tation of the wild-type strain of HCV *in vivo* (9). In the present study, ultradeep sequencing did not detect any telaprevir-resistant variants at baseline in 11 patients, although *de novo* resistant variants emerged in all 11 patients over time. The present clinical results based on patients who did not achieve an SVR provide evidence in support of a *de novo* emergence of telaprevir resistance that is induced by viral mutation.

The results of the present study should be interpreted with caution, since the study was performed in a small number of Japanese patients infected with HCV-1b. Any generalization of the results should await confirmation by a multicenter randomized trial based on a larger number of patients, including patients of other races and those infected with HCV-1a. Furthermore, the other limitation of the present study is that the existence of very low-frequency telaprevir-resistant variants was not investigated long after the cessation of therapy by ultradeep sequencing. Further large-scale studies using ultradeep sequencing should be performed to investigate the effects of telaprevir-resistant variants on the response to treatment using new drugs, including direct-acting antiviral agents.

In conclusion, this study, which is based on Japanese patients infected with HCV genotype 1b, indicated that the efficacy of triple therapy could be predicted by the combination of host, viral, and treatment factors. However, the present results show that it might be difficult to predict at baseline the emergence of telaprevir-resistant variants during triple therapy, even with the use of ultradeep sequencing. Further large-scale prospective studies are needed to investigate the pretreatment predictors of treatment efficacy and the emergence of telaprevir-resistant variants after triple therapy, and to develop more effective therapeutic regimens in patients infected with HCV genotype 1.

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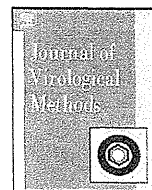

Protocols

Comparative quantitative analysis of hepatitis C mutations at amino acids 70 and 91 in the core region by the Q-Invader assay

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ABSTRACT

Hepatitis C virus (HCV) is a major worldwide public health problem, and mutations at amino acids 70 and 91 in the genotype 1b core region predict the effectiveness of combination therapy with peginterferon and ribavirin. An assay based on the Q-Invader technology was developed to determine the relative ratios of the mutant to wild-type virus with high sensitivity. The assay detected a minor type plasmid that constituted only 1% of a mixture of plasmids containing wild-type and mutant sequences. The calculated ratios agreed with those of the template DNA. A total of 123 serum samples of HCV in Japan were examined with the Q-Invader assay. The Q-Invader assay detected all of the mutations that were detected by direct sequencing and even some mutants that direct sequencing could not. PCR with mutant specific primers confirmed those mutations found by the Q-Invader assay and not by direct sequencing. The Q-Invader assay, thus, is a useful tool for detecting mutations at positions 70 and 91 in the HCV-1b core region.
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Table 1
Primer and Invader probe with the Q-Invader assay.

Target		Sequence (5′–3′)
Core 70	F-primer	CCTCGTGGAAGGCGACAACCTAT
	R-primer	GGCCADGGRTACCCRGGCTG
	p1 probe	CGCGCCGAGG**C**GRMRAKCYTTGG
	p2 probe	ACGGACGCGGAG**T**GRMRAKCYTTGG
	io probe	GCCCAGGHYCTRCCCTCGKBNA
core 91	F-primer	CCTGGGCTCAGCCYGGGTA
	R-primer	CGGGGTGACAGGAGCCATC
	p1 probe	CGCGCCGAGG**Y**TRGGRTGGRCAG
	p2 probe	ACGGACGCGGAG**A**TRGGRTGGRCAGGAT
	io probe	TTGGCCCCTCTAYGGCAAYKAGGGYT

F-primer, forward primer; R-primer, reverse primer; p1 probe, primary probe (FAM); p2 probe, primary probe (RED); io probe, Invader oligo.
Underlined sequence represents the 5′ flap of probe. Amino-blocked 3′ end of all primary probes. Boldfaced sequences denote the cleavage site of primary probes.

Table 2
Comparison of the number of mutant by the Q-Invader assay with those of sequencing.

Core 70	Sequencing		
	Wild	Mixed	Mutant
Q-Invader (Mutant%)			
Less than 1%	43		
1–99%	12	21	4
More than 99%			43
Core 91	**Sequencing**		
	Wild	Mixed	Mutant
Q-Invader (Mutant%)			
Less than 1%	55		
1–99%	9	9	4
More than 99%			46

1. Introduction

Hepatitis C virus (HCV) affects approximately 170 million people and is a major worldwide public health problem. It is responsible for chronic liver disease and increases the risk for severe diseases, such as cirrhosis and hepatocellular carcinoma (HCC) (Seeff, 2002). Interferon (IFN) therapy in chronic hepatitis C infection reduces the risk of developing HCC and liver-related death by clearing the virus. Since 2004, combination therapy with peginterferon (PEG-IFN) and ribavirin (RBV) has been the standard treatment (Fried et al., 2002). Recently, a triple therapy of peginterferon, ribavirin and a direct-acting antiviral (DAA), such as the protease inhibitor boceprevir or telaprevir, was approved and has been routinely used since 2011. However, for some patients, a sustained virological response does not last long. In Japan, the most common HCV genotype is genotype 1b (about 70%), but no virological response was found in 26.3% of patients infected with genotype 1b (Akuta et al., 2006).

In the present study, two key factors were related to the lack of a virological response in patients with the HCV genotype 1b: host polymorphisms at the neighboring IL28B gene (Ge et al., 2009; Tanaka et al., 2009; Thomas et al., 2009) and mutations in the HCV-1b core region (Akuta et al., 2005, 2006). The HCV-1b mutations (*e.g.*, arginine to glutamine or histidine at position 70 and/or from leucine to methionine at position 91) were significantly more common in virological non-responders, and the rate of decline in the HCV load during combination therapy in patients with mutant variants was less than those of patients with wild-type virus (Akuta et al., 2005). With the triple therapy, the proportions of sustained virological response were predicted by the prevalence of mutations at position 70 and the polymorphisms at the neighboring IL28B gene (Akuta et al., 2010). In addition, the prevalence of mutations at position 70 was increased by positive selection during combination therapy (Kurbanov et al., 2010). Therefore, for HCV therapy, monitoring the ratios of mutant variant to wild-type at position 70/91 may be as important as monitoring the HCV load. However, measuring the ratios of one point mutation with high sensitivity is difficult because only one quantitative method is available (Nakamoto et al., 2009).

This report describes a novel approach that uses the real-time PCR monitoring Invader reaction (Q-Invader assay) in a comparative quantitative assay for mutations at positions 70/91 in HCV-1b core region. The Invader technology has high specificity for detecting single-nucleotide differences in genomic DNA or PCR products (Lyamichev et al., 2000), and a highly sensitive quantification assay can be attained by adding real-time PCR (Tadokoro et al., 2009, 2010). To demonstrate its sensitivity and effectiveness, the method was used to detect mutations in clinical samples, and the results were compared to those from sequencing (Akuta et al., 2005) and PCR with mutation-specific primers (Okamoto et al., 2007).

2. Materials and methods

2.1. Source of patients

Serum samples were obtained from 123 patients infected with HCV-1b at the Toranomon Hospital (Kanagawa, Japan). The study was conducted in accord with the ethical principles of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethical Committee. Written informed consent was obtained from each patient.

2.2. Design of Invader probes

The primary probe and Invader oligo to detect mutations (*i.e.*, R70H/Q and L91M in the core region) were designed with the Invader technology creator (TWT, Madison, WI, USA) (Fig. 1 and Table 1). Variations in neighboring regions were confirmed by analyzing 55 individual sequences of the HCV core region from a database at the National Center for Biotechnology Information (NCBI) (see Table 1).

2.3. Extraction HCV RNA and cDNA synthesis

HCV RNA was extracted from 200 μl of serum and eluted in 10 μl RNAse/DNAse-free water with the PureLink Viral RNA/DNA Mini Kit (Life Technologies, Carlsbad, CA, USA). cDNA was synthesized with random primers in 20-μl reactions with a commercial kit (SuperScript III cDNA Synthesis Kit, Life Technologies).

2.4. The Q-Invader assay

Comparative quantitative analysis of mutant and wild-type virus was completed by the Q-Invader assay. Two fluorescence signals (carboxyfluorescein or FAM for wild-type; REDmond RED or RED for mutants) could be detected in a single reaction with a Universal General Purpose Reagent (TWT), including Cleavase and FRET mix with two common fluorescence probes. Template c-DNA was added to a 15-μl reaction mixture containing 500 nM primers for amplification HCV-1b core region, 300 nM of each primary probe, 700 nM Invader oligo, 2 U AmpliTaq gold (Life Technologies), Universal General Purpose Reagent (TWT) and FRET mix (Table 1). The reaction mixture was preheated in a 384-PCR plate (Roche, Basel, Switzerland) at 95 °C for 20 min, and a two-step PCR was carried out for 40 cycles (95 °C for 15 s, 65 °C for 60 s) in a LightCycler 480 (Roche) (Tadokoro et al., 2009). Fluorescence values of FAM

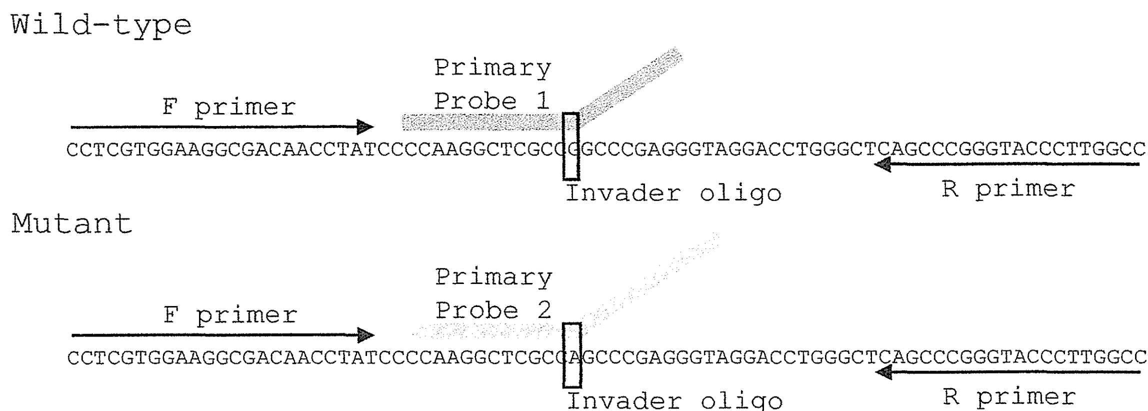

Fig. 1. Cleavage point for the Q-Invader assay at position 70 in the HCV-1b core region. White boxes represent cleavage points in PCR amplicon. Reference sequence: HCV-J.

