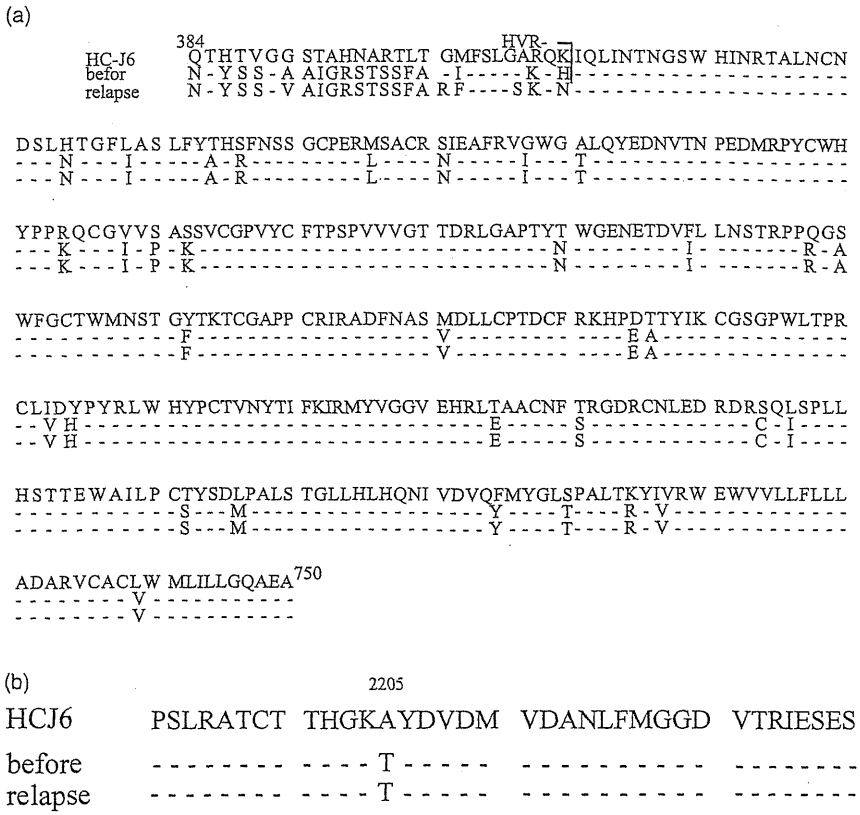

Figure 2 (a) Comparison of the hepatitis C virus (HCV) E2 (top) region amino acid sequences. Sequencing was performed for serum samples obtained before therapy on 3 February 2006 and after relapse on 25 January 2008. Sequences were aligned against the HCJ6 HCV genotype 2a reference sequence (GenBank accession no. D00944). Hypervariable region (HVR)1 positions of E2 are indicated by numbers corresponding to the amino acid positions within the HCV genotype 2a polyprotein of the reference sequence. There were five amino acid mutations in these regions between the two samples. (b) IFN sensitivity-determining region (ISDR) sequences before therapy and after relapse showed the same single mutation at codon 2205. Sequences were aligned against the HCJ6 HCV genotype 2a reference sequence (GenBank accession no. D00944). ISDR positions are indicated as numbers corresponding to the amino acid positions within the HCV genotype 2a polyprotein of the reference sequence.

The first PCR primer sequences for ISDR were: sense (6866, 6885) 5′-ACGTCCATGCTAACAGACCC-3′ and antisense (7185, 7166) 5′-GGGAATCTCTTCTTGGG GAG-3′. The second PCR primer sequences for ISDR were the sense primer from the first-round PCR and a new antisense primer (7109, 7090) 5′-CGAGAG AGTCCAGAACGACC-3′.[7]

Polymerase chain reaction products were separated by electrophoresis on 1% or 2% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light. The products were purified and sequenced with second-round PCR primers, using a dye terminator sequencing kit (BigDye Terminator ver. 1.1 cycle sequencing kit; Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 genetic analyzer (Applied Biosystems). Sequence alignments and phylogenetic analyses were performed with MEGA4 software.[8] Nucleotide sequences obtained from the two samples were compared to 23 published HCV genotype 2a sequences. A phylogenetic tree was constructed by the neighbor-joining method[9] based on the nucleotide sequence of the E2 region, with pairwise distances being estimated using the Kimura two-parameter method. Bootstrap values were determined on 1000 re-samplings of data sets.[10]

The E2 nucleotide sequences before treatment and after relapse were 98.6% similar. Except HVR1, two of the samples were 99.0% similar. The amino acid sequences in the E2 region, except HVR1, were identical between the two samples. There was a difference of five amino acids in HVR1 (Fig. 2a).

