is a prospective cohort study for surveillance of AHB throughout Japan in a national research programme.
- ▶ The incidence of AHB in Japan has not decreased, because genotype A infections have increased over time.
- ▶ Genotype A infections started to increase in the capital region of Japan, and then spread to other regions 5–6 years later.
- ▶ About 90% of genotype A found in AHB patients in Japan is subgenotype A2.
- ▶ Subgenotype A2 isolates from patients with AHB tend to preserve sequence identity over time, indicating that particular subgenotype A2 strains have been transmitted without undergoing mutations.

Hepatitis B virus (HBV) has been classified into 10 genotypes, designated A–J, based on a >8% divergence in the full-genome sequence.^{1–7} Different genotypes are associated with distinct clinical manifestations, such as severity and progression of

liver disease, as well as response to antiviral treatments.^{8–10} Some genotypes are subclassified: genotype A into at least two subgenotypes, A1 (Asian/African type) and A2 (European type)^{11–13};

Viral hepatitis

Significance of this study

How might it impact on clinical practice in the foreseeable future?

- ▶ It needs to be noted that subgenotype A2 infections are spreading among sexually active generations in Japan.
- ▶ Although selective vaccination has prevented mother-to-baby transmission of HBV since 1986, it does not contain sporadic infections in Japan.
- ▶ Herd vaccination of younger generations needs to be considered in Japan.

B into B1 (Japanese type) and B2 (Asian type)^{14 15}; and C into C1 (Southeast-Asian type) and C2 (East-Asian type).¹⁶ Subgenotypes also influence the replication of HBV and clinical manifestation.^{15 17 18}

According to a report from Japan in 2001,¹⁹ genotype C was the most prevalent (84.7%), followed by genotype B (12.2%) and A (1.7%), among patients with chronic hepatitis B. In 2002, genotype A became the most prevalent in patients with acute hepatitis B (AHB) around Tokyo, the capital region of Japan.^{20 21} Several reports have shown that infection with HBV genotype A is associated with particular sexual behaviours, such as homosexual activity and promiscuous sexual contacts, and tends to persist longer than that with HBV genotype C.^{22 23} These reports have raised concerns about the horizontal HBV infection in adults, which, in general, is considered to resolve spontaneously. However, adult-acquired HBV infection may result in chronic HBV infection in some instances.

Information on changes in genotype distribution over time, as well as genotype-specific clinical manifestations, may help in planning preventive measures and antiviral therapy strategies. Therefore it is important to examine how genotype A infection has spread in Japan, and what clinical and virological characteristics it possesses.

We have been conducting a nationwide, sentinel surveillance on acute viral hepatitis for more than 30 years. As part of this surveillance, a prospective cohort study has been conducted on 547 patients with AHB in 28 medical centres over the 19 years from 1991 to 2009. Geographical and longitudinal distributions of HBV genotypes/subgenotypes were surveyed, and their influence on clinical outcome was evaluated.

PATIENTS AND METHODS

Patients

A total of 681 patients with sporadic AHB were enrolled consecutively in a survey carried out by the Japan National Hospital Acute Hepatitis Study Group (JNHAHSG). They were admitted to 28 national hospitals from January 1991 to the end of December 2009. They were grouped geographically into two areas: the capital region (Gunma, Saitama, Tokyo and Kanagawa) and other regions (figure 1). Patients were also longitudinally categorised into three periods: 1st (1991–1996), 2nd (1997–2002) and 3rd (2003–2008). In addition, the year 2009 provided the most recent data. Of the 681 patients, 547 (80.3%) entered the study, for whom serum samples were available on admission and had been stored at -20°C .

The diagnosis of AHB was based on the following criteria: (1) acute onset of liver injury without a history of liver dysfunction; (2) detection of hepatitis B surface antigen (HBsAg) in the