(wavelength/bandwidth: excitation, 465 nm; emission, 510 nm) and RED (excitation, 533 nm; emission, 610 nm) were measured at end of the incubation/extension step at 65 °C for each cycle and by standard real-time PCR. By analyzing the results, a crossing point (Cp) can be obtained by a fit point method (Luu-The et al., 2005) in the LightCycler 480 software.

2.5. Sequencing

The sequence at position 70/91 in clinical samples was determined as described (Akuta et al., 2005). The amplified products were sequenced by the dideoxy method with the BigDye Terminator v1.1 Cycle Sequencing Kit (Life Technologies) in a model 3130 fluorescent DNA sequencer (Life Technologies).

2.6. Manufacture of control plasmid DNA by cloning clinical HCV sequence

HCV RNA, wild-type and that with mutations at positions 70/91, was isolated from clinical samples and amplified by. PCR amplicons were cloned into the pCRII-TOPO vector (Life Technologies) and sequenced (Tadokoro et al., 2009).

2.7. Mutation analysis by wild-type/mutant-specific primers

The mutations of positions 70/91 in patient c-DNA were determined by PCR with wild-type/mutant specific primer (Okamoto et al., 2007). The PCR amplicon was confirmed by

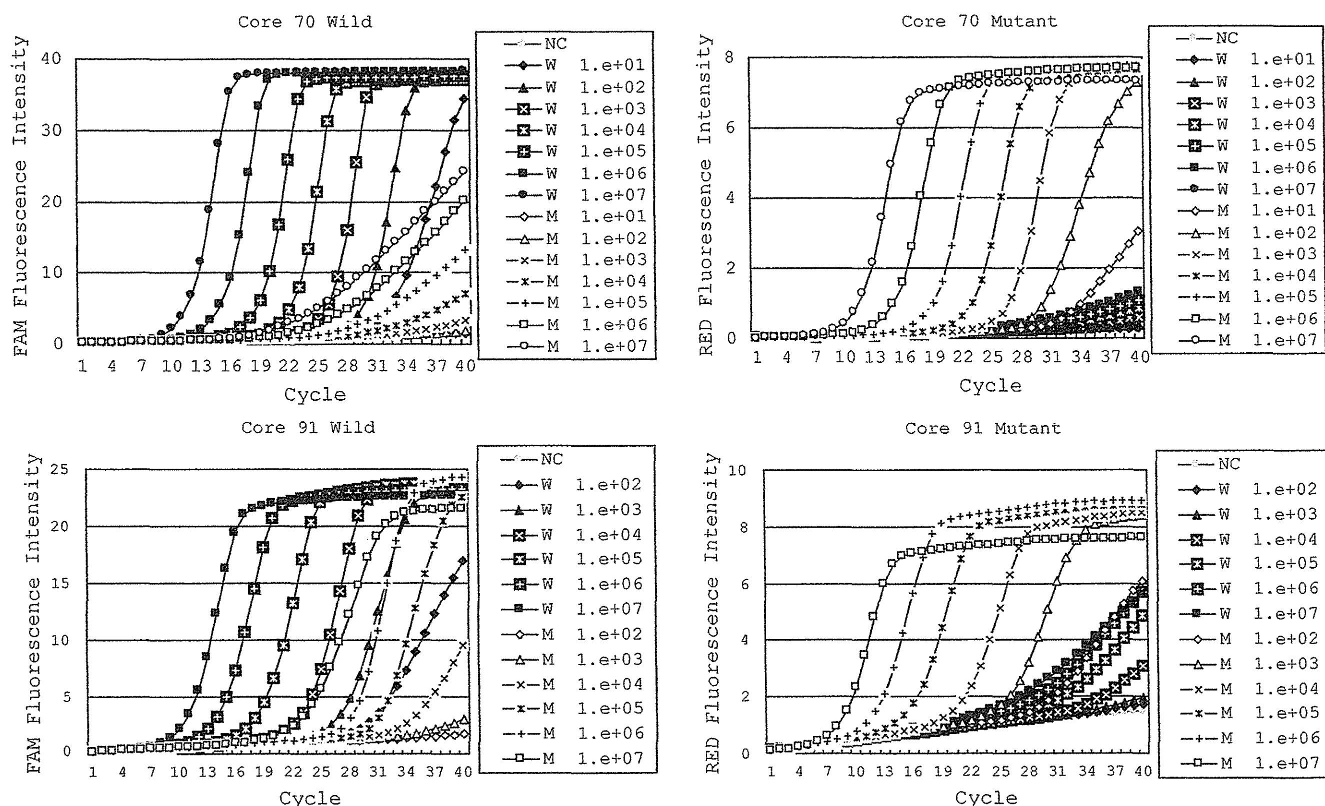

Fig. 2. Amplification plots generated by the Q-Invader assay. Fluorescence intensity was plotted against the number of cycles for a 10^{-7} dilution of plasmid (W: wild-type plasmid DNA, M: mutant plasmid DNA).

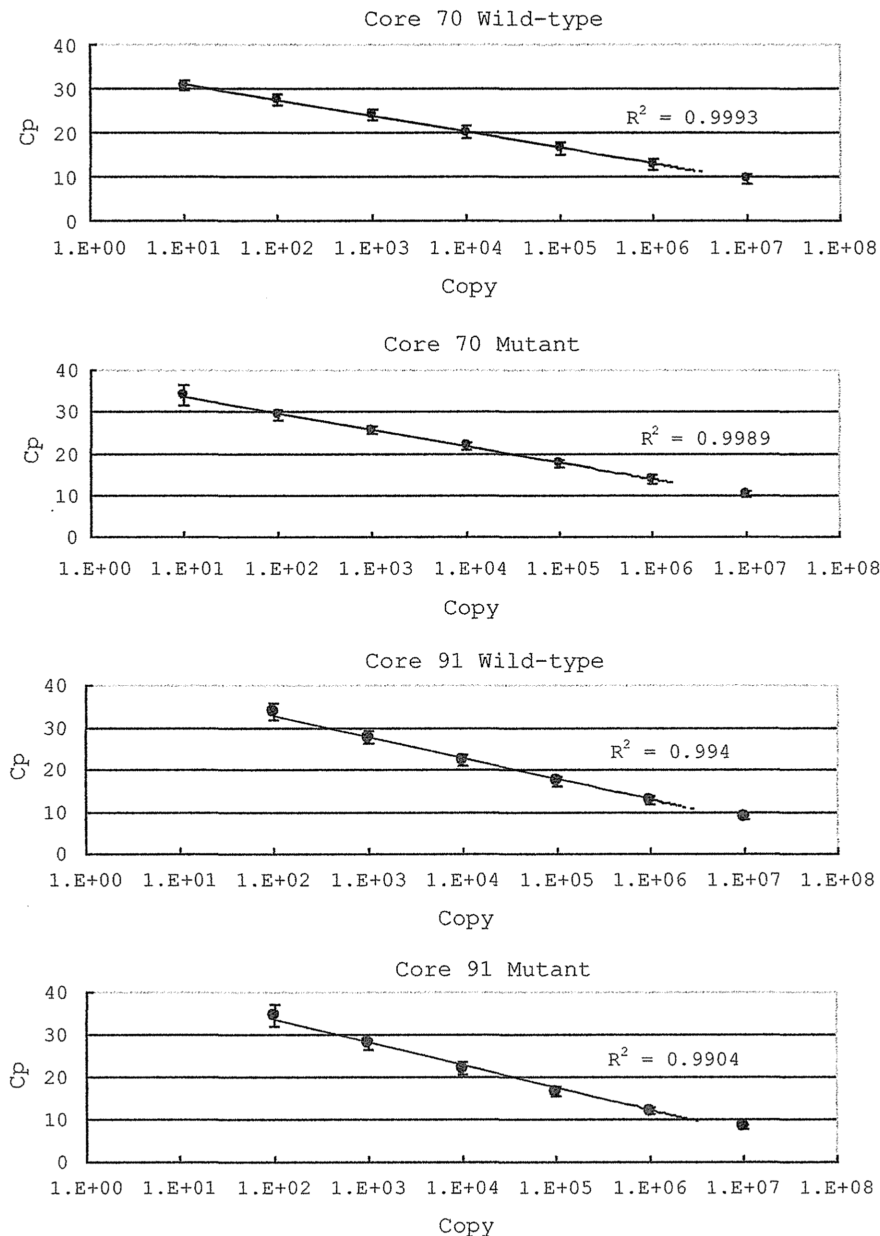

Fig. 3. Standard curves generated by the Q-Invader assay. The Cp was calculated by the fit point method with a 10^{-7} dilution of plasmid.

electrophoresis with a MultiNA microchip electrophoresis system (Shimadzu, Kyoto, Japan).