When compared to known HCV isolates of various genotypes whose entire coding region sequence has

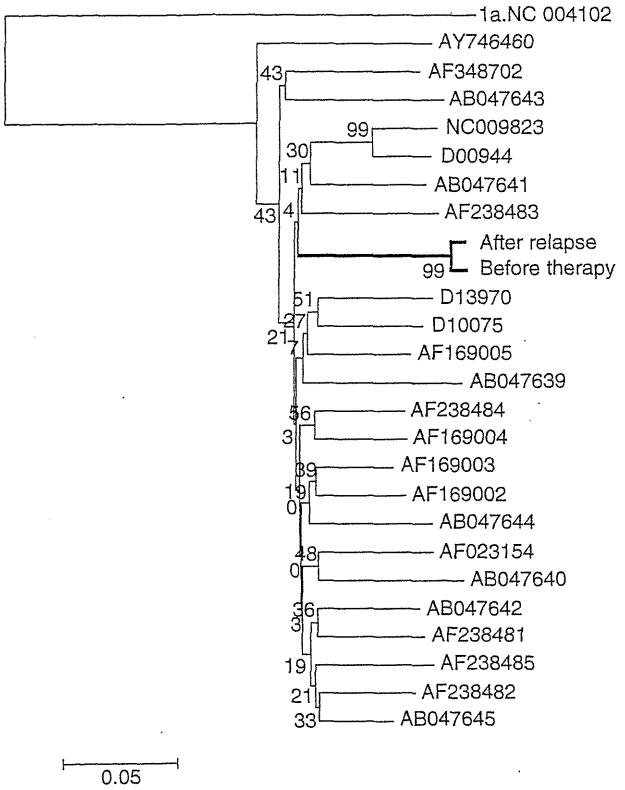

Figure 3 A phylogenetic tree was constructed by the neighbor-joining method based on the nucleotide sequence of the E2 region (1101 nt) of 23 genotype 2a strains using the genotype 1a hepatitis C virus (HCV) isolate (NC004102) as an outgroup. The isolates obtained in the present study before therapy and after relapse are indicated in bold letters for clarity. Twenty-three reported genotype 2a HCV isolates, whose entire coding region sequence is known, are included for comparison, and their accession numbers are shown. Bootstrap values were determined on 1000 re-samplings of datasets.

been determined, the two isolates were the closest to a particular genotype 2a HCV isolate (accession no. AB047645) with 87.3% nucleotide sequence similarity; however, the isolates were only 64.4–72.7% similar to known genotype non 2a (1a NC004102, 1b D90208, 2b D01221, 3a D17763, 4a Y11604, 5a Y13184 and 6a Y12083). The phylogenetic tree of 23 genotype 2a HCV isolates, constructed based on the E2 1101-nucleotide sequence, indicated that the sample obtained after relapse bifurcated from a common trunk with the sample before treatment, and we confirmed that these two samples were the closest to each other among all known genotype 2a HCV isolates (Fig. 3). The results of sequencing analysis before therapy and after re-emergence of viremia ruled out the possibility of a re-infection and strongly suggested a late relapse of chronic hepatitis C.

Interferon sensitivity-determining region sequences before treatment and after relapse showed 98.9% similarity. The amino acid sequences of the two ISDR regions were completely identical. The sequences of the HCJ6 (accession no. D00944) strain were defined as wild-type ISDR, and those that deviated from this strain were defined as the mutant type. ISDR sequences before treatment and after relapse were different in only one codon (2205) when compared with the reference HCJ6 sequence (Fig. 2b).

DISCUSSION

IN THIS STUDY, we clarified that E2 1101-nucleotide sequences of HCV isolated from sera before treatment and after relapse shared a 98.6% homology. Furthermore, phylogenetic analyses classified these two samples as the same strain. These results ruled out the possibility of a re-infection and strongly suggested a late relapse of chronic hepatitis C.

Hepatitis C virus is an RNA virus belonging to the genus *Hepacivirus* in the Flaviviridae family. Similar to other RNA viruses, HCV circulates as a genetically distinct population, demonstrating a quasispecies.[11] HCV HVR1, which is composed of 27 amino acids and is located at the 5′ terminus of the E2 gene, is highly variable among and within infected patients,[12–14] so it can be used to identify individual HCV isolates.[15,16] HCV HVR1 changes rapidly over time in the same individual. Our pairwise sequences were not completely identical but shared a high homology, which was equal to the homology reported previously.[16] These results suggest that the patient achieved SVR but suffered a relapse of hepatitis C after 1.5 years.