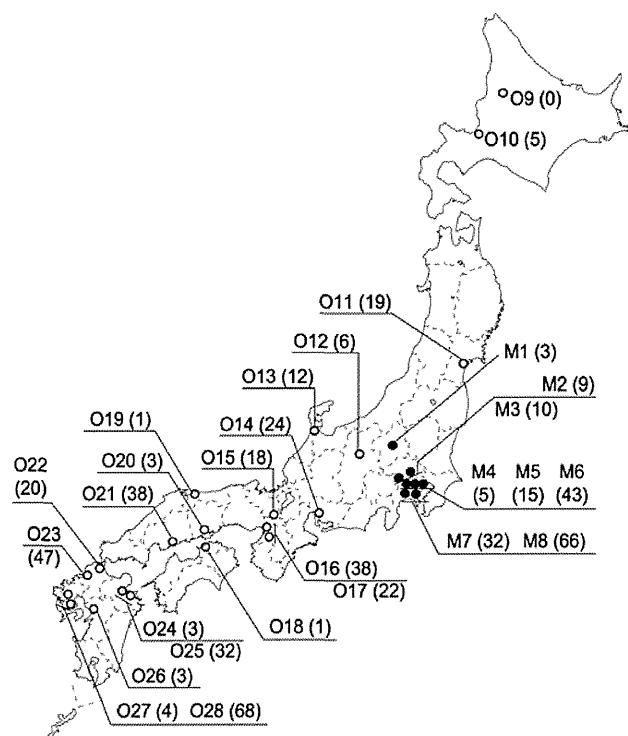


Figure 1 Locations of participating hospitals in Japan. Hospitals in the capital region (M1–M8) are indicated by eight closed circles, and those in other regions (O9–O28) by 20 open circles. Numbers in parentheses indicate the total number of enrolled subjects for each site. The hospitals are: M1, Nishigunma Hospital, Gunma; M2, Nishisaitama-Chuo Hospital, Saitama; M3, National Disaster Medical Center, Tokyo; M4, Tokyo Hospital, Tokyo; M5, Tokyo Medical Center, Tokyo; M6, National Center for Global Health and Medicine, Tokyo; M7, Sagami Hospital, Kanagawa; M8, Yokohama Medical Center, Kanagawa; O9, Asahikawa Medical Center, Hokkaido; O10, Hokkaido Medical Center, Hokkaido; O11, Sendai Medical Center, Miyagi; O12, Matsumoto Medical Center, Nagano; O13, Kanazawa Medical Center, Ishikawa; O14, Nagoya Medical Center, Aichi; O15, Kyoto Medical Center, Kyoto; O16, Osaka National Hospital, Osaka; O17, Osaka-Minami Medical Center, Osaka; O18, Zentsuji Hospital, Kagawa; O19, Yonago Medical Center, Tottori; O20, Okayama Medical Center, Okayama; O21, Kure Medical Center and Chugoku Cancer Center, Hiroshima; O22, Kokura Medical Center, Fukuoka; O23, Kyushu Medical Center, Fukuoka; O24, Beppu Medical Center, Oita; O25, Oita Medical Center, Oita; O26, Kumamoto Medical Center, Kumamoto; O27, Ureshino Medical Center, Saga; and O28, Nagasaki Medical Center, Nagasaki.

serum; (3) positivity for IgM antibody to HBV-core antigen (IgM anti-HBc) in high titres (detectable in sera diluted 10-fold); and (4) absence of past or family history of chronic HBV infection. Severe acute hepatitis (SAH) was defined as prothrombin time (PT) $\leq 40\%$ and hepatic encephalopathy of grade $\leq \text{I}$. Fulminant hepatitis (FH) was diagnosed from PT $\leq 40\%$ and hepatic encephalopathy of grade $\geq \text{II}$. Patients in whom HBsAg remained in the serum for >6 months after onset were considered to have acquired chronic HBV infection. The following information was collected from each patient: year and age at onset, gender, residential area, HBsAg, IgM anti-HBc, alanine aminotransferase, total bilirubin, PT, severity of liver disease, mortality, routes of transmission, sexual behaviours, travelling abroad in recent past, HBV genotype, mutations in precore (PreC) and core promoter (CP) regions, and RNA of hepatitis D virus. Antibody to HIV type 1 (anti-HIV) was

determined in patients who were at high risk and gave consent to testing.

Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and the Ministry of Education, Culture, Sports Science and Technology of Japan, and was approved by the ethics committee of each institution.

Extraction of HBV DNA

HBV DNA was extracted from serum (100 μ l) by the SMITEST EX-R&D Nucleic Acid Extraction Kit (MBL Co, Nagoya, Japan) and used for genotyping/subgenotyping and detecting mutations in PreC and CP regions.

HBV genotypes

Genotypes were determined in Nagasaki Medical Center with the SMITEST HBV Genotyping Kit (MBL) by hybridisation with type-specific probes immobilised on a solid-phase support.²⁴

Determination of HBV subgenotypes

For subgenotyping, HBV DNA was amplified by PCR with TaKaRa Ex Taq (Takara Bio, Shiga, Japan). PCR was performed with appropriate nested primers to amplify a ~1.2 kb sequence in the preS1/S2/S gene (nucleotides 2854–835 in the reference isolate (AB116077)). PCR products were purified, subjected to cycle sequencing reaction with the BigDye Terminator v1.1 (Applied Biosystems, Tokyo, Japan), and applied to the DNA sequencer (3100-Avant; Applied Biosystems).