2.8. *Detection sensitivity and assay variation for the Q-Invader assay*

Detection sensitivity and assay variation for the Q-Invader assay were examined with single or mixed plasmids for template DNA. The detection limits of the Q-Invader assay in each measurement were determined with a 10^{-7} dilution of plasmid. Mixtures of plasmids were prepared in various ratios and determined in the Q-Invader assay for comparative quantitative analysis. A fivefold measurement was performed three times each to examine assay variation.

3. Results

3.1. *Detection threshold and assay variation*

Measurements of the mutant and wild-type virus at position 70 were both effective between 10^1 and 10^7 copies. The detection thresholds of both measurements were 10^1 copies of HCV c-DNA. Measurements at position 91 (mutant and wild-type) were effective between 10^2 and 10^7 copies, and the detection threshold was 10^2 copies (Figs. 2 and 3).

3.2. *Comparative quantitative analysis for calculating relative ratios*

Comparative quantitative analysis was used to determine the relative ratios in competitive infections by mutant variant and

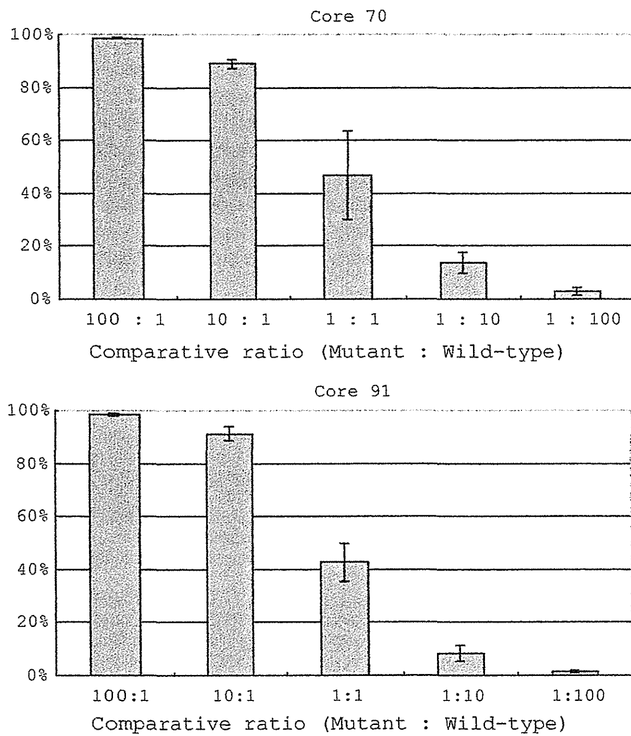

Fig. 4. Detection threshold in the Q-Invader assay with plasmid DNA at ratios of 100:1 to 1:100. At position 70, the minimum number of minor types was 10 copies. Position 91 was 100 copies.

wild-type virus. Copy numbers of mutant and wild-type virus were calculated separately by fit point analysis with each standard plasmid. To determine the relative ratios, the number of mutants was divided by those of total (mutant + wild-type). When the mutant and wild-type plasmids were mixed in various ratios for template DNA, the minor plasmid (mutant or wild-type) in template DNA could be detected down to 1:100 (Fig. 4), and the calculated relative ratios agreed with those of template DNA (Fig. 5).

3.3. *Determination of relative ratios in clinical samples*

Sequences from the HCV core region of 123 serum samples were confirmed by direct sequencing. The Q-Invader assay was used to determine the relative ratios of mutants at positions 70/91. The relative ratios identified three categories to compare by direct sequencing: less than 1% (wild-type), 1–99% (mixed) and more than 99% (mutant). Mixed types were found in 37 samples (30.1%) at position 70, and only 43.2% (16 of 37) of the cases were detected by the Q-Invader assay. At position 91, 22 samples (17.9%) were found to be of the mixed type, and only 59.1% (13 of 22) of the cases were detected by Q-Invader assay (Table 2).

3.4. *Confirming mixed type by PCR with specific primer*

To confirm the mixed type decided by the Q-Invader assay, but not by direct sequencing, PCR with wild-type/mutant specific primer was performed for each four samples. At position 70, a minor mutant variant was detected in samples 99 and 118 (4% and 5%). In samples 80 and 18, mutant variant held most (98% and 99%). By PCR with specific primer, both wild-type and mutant were detected in all samples. A similar result was obtained at position 91 (Table 3).

4. Discussion

Accurate, rapid determination of the relative ratios of mutations at positions 70/91 in the HCV-1b core region is important to insure

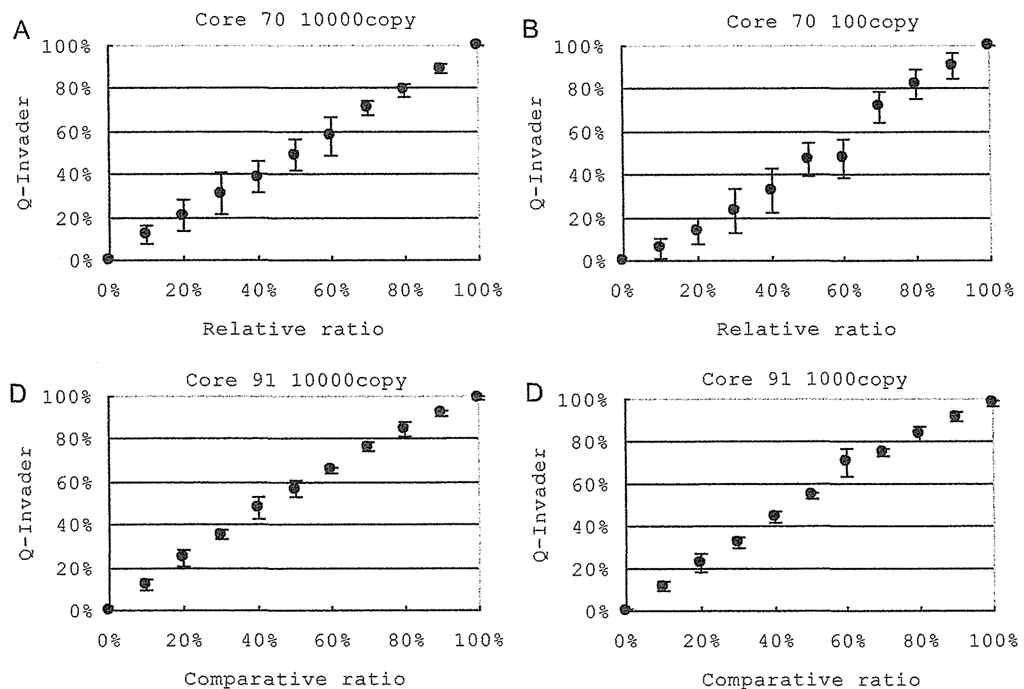

Fig. 5. Assay variation in the Q-Invader assay with 0–100% mixed plasmid DNA. Plasmid DNA was mixed to 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% with the mutation. At position 70, total numbers of plasmid DNA copies were 10,000 (A) and 100 (B). Position 91 was also 10,000 (C) and 1000 (D) copies.

Table 3
Confirmation for mixed type by PCR with specific primers.

Core 70	Q-Invader mutant (%)	Sequencing	PCR with specific primers	
			Wild	Mutant
Sample 099	4%	Wild	+	+
Sample 118	5%	Wild	+	+
Sample 080	98%	Mutant	+	+
Sample 018	99%	Mutant	+	+
Core 91	Q-Invader mutant (%)	Sequencing	PCR with specific primers	
			Wild	Mutant
Sample 088	2%	Wild	+	+
Sample 067	2%	Wild	+	+
Sample 090	98%	Mutant	+	+
Sample 071	98%	Mutant	+	+

the effectiveness of the combination therapy with peginterferon and ribavirin. A highly sensitive method with comparative quantitative analysis was developed in this study. The Q-Invader assay was examined for detection sensitivity and accuracy and compared with direct sequencing and PCR with type-specific primers.

Multiple reaction systems with two florescence probes were examined for detection of wild-type/mutant sequences at two positions (70 and 91) in the HCV-1b core region. The detection sensitivities at position 70, both wild-type and mutant, were 10 copies of template DNA, and those at position 91 were 100 copies (Fig. 3). In examinations of mixtures of plasmids with wild-type and mutant sequences in various ratios, the Q-Invader assay could be determined down to 1% of the minor type (Fig. 4), and the calculated relative ratios agreed with those determined by use of template DNA. The relative ratios were not influenced by total number of DNA templates (Fig. 5).

To validate the Q-Invader assay, 123 clinical samples from patients were analyzed for mutations at positions 70/91 in HCV-1b core region by direct sequencing and the Q-Invader assay. All of mutations found by direct sequencing were also detected by the Q-Invader assay. Moreover, The Q-Invader assay could detect wild-type/mutant that slightly included in sample. At position 70, both wild-type and mutant were detected in 37 samples by the Q-Invader assay and 56.8% (21 of 37) of the cases could detect only either one by direct sequencing. To confirm the existence of the minor type detected by the Q-Invader assay, the PCR with specific primer was performed (Table 3).

The relevance of amino acid mutations at positions 70/91 in HCV-1b core region to the effectiveness of combination or triple therapy was reported in many studies (Akuta et al., 2005, 2010; Nakamoto et al., 2010). In addition, amino acid mutations at the positions 70/91 were resistant to interferon *in vitro* (Funaoka et al., 2011). The expression levels of IL-6, which upregulates SOCS3, in cells transfected with the core mutant were significantly higher than with wild type. These mechanisms may explain the clinical resistance of amino acid mutations at positions 70/91 for interferon therapy. Furthermore, the amino acid mutations at position 70 in the HCV-1b core region are significant as an independent predictor of HCC in virological non-responders (Seko et al., 2013).

In studies of interferon therapy and HCC development, the importance of the amino acid mutations at positions 70/91 is increasing. In addition, the relative ratios of mutants varied in individual patients (Okamoto et al., 2007) and changed between therapies (Kozuka et al., 2012; Kurbanov et al., 2010). Therefore, monitoring the relative ratios of mutant variants should contribute to new knowledge and efficacy prediction for HCV therapy. A high-quality quantitative system is required to push forward a study. The Q-Invader assay, which is more high sensitive method than existing method, would provide detailed dynamic change of relative ratios.

In summary, the Q-Invader assay had a high sensitivity for calculating the relative ratios of mutant variants at positions 70 and 91 in HCV-1b core region. The mutant variants were detected down to 1% of the total, and mixed template DNAs in various ratios were quantified accurately by the Q-Invader assay. The Q-Invader assay will be useful for patients with HCV in clinical setting.

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Effect of Type 2 Diabetes on Risk for Malignancies Includes Hepatocellular Carcinoma in Chronic Hepatitis C

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The aim of this retrospective cohort study was to assess the cumulative development incidence and predictive factors for malignancies after the termination of interferon (IFN) therapy in Japanese patients for hepatitis C virus (HCV). A total of 4,302 HCV-positive patients treated with IFN were enrolled. The mean observation period was 8.1 years. The primary outcome was the first onset of malignancies. Evaluation was performed using the Kaplan-Meier method and Cox proportional hazard analysis. A total of 606 patients developed malignancies: 393 developed hepatocellular carcinoma (HCC) and 213 developed malignancies other than HCC. The cumulative development rate of HCC was 4.3% at 5 years, 10.5% at 10 years, and 19.7% at 15 years. HCC occurred significantly ($P < 0.05$) when the following characteristics were present: advanced histological staging, sustained virological response not achieved, male sex, advanced age of ≥50 years, total alcohol intake of ≥200 kg, and presence of type 2 diabetes (T2DM). T2DM caused a 1.73-fold enhancement in HCC development. In patients with T2DM, HCC decreased when patients had a mean hemoglobin A1c (HbA1c) level of <7.0% during follow-up (hazard ratio, 0.56; 95% confidence interval, 0.33-0.89; $P = 0.015$). The cumulative development rate of malignancy other than HCC was 2.4% at 5 years, 5.1% at 10 years, and 9.8% at 15 years. Malignancies other than HCC occurred significantly when patients were of advanced age of ≤50 years, smoking index (package per day × year) was ≥ 20, and T2DM was present. T2DM caused a 1.70-fold enhancement in the development of malignancies other than HCC. *Conclusion:* T2DM causes an approximately 1.7-fold enhancement in the development of HCC and malignancies other than HCC in HCV-positive patients treated with IFN. In T2DM patients, maintaining a mean HbA1c level of <7.0% reduces the development of HCC. (HEPATOLOGY 2013;57:964-973)

Hepatitis C virus (HCV) is one of the more common causes of chronic liver disease worldwide. Chronic hepatitis C is an insidiously progressive form of liver disease that relentlessly but silently progresses to cirrhosis in 20%-50% of cases over a period of 10-30 years.[1,2] In addition, HCV is a major risk factor for hepatocellular carcinoma (HCC).[3-7]

On the other hand, the prevalence of patients with type 2 diabetes mellitus (T2DM) is increasing in many nations, including Japan.[8] Thus, the management of T2DM patients who are chronically infected with HCV is one of the most important issues confronted by physicians. Few studies have reported relationships between T2DM and total malignancies, including HCC in HCV patients. In addition, it is not clear whether the stringent control of T2DM is necessary for protecting the development of malignancies in HCV patients. This issue needs to be confirmed via long-term follow-up of a large cohort of patients at high risk of developing malignancy.

Abbreviations: CH, chronic hepatitis; CI, confidence interval; HbA1c, hemoglobin A1c; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HR, hazard ratio; IFN, interferon; LC, liver cirrhosis; SVR, sustained virological response; T2DM, type 2 diabetes mellitus; TAI, total alcohol intake.

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With this background in mind, the present study was initiated to investigate the cumulative incidence and risk factors of malignancies, including HCC after prolonged follow-up in HCV patients treated with interferon (IFN) monotherapy or combination therapy of IFN and ribavirin. The strengths of the current study are the large numbers of patients included and the long-term follow-up of patients.

Patients and Methods

Patients. The number of patients who were diagnosed with chronic HCV infection and treated for the first time with IFN monotherapy or combination therapy between September 1990 and March 2009 in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan, was 7,205. Of these, 4,302 patients met the following enrollment criteria: (1) no evidence of malignancies by physical examination, biochemical tests, abdominal ultrasonography, gastrofiberscope (or gastrography), or chest X-ray (or computed tomography); (2) features of chronic hepatitis or cirrhosis diagnosed via laparoscopy and/or liver biopsy within 1 year before the initiation of IFN therapy; (3) positivity for serum HCV-RNA before the initiation of IFN therapy; (4) period of $\geq$1 month to $\leq$1 year of IFN therapy; (5) negativity for hepatitis B surface antigens, antibody to hepatitis B core, or antimitochondrial antibodies in serum, as determined by radioimmunoassay, enzyme-linked immunosorbent assay, or indirect immunofluorescence assay; (6) age of $\geq$30 years to $\leq$80 years; (7) no underlying systemic disease, such as systemic lupus erythmatosus or rheumatic arthritis; and (8) repeated annual examinations during follow-up. Annual examinations included biochemical tests, tumor marker (carcinoembryonic antigen, alpha-fetoprotein, and prostate-specific antigen [only in men]), and abdominal ultrasonography. Patients with were excluded from the study if they had illnesses that could seriously reduce their life expectancy or if they had a history of carcinogenesis.

The primary outcome was the first development of malignancy. The development of malignancies was diagnosed by clinical symptoms, tumor marker, imaging (ultrasonography, computed tomography, or magnetic resonance imaging), and/or histological examination.[9-15] All of the studies were performed retrospectively by collecting and analyzing data from the patient records. The physicians in charge explained the purpose, method, and side effects of IFN therapy to each patient and/or the patient's family. In addition, the physicians in charge received permission for the use of serum stores and future use of stored serum. Informed consent for IFN therapy and future use of stored serum was obtained from all patients. The study was approved by the Institutional Review Board of our hospital.

Medical Evaluation. Body weight was measured in light clothing and without shoes to the nearest 0.1 kg. Height was measured to the nearest 0.1 cm. Height and weight were recorded at baseline, and body mass index was calculated as kg/m^2. All patients were interviewed by physicians or nurse staff in the Toranomon Hospital using a questionnaire that gathered information on demographic characteristics, medical history, and heath-related habits, including questions on alcohol intake and smoking history.

The value for hemoglobin A_{1C} (HbA_{1C}) was estimated as a National Glycohemoglobin Standardization Program equivalent value (%). Patients were defined as having T2DM when they had a fasting plasma glucose level of $\geq$126 mg/dL and/or HbA_{1C} level of $\geq$6.5%.[16]

Patients were regarded as hypertensive when systolic blood pressure was $\geq$140 mm Hg and/or diastolic blood pressure was $\geq$90 mm Hg for at least three visits. Smoking index (packs per day $\times$ year) and total alcohol intake (TAI) were evaluated by the sum of before, during, and after the IFN therapy.

Laboratory Investigation. Diagnosis of HCV infection was based on detection of serum HCV antibody and positive RNA. Anti-HCV was detected using an enzyme-linked immunosorbent assay (ELISA II; Abbott Laboratories, North Chicago, IL). HCV genotype was examined via polymerase chain reaction assay, using a mixture of primers for the six subtypes known to exist in Japan, as reported.[17] HCV-RNA was determined using the COBAS TaqMan HCV test (Roche Diagnostics, Basel, Switzerland). The serum samples stored at $-80°C$ before IFN therapy were used. The linear dynamic range of the assay was 1.2-7.8 log IU/

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Potential conflict of interest: Dr. Suzuki is on the speakers' bureau of Bristol-Myers Squibb. Dr. Akuta is on the speakers' bureau of MSD and holds intellectual property rights with SRL. Dr. Kumada is on the speakers' bureau of MSD, Mitsubishi Tanabe Pharma, Dainippon Sumitomo Pharma, Bristol-Myers Squibb. He also holds intellectual property rights with SRL. Dr. Arase is on the speakers' bureau of MSD.