Mutations in the PreC and CP regions

The A1896 mutation in the PreC region was detected by the enzyme-linked minisequence assay (SMITEST HBV PreC ELMA; Roche Diagnostics, Tokyo, Japan), and mutations in the CP region for T1762/A1764 by the enzyme-linked specific probe assay (SMITEST HBV Core Promoter Mutation Detection Kit; Roche Diagnostics). The results were recorded as 'wild-type' and 'mutant types' dominantly expressed by HBV isolates.²⁵

Phylogenetic analyses

Nucleotide sequences were aligned, and phylogenetic trees were constructed by the CLUSTAL W program v1.83 (DDBJ homepage: <http://clustalw.ddbj.nig.ac.jp/top-j.html>). The statistical validity was assessed by bootstrap resampling with 1000 replicates. Reference HBV strains were retrieved from the GenBank database.

Statistical analysis

Results were expressed as percentage or mean \pm SD. Statistical differences were evaluated by χ^2 and Fisher exact tests for categorical variables, and analysis of variance and Scheffe's test for quantitative variables, using the SPSS software. The 95% CI, for the difference in means, was calculated in analyses for quantitative variables. $p < 0.05$ was considered significant.

RESULTS

Distribution of HV genotypes

HBV genotypes were determined in the 547 patients with AHB. The genotype was A in 137 (25.0%) patients (accompanied by G in one (0.2%)), B in 48 (8.8%), C in 359 (65.6%), D in one (0.2%), E in one (0.2%), and H in one (0.2%). Because HBV genotype G is a defective virus and cannot replicate by itself,^{26 27} the single patient with mixed genotypes A and G was included in the 137 patients with genotype A in further analyses. RNA of hepatitis

D virus was detected in three of the 453 (0.7%) patients. Anti-HIV was examined in patients at high risk of infection and detected in 14 of the 53 (26.4%) who gave consent to testing.

Demographic and clinical differences among patients infected with HBV of distinct genotypes

Demographic and clinical characteristics of patients with different genotypes are compared in table 1. There was no difference in mean age among patients with genotypes A, B and C. The proportion of men was higher in patients with genotype A than B or C (94.2% vs 79.2%, $p < 0.05$; or 56.0%, $p < 0.0001$), and in those with genotype B than C (79.2% vs 56.0%, $p < 0.05$).

Maximum levels of total bilirubin were higher in patients with genotype A than C (9.6 ± 7.6 vs 7.1 ± 6.2 mg/dl, $p < 0.05$), with a difference of 2.5 mg/dl (95% CI 0.93 to 4.08), whereas the highest alanine aminotransferase activity and lowest PT values did not differ among patients with distinct genotypes.

SAH developed in four (2.9%) patients with genotype A, four (8.3%) with genotype B, and 26 (7.2%) with genotype C. FH developed in one (2.1%) patient with genotype B and eight (2.2%) with genotype C; no patients with genotype A developed FH. Eight (1.5%) patients died, including one with genotype B and seven with genotype C. There were no significant differences among patients with different genotypes in the frequency of SAH or FH or mortality.

The outcome of AHB was traceable in 514 of the 547 (94.0%) patients. Chronic infection with persistence of HBsAg for >6 months developed in five of the 123 (4.1%) patients with genotype A (including the one accompanied by genotype G), none of the 46 (0%) with genotype B, and none of the 342 (0%) with genotype C; it was more common in patients with genotype A than C ($p < 0.05$). HBV infection persisted exclusively in the patients with genotype A, either alone (four patients) or together with genotype G (one).

Among the five patients who acquired chronic HBV infection, four (three with genotype A and one with mixed genotypes A and G) were examined for anti-HIV, and one with genotype A was found to be positive. HBV infection persisted in three (including the one with anti-HIV) of the five patients for >1 year after the onset, and the remaining two (both without anti-HIV) cleared HBsAg from the serum after retaining it for >6 months.

Mutations in the PreC and/or CP region were detected in 3.7% (4/109) of patients with genotype A, 15.4% (6/39) of those with genotype B, and 25.5% (79/310) of those with genotype C. They were significantly less common in patients with genotype A than B or C (A vs B, $p < 0.05$; A vs C, $p < 0.0001$). The only patient with genotype A who had the PreC mutation was simultaneously infected with genotype G.

Routes of transmission were identifiable in 275 of the 547 (50%) patients, and the main route was heterosexual contacts; those in the remaining patients could not be disclosed. The frequency of heterosexual activity did not differ among patients with distinct genotypes. However, homosexual activity was more common in patients with genotype A than B or C (21.2%, 0% and 0.8%, respectively (A vs B, $p < 0.001$; A vs C, $p < 0.0001$)). Among the 32 homosexual men, HBV genotype A was detected in 29 (91%). Consent to anti-HIV testing was given by 10 of the 29 patients, and four of these (40%) were positive.

Longitudinal changes in the distribution of genotypes

Figure 2 illustrates changes in the distribution of HBV genotypes through three 6-year periods over 18 years (1991–2008). In addition, data from 2009 are shown. HBV genotype A accounted