Table 1. Clinical Backgrounds at Initiation of Follow-up in Enrolled Patients

Variable	Total	HCC Group	Non-HCC Malignancy Group	Without Events Group	P
No. of patients	4,302	393	213	3,696	
Age, years	52.0 ± 11.8	55.8 ± 7.9	57.9 ± 9.1	51.3 ± 12.1	<0.001
Sex, male/female	2528/1774	272/121	129/84	2127/1569	<0.001
Height, cm	163.0 ± 9.2	162.8 ± 8.3	163.3 ± 9.1	163.0 ± 9.3	0.772
Weight, kg	61.4 ± 13.0	62.3 ± 10.6	60.8 ± 10.1	61.3 ± 13.4	0.142
BMI	23.0 ± 4.0	23.4 ± 3.0	22.8 ± 2.8	23.0 ± 4.1	0.012
Blood pressure, mm Hg					
Systolic	128 ± 18	132 ± 19	133 ± 20	127 ± 17	<0.001
Diastolic	77 ± 13	80 ± 12	80 ± 13	77 ± 13	<0.001
TAI, kg*	95 ± 92	151 ± 101	135 ± 81	85 ± 89	<0.001
Smoking index*	6.4 ± 9.4	10.8 ± 11.1	12.5 ± 11.8	5.5 ± 8.7	<0.001
AST, IU/L	42 ± 44	64 ± 55	42 ± 31	40 ± 42	<0.001
ALT, IU/L	44 ± 53	72 ± 63	43 ± 43	42 ± 52	<0.001
GGT, IU/L	54 ± 61	63 ± 65	56 ± 45	53 ± 38	0.007
Albumin, g/dL	4.1 ± 0.3	4.1 ± 0.3	4.1 ± 0.2	4.1 ± 0.2	0.310
Triglyceride, mg/dL	101 ± 53	104 ± 54	105 ± 50	100 ± 52	0.329
Cholesterol, mg/dL	170 ± 32	165 ± 31	169 ± 33	171 ± 32	0.025
FPG, mg/dL	100 ± 22	110 ± 26	104 ± 22	98 ± 21	<0.001
HbA1c, %, NSPG	5.6 ± 1.2	5.9 ± 1.4	5.7 ± 1.4	5.5 ± 1.1	<0.001
T2DM, +/−	267/4,035	63/330	34/179	170/3,526	<0.001
Platelet count, $\times 10^4/mm^3$	17.1 ± 5.1	13.7 ± 4.9	16.5 ± 5.4	17.5 ± 5.4	<0.001
Staging, LC/non-LC	433/3,869	113/285	27/189	293/3,395	<0.001
HCV genotype, 1b/2a/2b/other	2,721/995/458 /128	283/52/20/38	121/62/18/12	2,317/881/420/78	<0.001
HCV RNA, log IU/mL	6.06 ± 1.05	6.22 ± 0.52	6.05 ± 0.86	6.04 ± 1.05	0.003
IFN monotherapy†/combination therapy‡	2,861/1,441	358/35	175/38	2,328/1,368	<0.001
Efficacy, SVR/non-SVR	1,900/2,402	44/349	88/125	1,768/1,928	<0.001

Data are presented as no. of patients or mean ± SD.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; F, female; FPG, fasting plasma glucose; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein; M, male; NGSP, National Glycohemoglobin Standardization Program.

*Smoking index is defined as packs per day × year. TAI and smoking index indicate the sum before and after first consultation.

†Outbreak of IFN monotherapy: recombinant IFN-α2a, n = 220, recombinant IFN-α2b, n = 183, natural IFN-α, n = 1,678, natural IFN-α, n = 691, total dose of IFN = 560 ± 164 megaunit. Outbreak of pegylated IFN monotherapy: pegylated IFN-α2a, n = 89, total dose of pegylated IFN = 7.52 ± 2.24 mg.

‡Outbreak of combination therapy: recombinant IFN-α2b + ribavirin, n = 335, total dose of IFN = 508 ± 184 megaunit, total dose of ribavirin = 160 ± 68 g; natural IFN-β + ribavirin, n = 101, total dose of IFN = 502 ± 176 megaunit, total dose of ribavirin = 156 ± 67 g; pegylated IFN-α2b+ribavirin, n = 1,005 cases, total dose of pegylated IFN = 4.14 ± 1.10 mg, total dose of ribavirin = 206 ± 58 g.

mL, and the undetectable samples were defined as negative. A sustained virological response (SVR) was defined as clearance of HCV-RNA using the COBAS TaqMan HCV test 6 months after the cessation of IFN therapy.

Evaluation of Liver Cirrhosis. Status of liver was mainly determined on the basis of peritoneoscopy and/or liver biopsy. Liver biopsy specimens were obtained using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style; Kakinuma Factory, Tokyo, Japan), fixed in 10% formalin, and stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. The size of specimens for examination was more than six portal areas.[18]

Follow-up. The observation starting point was 6 months after the termination of IFN therapy. After that, patients were followed up at least twice a year in our hospital. Physical examination and biochemical tests were conducted at each examination together with a regular checkup. In addition, annual examinations during follow-up were undertaken. When a patient had complaints during follow-up, the physician in charge performed additional examinations based on symptoms. Four hundred eighteen patients were lost to follow-up. The final date of follow-up in 418 patients with loss of follow-up was regarded as the last consulting day. In addition, 881 patients were retreated with IFN. The final date of follow-up in 881 patients re-treated with IFN were regarded as the time of the initiation of IFN retreatment. Thus, 418 patients with loss of follow-up and 881 patients retreated with IFN were counted censored data in statistical analysis.[19] The mean follow-up period was 6.8 (SD 4.3) years in 418 patients with loss of follow-up and 7.5 (SD 4.8) years in 881 patients retreated with IFN. Censored patients were counted in the analysis.

Statistical Analysis. Clinical differences among three groups of patients with HCC with malignancies other than HCC without events were evaluated using the Kruskal-Wallis test. The cumulative development rates of malignancies were calculated using the Kaplan-Meier technique, and differences in the curves were

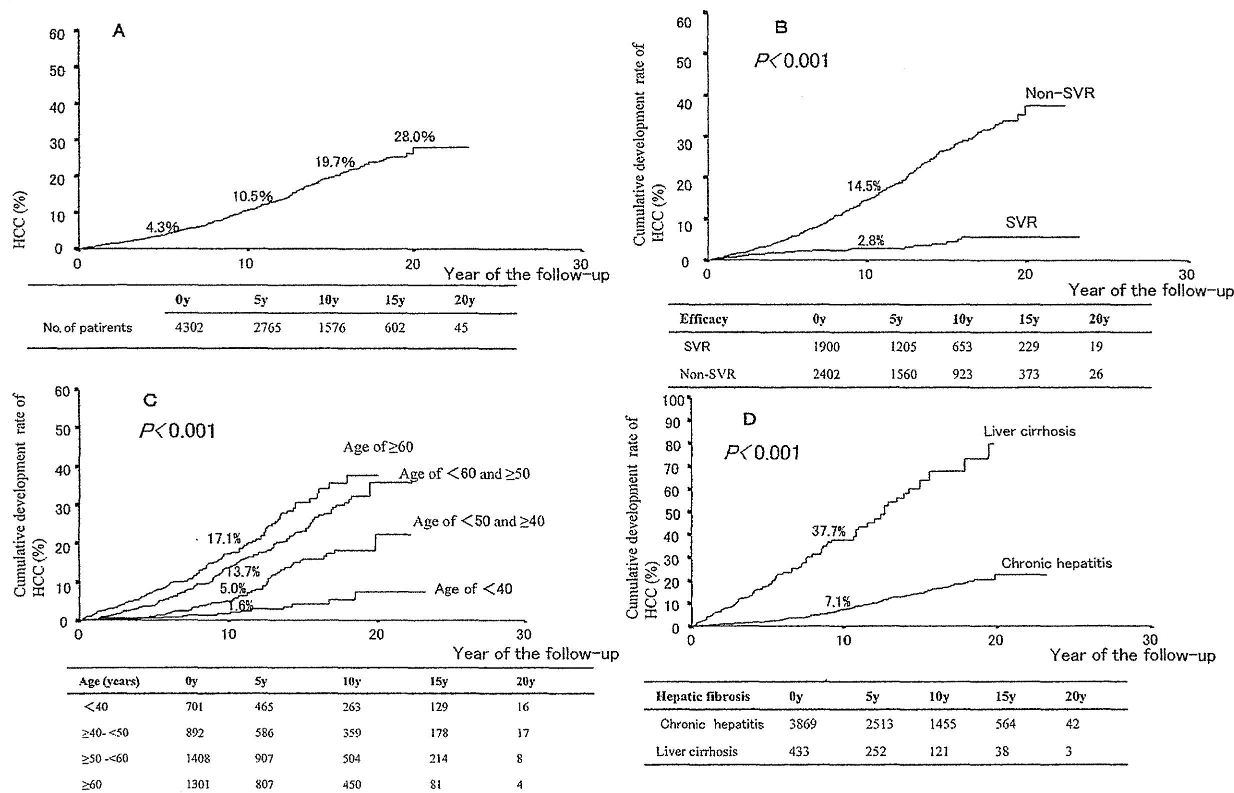

Fig. 1. Cumulative development rate of HCC (A) in total HCV patients treated with IFN therapy and based on the difference of (B) efficacy, (C) age, and (D) hepatic fibrosis.

tested using the log-rank test.[20,21] Independent risk factors associated with malignancies were studied using the stepwise Cox regression analysis.[22] The following variables were analyzed for potential covariates for incidence of primary outcome: (1) age, sex, T2DM, and hypertension at the initiation time of follow-up; (2) HCV genotype, HCV load, and hepatic fibrosis before IFN therapy; (3) average value of body mass index, aspartate aminotransferase, alanine aminotransferase, triglyceride, total cholesterol, and platelet count during follow-up; (4) sum value of smoking and alcohol before, during, and after the IFN therapy; and (5) efficacy of IFN therapy, combination of ribavirin, type of IFN, and total dose of IFN. A $P < 0.05$ was considered statistically significant. Data analysis was performed using SPSS 11.5 for Windows (SPSS, Chicago, IL).

Results

Patient Characteristics. Table 1shows the baseline characteristics of the 4,302 enrolled patients at initiation of follow-up. The patients were divided into three groups: with HCC, with malignancies other than HCC, and without events. There were significant differences in several baseline characteristics among the three groups. The SVR rate was 34.4% (985/2,861) in IFN monotherapy and 63.5% (915/1,441) in combination therapy of IFN and ribavirin. Thus, the number of patients with SVR was 1,900. The mean follow-up was 8.1 (SD 5.0) years.

Development and Breakdown of Malignancies. As shown in Table 1, 606 of 4,302 patients developed malignancies: 393 developed HCC and 213 developed malignancies other than HCC. HCC accounted for 33.3% (44/132) of malignancies in patients with SVR and 73.6% (349/474) in patients without SVR. The breakdown of malignancies other than HCC was as follows: stomach cancer, n = 36; colon cancer, n = 35; lung cancer, n = 20; malignant lymphoma, n = 19; pancreatic cancer, n = 12; prostatic cancer, n = 16; breast cancer, n = 15; other cancers, n = 60.

Predictive Factors for the Development of HCC. The cumulative development rate of HCC was 4.3% at 5 years, 10.5% at 10 years, 19.7% at 15 years, and 28.0% at 20 years (Fig. 1A). The factors associated with the development of HCC are shown in Table 2. Multivariate Cox proportional hazards analysis

Table 2. Predictive Factors for Development of HCC in Enrolled Patients

Variable	Univariate Analysis		Cox Regression Analysis	
	HR (95% CI)	P	HR (95% CI)	P
Age, years (per 10)	1.84 (1.64-2.06)	<0.001	1.97 (1.71-2.28)	<0.001
Sex, male/female	1.47 (1.18-1.83)	<0.001	1.67 (1.24-2.23)	0.001
BMI, ≥22/<22	1.37 (1.12-1.66)	0.002		
T2DM, +/−	2.77 (2.13-3.60)	<0.001	1.73 (1.30-2.30)	<0.001
Hypertension, +/−	1.32 (1.02-1.71)	0.036		
Smoking index, ≥20/<20*	1.43 (1.14-1.79)	0.002		
TAI, kg, ≥200/<200*	2.13 (1.74-2.61)	<0.001	1.45 (1.11-1.88)	0.007
AST, IU/L, ≥34/<34	3.00 (2.40-3.89)	<0.001		
ALT, IU/L, ≥36/<36	2.74 (2.16-3.42)	<0.001		
GGT, IU/L, ≥109/<109	1.79 (1.19-2.46)	0.039		
Albumin, g/dL, <3.9/≥3.9	1.92 (1.37-2.55)	0.015		
Triglyceride, mg/dL, ≥100/<100	1.14 (0.94-1.37)	0.179		
Cholesterol, mg/dL, <150/≥150\	1.38 (1.10-1.72)	0.004		
Platelet count, $\times 10^4$/mm^3, <15/≥15)	3.27 (2.56-4.17)	<0.001		
Histological diagnosis, LC/non-LC	7.09 (5.59-9.01)	<0.001	5.01 (3.92-6.40)	<0.001
Combination of ribavirin, +/−	0.66 (0.45-0.97)	0.033		
Type of IFN, α/β	1.10 (0.85-1.41)	0.474		
Total dose of IFN, MU, ≥500/<500	1.12 (0.91-1.38)	0.291		
HCV genotype, ½	1.67 (1.30-2.14)	<0.001		
HCV-RNA, log IU/mL, ≥5/<5	1.02 (0.98-1.05)	0.315		
Efficacy, non-SVR/SVR	4.78 (3.47-6.59)	<0.001	4.93 (3.53-6.89)	<0.001

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein.

*Smoking index is defined as packs per day × year. TAI and smoking index indicate the sum before and after first consultation.

showed that HCC occurred when patients had liver cirrhosis (hazard ratio [HR], 5.01; 95% confidence interval [CI], 3.92-6.40; $P < 0.001$), non-SVR (HR, 4.93; 95% CI, 3.53-6.89; $P < 0.001$), age increments of 10 years (HR, 1.97; 95% CI, 1.71-2.28; $P < 0.001$), T2DM (HR, 1.73; 95% CI, 1.30-2.30; $P < 0.001$), male sex (HR, 1.67; 95% CI, 1.24-2.23; $P = 0.001$), and TAI of ≥ 200 kg (HR, 1.45; 95% CI, 1.11-1.88; $P = 0.007$). Fig. 1B-D and Fig. 2A-C show the cumulative development rates of HCC based on difference of IFN efficacy, age, hepatic fibrosis, TAI, sex, and T2DM. The 10-year cumulative rates of HCC after IFN therapy was determined to be 7.1% in 3,869 patients with chronic hepatitis and 37.7% in 433 patients with cirrhosis by using the Kaplan-Meier Method (Fig. 1D). Fig. 2D shows the development rates of HCC in T2DM patients according to difference of mean hemoglobin A1c (HbA1c) level during follow-up. HCC decreased when T2DM patients had a mean HbA1c level of <7.0% during follow-up (HR, 0.56; 95% CI, 0.33-0.89; $P = 0.015$). The development of HCC was reduced by 44% in T2DM patients with a mean HbA1c level of <7.0% compared with those with a mean HbA1c level of ≥7.0%.

Table 3 shows the development rate of HCC and risk factors in four groups classified by the difference of hepatic fibrosis and efficacy of IFN therapy. The development rate of HCC per 1,000 person years was 1.55 in patients with chronic hepatitis (CH) at baseline and SVR (CH+SVR), 18.23 in patients with liver cirrhosis (LC) at baseline and SVR (LC+SVR), 13.53 in patients with chronic hepatitis at baseline and non-SVR (CH+non-SVR), and 50.43 in patients with LC at baseline and non-SVR (LC+non-SVR). The risk of HCC development in the CH+SVR group was advanced age, male sex, TAI of ≥200 kg, and T2DM. T2DM enhanced the development of HCC with statistical significance in three groups of CH+SVR, CH+non-SVR, and LC+non-SVR.

Predictive Factors for Development of Malignancies Other than HCC. The cumulative development rate of malignancies other than HCC was 2.4% at 5 years, 5.1% at 10 years, 9.8% at 15 years, and 18.0% at 20 years (Fig. 3A). The factors associated with the development of malignancies other than HCC are shown in Table 4. Malignancies other than HCC occurred when patients had age increments of 10 years (HR, 2.19; 95% CI, 1.84-2.62; $P < 0.001$), smoking index of ≥20 (HR, 1.89; 95% CI, 1.41-2.53; $P < 0.001$), and T2DM (HR, 1.70; 95% CI, 1.14-2.53; $P = 0.008$). Fig. 3B-D shows the cumulative development rates of malignancies other than HCC based on difference of age, smoking index, and T2DM. Fig. 3E shows the risk of malignancies other than HCC in T2DM patients according to mean HbA1c level during follow-up. The HR of HCC development in

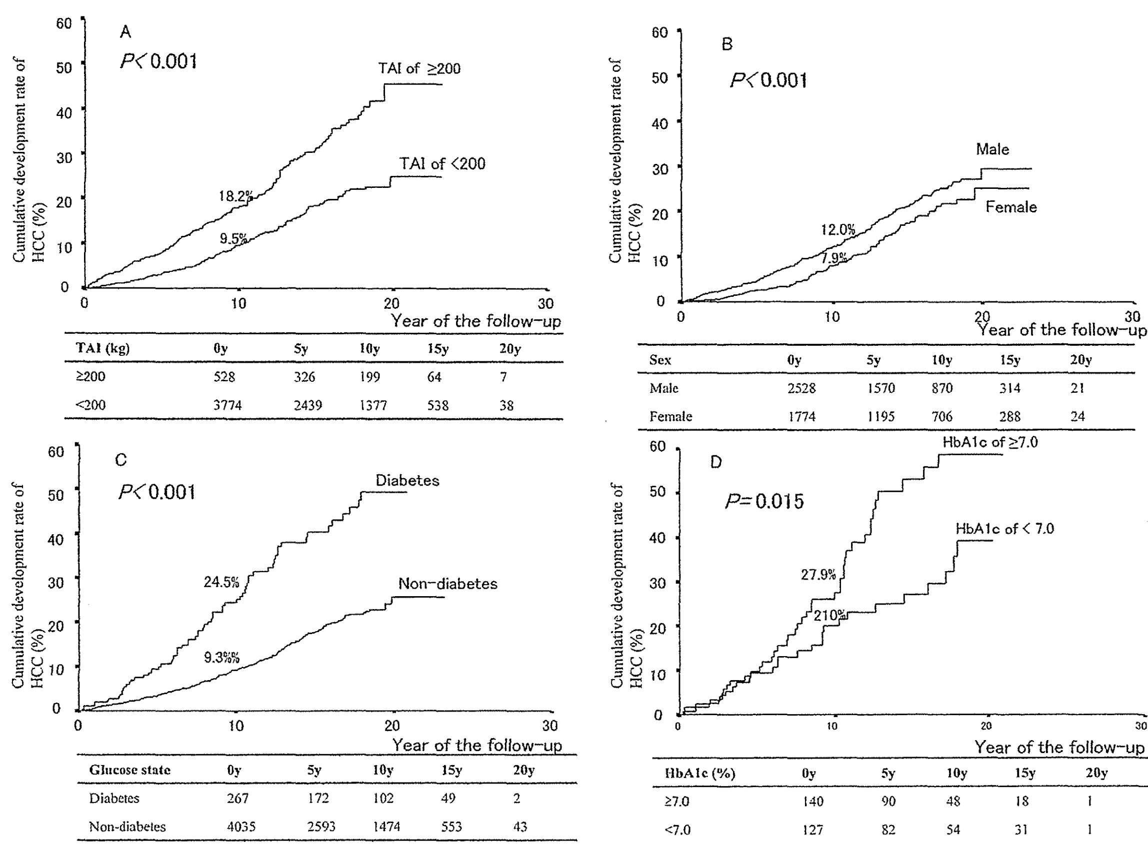

Fig. 2. Cumulative development rate of HCC based on the difference of (A) TAI, (B) sex, (C) diabetic state, and (D) mean HbA1c level during follow-up in T2DM patients.

patients with mean HbA1c level of <7.0% versus those with mean HbA1c level of ≥7.0% was 0.62 (95% CI, 0.31-1.23; $P = 0.170$). There was no significant difference in development of malignancies other than HCC based on the difference of mean HbA1c level during follow-up. Table 5 shows the impact based

Table 3. Development Rate of HCC Based on Hepatic Fibrosis and Efficacy of IFN Therapy

Variable	CH + SVR	LC + SVR	CH + Non-SVR	LC + Non-SVR
No. of patients	1,751	149	2,118	284
Age, years	51.7 ± 12.1	56.9 ± 9.8	51.5 ± 11.7	57.2 ± 9.9
Sex, male/female	1,082/669	91/58	1,190/928	165/119
HbA1c (%, NSPG)	5.5 ± 0.7	5.8 ± 0.8	5.7 ± 0.7	6.1 ± 0.8
TAI, kg	86 ± 91	104 ± 99	97 ± 90	129 ± 102
Patients with T2DM	74	13	133	47
Patients with HCC	22	22	233	116
1,000 person years of HCC	1.55	18.23	13.53	50.43
Age, years (per 10)*	2.60 (1.48-4.58)	1.83 (0.95-3.55)	2.07 (1.75-2.46)	1.09 (0.87-1.37)
P value	0.001	0.070	<0.001	0.477
Sex, male/female*	3.42 (1.01-11.63)	3.41 (1.00-11.63)	1.34 (0.99-1.81)	1.93 (1.25-3.00)
P value	0.049	0.050	0.058	0.003
TAI, kg, ≥200/<200*	2.68 (1.14-6.34)	3.84 (1.83-9.85)	2.21 (1.65-2.95)	1.54 (1.03-2.31)
P value	0.024	0.004	<0.001	0.038
T2DM, +/−*	4.76 (1.60-14.10)	2.48 (0.57-10.86)	2.53 (1.76-3.65)	1.87 (1.16-3.01)
P value	0.005	0.228	<0.001	0.010

Abbreviations: CH + Non-SVR, patients with CH at baseline and non-SVR 6 months after IFN therapy; CH + SVR, patients with CH at baseline and SVR 6 months after IFN therapy; LC + Non-SVR, patients with LC at baseline and non-SVR 6 months after IFN therapy; LC + SVR, patients with LC at baseline and SVR 6 months after IFN therapy.

*Hazard ratio (95% confidence interval) and *P* value by Cox proportional hazards analysis.

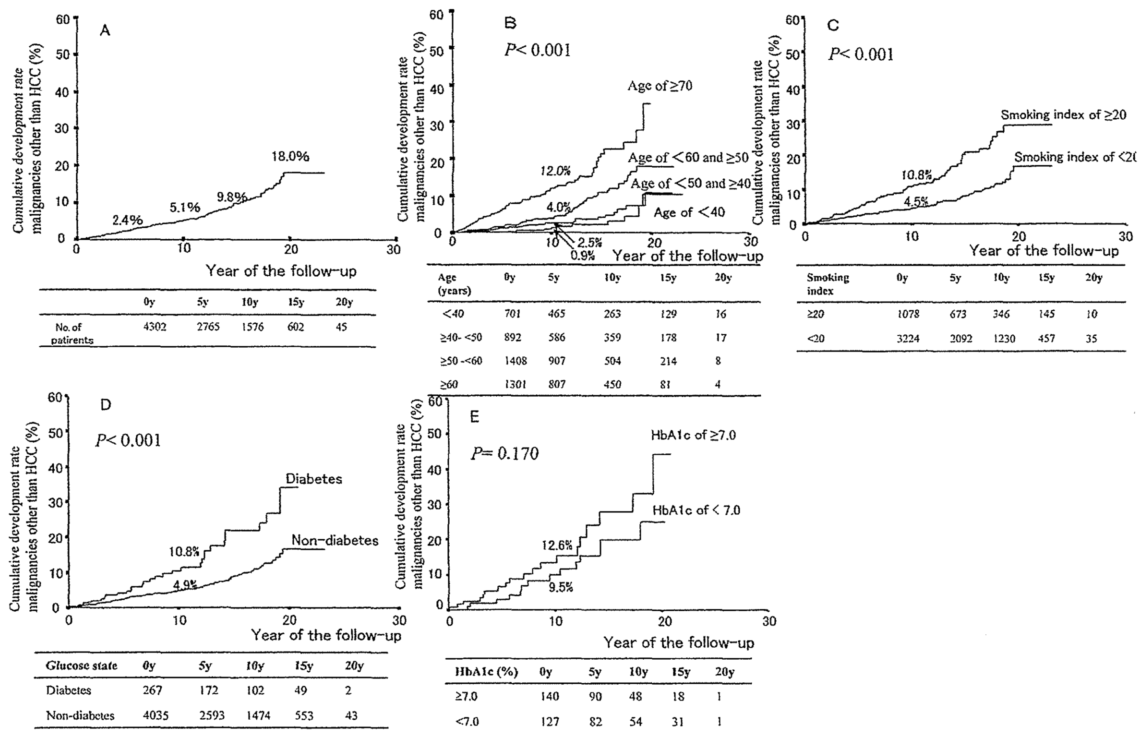

Fig. 3. Cumulative development rate of malignancies other than HCC (A) in total HCV patients treated with IFN therapy and based on the difference of (B) age, (C) smoking index, (D) diabetic state, and (E) mean HbA1c level during follow-up in T2DM patients.

on three factors of age, smoking index, and T2DM for the development of each malignancy other than HCC by using Cox regression analysis. Aging enhanced carcinogenesis of stomach, colon, lung, prostate, breast, and pancreas with statistical significance. Smoking enhanced lung cancer and colorectal cancer

Table 4. Predictive Factors for Development of Malignancies Other than HCC

	Univariate Analysis		Cox-Regression Analysis	
Variables	HR (95% CI)	P	HR (95% CI)	P
Age, years (per 10)	2.23 (1.88-2.65)	< 0.001	2.19 (1.84-2.62)	<0.001
Sex, male/female	1.06 (0.79-1.40)	0.759		
BMI, ≥22/<22	0.97 (0.75-1.24)	0.767		
T2DM, +/−	2.56 (1.76-3.72)	<0.001	1.70 (1.14-2.53)	0.008
Hypertension, +/−	2.33 (1.70-3.18)	<0.001		
Smoking index, ≥20/<20*	2.74 (2.06-3.65)	<0.001	1.89 (1.41-2.53)	<0.001
TAI, kg, ≥200/<200*	1.77 (1.33-2.37)	<0.001		
AST, IU/L, ≥34/<34	0.89 (0.65-1.20)	0.412		
ALT, IU/L, ≥36/<36	0.98 (0.72-1.34)	0.891		
GGT, IU/L, ≥109/<109	1.26 (0.79-2.01)	0.350		
Albumin, g/dL, <3.9/≥3.9	1.41 (0.90-2.04)	0.145		
Triglyceride, mg/dL, ≥100/<100	1.28 (1.03-1.60)	0.030		
Total cholesterol, mg/dL, <150/≥150	1.10 (0.82-1.46)	0.548		
Platelet count, × 10^4/mm^3, <15/≥15	1.39 (1.02-1.91)	0.038		
Histological diagnosis, LC/non-LC	1.77 (1.13-2.75)	0.012		
Combination of ribavirin, +/−	0.66 (0.44-0.97)	0.034		
Type of IFN, α/β	1.05 (0.75-1.47)	0.789		
Total dose of IFN, MU, ≥500/<500	1.31 (0.96-1.77)	0.084		
HCV genotype, ½	1.30 (0.80-2.93)	0.432		
HCV RNA, log IU/mL, ≥5/<5	0.89 (0.50-1.23)	0.612		
Efficacy, non-SVR/SVR	0.85 (0.64-1.12)	0.232		

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGT, gamma-glutamyl transferase.
*Smoking index is defined as packs per day × year. TAI and smoking index indicate the sum before and after first consultation.

Table 5. Impact Based on Age, Smoking Index, and Diabetes for Development of Malignancies Other than HCC

	Age, Years (per 10)		Smoking Index, $\geq$20/<20		Diabetes, +/−	
Malignancy	HR (95% CI)	P	HR (95% CI)	P	HR (95% CI)	P
Gastric cancer (n = 36)	2.48 (1.62-3.78)	<0.001	1.69 (0.83-3.43)	0.146	2.29 (0.95-5.52)	0.065
Colorectal cancer (n = 35)	1.91 (1.28-2.86)	0.002	2.27 (1.13-4.58)	0.022	1.78 (0.68-4.66)	0.240
Lung cancer (n = 20)	2.33 (1.35-4.01)	0.002	2.90 (1.25-6.74)	0.013	1.53 (0.45-5.24)	0.496
Prostatic cancer (n = 16)	2.84 (1.32-6.13)	0.008	1.89 (0.88-3.15)	0.266	0.71 (0.09-5.47)	0.735
Breast cancer (n = 15)	2.86 (1.30-6.29)	0.009	1.29 (0.17-10.19)	0.808	1.20 (0.16-9.39)	0.859
Malignant lymphoma (n = 19)	2.21 (1.26-3.88)	0.006	1.25 (0.44-3.56)	0.671	1.39 (0.32-6.12)	0.663
Pancreatic cancer (n = 12)	3.32 (1.44-7.65)	0.005	1.41 (0.45-4.82)	0.578	3.75 (1.02-13.88)	0.046

with statistical significance. In addition, T2DM enhanced the pancreatic cancer with statistical significance and tended to enhance the gastric cancer.

Discussion

This study describes the development incidence of HCC or malignancies other than HCC after the termination of IFN therapy in HCV patients. Patients at Toranomon Hospital comprised mainly government employees, office workers, and business persons. Most patients were regularly recommended to undergo annual multiphasic health screening examinations. In the present study, patients who had undergone annual multiphasic health screening examinations were enrolled. The strengths of the present study are a prolonged follow-up in the large numbers of patients included.

The present study shows several findings with regard to the development incidence and predictive factors for total malignancies after IFN therapy for HCV patients. First, the 10-year cumulative rates of HCC after IFN therapy was determined to be 7.1% in 3,869 patients with chronic hepatitis and 37.7% in 433 patients with cirrhosis using the Kaplan-Meier method. Our previous studies showed via retrospective analysis that the 10-year cumulative rates of HCC were 12.4% for 456 patients with chronic hepatitis and 53.2% for 349 patients with cirrhosis.[7,23] Although patient selection bias for IFN treatment versus no treatment had been noted in the previous studies, the results suggest the possibility that IFN therapy reduces the development of HCC in HCV patients. Several historical data in Japan suggest that IFN therapy reduces the development of HCC in HCV patients.[24-26]

Second, HCC occurred with statistical significance when the following characteristics were present: non-SVR, advanced age, cirrhosis, TAI of $\geq$200 kg, male sex, and T2DM. T2DM caused a 1.73-fold enhancement in HCC development. Several authors have reported an increased risk of HCC among patients with the following characteristics: non-SVR, cirrhosis, male sex, advanced age, and T2DM.[24-28] Our results show that physicians in charge of aged male patients with non-SVR, advanced fibrosis, TAI of $\geq$200 kg, and T2DM should pay attention to the development of HCC after IFN therapy. In addition, maintaining a mean HbA1c level of <7.0% during follow-up reduced the development of HCC. This result indicates that stringent control of T2DM is important for protecting the development of HCC.

Third, the development rate of HCC per 1,000 person years was about 1.55 in 1,751 patients with chronic hepatitis at baseline and SVR. In these patients, the risk factors associated with HCC were advanced age, male sex, TAI, and T2DM. We compared the HCC development rate in patients with chronic hepatitis at baseline and SVR to the general population. A total of 5,253 individuals without HCV antibody and hepatitis B surface antigen, who underwent annual multiphasic health screening examinations in our hospital were evaluated as controls. Individuals with either of the following criteria were excluded: (1) illness that could seriously reduce their life expectancy or (2) history of carcinogenesis. They were selected by matching 3:1 with patients who had chronic hepatitis at baseline and SVR for age, sex, T2DM, and follow-up periods. In control individuals, the mean age was 51.7 years; the prevalence (number) of male patients was 61.8% (3,246); the prevalence (number) of T2DM patients was 4.2% (222); the mean follow-up period was 8.0 years. The number of development of HCC in control individuals was only five. This result suggests that the development rate of HCC in patients with chronic hepatitis at baseline and SVR is higher than that in the general population.

Fourth, HCC accounted for 33.3% in SVR patients and 73.6% in non-SVR patients. According to Matsuda et al.,[29] the outbreak of malignancies in the Japanese male population was observed in the following order in 2005: gastric cancer 20.4% > colon

cancer 16.0% > lung cancer 15.4% > prostate cancer 10.9% > HCC 7.4%. On the other hand, the outbreak of malignancies in the Japanese female population was observed in the following order in 2005: breast cancer 18.0% > colon cancer 16.2% > gastric cancer 13.6% > lung cancer 9.3% > uterine cancer 6.8%. Our results show that HCC is the most common cause of malignancy, not only in the non-SVR group but also in the SVR group.

Finally, malignancies other than HCC occurred with statistical significance when patients were of advanced age, were smokers, and had T2DM. Our result indicates that smoking enhances lung cancer and colorectal cancer. Many authors have reported that smoking is a direct cause of cancers of the oral cavity, esophagus, stomach, pancreas, larynx, lung, bladder, kidney, and colon.[30,31] In addition, the present study indicates that T2DM enhances pancreatic cancer with statistical significance and tends to enhance gastric cancer. T2DM showed up to about 1.7-fold increase in development of malignancies other than HCC. A recent meta-analysis of cohort studies have revealed that diabetic patients increase risk of pancreatic cancer, HCC, bladder cancer, non-Hodgkin's lymphoma, colorectal cancer, and breast cancer.[32-39]

Although the role of T2DM in carcinogenesis remains speculative, the following possible mechanisms have been reported: (1) hyperglycemia increases malignancy risk via increasing oxidative stress and/or activating the rennin-angiotensin system[40]; (2) insulin resistance increases malignancy risk via down-regulation of serine/threonine kinase II to adenosine monophosphate–activated protein kinase pathway[41]; (3) reduced insulin secretion increases malignancy risk via down-regulation of sterol regulatory element-binding protein-1c with consequent up-regulation of insulin-like growth factor.[42]

T2DM is increasing dramatically worldwide over the past decades.[8] It is estimated that about 7 million people are affected by diabetes mellitus in Japan. Approximately 8%-10% of adults in Japan have T2DM. The risk factors associated with T2DM include family history, age, sex, obesity, smoking, physical activity, and HCV.[43-46] In the near future, T2DM will be increasing in HCV-positive patients.

This study is limited in that it was a retrospective cohort trial. Another limitation is that patients were treated with different types of antivirus therapy for different durations. In addition, T2DM patients were treated with different types of drugs during follow-up. Finally, our cohort contains Japanese subjects only. On the other hand, the strengths of the present study are a long-term follow-up in the large numbers of patients included.

In conclusion, T2DM causes an approximately 1.7-fold enhancement in the development of HCC and malignancies other than HCC after IFN therapy. Additionally, in T2DM patients, maintaining a mean HbA1c level of <7.0% during follow-up reduced the development of HCC.

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Research Article

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Characterization of virologic escape in hepatitis C virus genotype 1b patients treated with the direct-acting antivirals daclatasvir and asunaprevir

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Background & Aims: Daclatasvir and asunaprevir are NS5A and NS3 protease-targeted antivirals currently under development for treatment of chronic hepatitis C virus infection. Clinical data on baseline and on-treatment correlates of drug resistance and response to these agents are currently limited.

Methods: Hepatitis C virus genotype 1b Japanese patients (prior null responders to PegIFN-α/RBV [n = 21] or PegIFN-α/RBV ineligible or intolerant [n = 22]) were administered daclatasvir/asunaprevir for 24 weeks during a phase 2a open-label study. Genotypic and phenotypic analyses of NS3 and NS5A substitutions were performed at baseline, after virologic failure, and post-treatment through follow-up week 36.

Results: There were three viral breakthroughs and four relapsers. Baseline NS3 polymorphisms (T54S, Q80L, V170M) at amino acid positions previously associated with low-level resistance (<9-fold) to select NS3 protease inhibitors were detected in four null responders and three ineligibles, but were not associated with virologic failure. Baseline NS5A polymorphisms (L28M, L31M, Y93H) associated with daclatasvir resistance (<25-fold) were detected in five null responders and six ineligibles. All three viral breakthroughs and 2/4 relapsers carried a baseline NS5A-Y93H polymorphism. NS3 and NS5A resistance-associated variants were detected together (NS3-D168A/V, NS5A-L31M/V-Y93H) after virologic failure. Generally, daclatasvir-resistant substitutions persisted through 48 weeks post-treatment, whereas asunaprevir-resistant substitutions were no longer detectable. Overall, 5/10 patients with baseline NS5A-Y93H experienced virologic failure, while 5/10 achieved a sustained virologic response.

Conclusions: The potential association of a pre-existing NS5A-Y93H polymorphism with virologic failure on daclatasvir/asunaprevir combination treatment will be examined in larger studies. The persistence of treatment-emergent daclatasvir- and asunaprevir-resistant substitutions will require assessment in longer-term follow-up studies.

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Introduction

The introduction of direct-acting antivirals (DAAs) targeting hepatitis C virus (HCV) NS3 protease activity has substantially increased sustained virologic response (SVR) in chronic HCV genotype 1 (GT1) infection. In combination with peginterferon-alfa and ribavirin (PegIFN-α/RBV), treatment with the recently approved protease inhibitors boceprevir or telaprevir results in SVR rates of around 70–75% in treatment-naive patients [1,2]. Despite these improvements, SVR rates vary by genotype and remain suboptimal in some patients, such as null responders to PegIFN-α/RBV [3], and patients for whom PegIFN-α/RBV is poorly tolerated or medically contraindicated. Furthermore, PegIFN-α/RBV is associated with frequent side effects [3], and the addition of these DAAs results in elevated rates of anemia and additional events such as dysgeusia (boceprevir), or rash, pruritus, and nausea (telaprevir) [4,5].

Daclatasvir (DCV) and asunaprevir (ASV) are currently undergoing clinical development for HCV infection. DCV (BMS-790052) is a first-in-class, highly selective NS5A replication complex inhibitor with picomolar potency and broad HCV genotypic coverage [6] that has demonstrated antiviral efficacy and good tolerability in combination with PegIFN-α/RBV [7]. ASV (BMS-650032) is a selective inhibitor of NS3 protease with antiviral activity *in vitro* against GT1 and GT4 [8]; it has also been shown to be

Keywords: Asunaprevir; Daclatasvir; Drug resistance; Direct-acting antivirals; Hepatitis C; Peginterferon-sparing.

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Abbreviations: DAA, direct-acting antiviral; HCV, hepatitis C virus; SVR, sustained virologic response; GT, genotype; PegIFN-α/RBV, peginterferon alfa and ribavirin; DCV, daclatasvir; ASV, asunaprevir; LLOQ, lower limit of quantitation; PCR, polymerase chain reaction; FU, follow-up; RAV, resistance-associated variant; BL, baseline; VBT, viral breakthrough; SD, standard deviation.