Some reports have indicated that in a majority of patients with SVR, low-level HCV RNA can be detected in lymphocytes, monocytes/macrophages and liver, despite constantly undetectable HCV RNA in sera.[17–19] This "occult" persistence of HCV replication could potentially play a role in late recurrence after treatment. However, the significance/mechanism of HCV RNA persistence in the liver or peripheral blood mononuclear cells is still uncertain, and data regarding occult persistence are conflicting.[20] Moreover, it is unclear as to how many of these late relapse patients were "true" relapsers and how many were re-infected. The relapse rates after SVR in IFN monotherapy are approximately 5–10%.[21,22] Nakayama *et al.* recently reported a late relapse of

hepatitis C after IFN-α plus ribavirin therapy and summarized late relapsing cases in Japan.[23] They indicated that compared to reports from foreign countries, late relapses were very rare in Japan, particularly after IFN and ribavirin therapy and that the relapse interval was principally restricted to within 2 years after therapy completion. Four hundred and fifty-five chronic hepatitis C patients were cured by PEG-IFN plus ribavirin therapy in the study group of Kyoto Prefectural University of Medicine and related hospitals, and this is the only case of late relapse to date (Itoh Y. *et al.*, 2009 unpublished data). This may be the first reported case of relapse after SVR with PEG-IFN plus ribavirin therapy.

Several host and viral characteristics are associated with the likelihood of response to IFN-based therapy. The HCV genotype and viral load are the most important viral predictors, and the ISDR sequence variation[24] and substitutions of amino acids 70 and/or 91 in the core region[25] within the HCV genome have been recently advocated in patients with genotype 1. It is interesting to note that only one amino acid varied in ISDR compared to the reference sequence in our case. For patients with HCV genotype 2a, Hayashi *et al.* reported that ISDR amino acid variations compared to the reference sequence and RVR as well as negative HCV at 4 weeks are important predictors of SVR in PEG-IFN monotherapy.[7] ISDR interacts with interferon-inducible double-stranded RNA-activated protein kinase (PKR) and inactivates HCV replication *in vitro*.[26] According to the report by Hayashi *et al.*,[7] an A-to-T mutation at codon 2205 (Fig. 2b) can be interpreted as wild type, and hence ISDR in this case contained no mutations, which may have influenced HCV RNA re-emergence after achieving SVR.

Patients with RVR, defined as a negative HCV RNA at 4 weeks, are more likely to have SVR.[7,27] In our case, HCV RNA was negative at 4 weeks, which indicated that this case may be cured; however, relapse of hepatitis C occurred after 1.5 years. The data concerning the efficacy of re-treatment of genotype 2 chronic hepatitis C are limited. According to the report by Moucari *et al.*,[28] there is a higher rate of SVR in genotype non-1 relapsers. Therefore, our patient could be retreated with a second PEG-IFN plus ribavirin combination therapy. However, because the patient is 41 years old and has stage F1 hepatic fibrosis, we will recommend that she wait for a new drug such as a protease inhibitor. Further research for unknown factors to predict late relapse after achieving SVR might be necessary.

In conclusion, SVR patients may have a potential risk of HCV reactivation. Annual surveillance including HCV RNA testing seems clinically reasonable for detecting spontaneous relapse and recurrence of hepatitis C in SVR patients.

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Insulin resistance raises the risk for recurrence of stage I hepatocellular carcinoma after curative radiofrequency ablation in hepatitis C virus-positive patients: A prospective, case series study

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Aim: Several studies have reported that insulin resistance raises the risk of primary hepatocellular carcinoma (HCC). We conducted a prospective, case series study to test the impact of insulin resistance on the recurrence after curative radiofrequency ablation (RFA) of stage I HCC in HCV-positive patients.

Methods: From January 2006 to December 2007, 226 consecutive patients underwent treatment for primary HCC at our institutions, including 37 stage I cases. Among them, 33 were HCV-positive, and three, six and 24 received curative surgery, transarterial chemoembolization or RFA, respectively. In the 24 patients treated with RFA, recurrence-free survival was analyzed using the Kaplan–Meier method. The factors contributing to recurrence of HCC were subjected to univariate and multivariate analyses using the Cox proportional hazards model. Insulin resistance was estimated by the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR).

Results: Kaplan–Meier analysis showed that the recurrence-free survival was lower in patients with higher HOMA-IR (>2.3, $P = 0.0252$) or with lower serum albumin level (<3.3 g/dL, $P = 0.0004$). In the univariate analysis, HOMA-IR ($P = 0.0420$) and albumin ($P = 0.0036$) were significantly associated with recurrence of HCC. Multivariate analysis revealed albumin (odds ratio = 0.01, 95% confidence interval = 0.0002–0.015, $P = 0.0001$) and HOMA-IR (odds ratio = 3.85, 95% confidence interval = 1.57–14.2, $P = 0.0015$) to be independent predictors for recurrence of HCC.

Conclusion: Serum albumin level and HOMA-IR were independent risk factors for recurrence of stage I HCC after curative RFA in HCV-positive patients. Patients with these factors require closer surveillance.

Key words: hepatitis C virus, hepatocellular carcinoma, Homeostatic Model Assessment of Insulin Resistance, insulin resistance, radiofrequency ablation, recurrence

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is prevalent worldwide, especially in Africa and the Western Pacific Region. HCC is the third most common cause of cancer death in men and the fifth most common in women; every year, more than 600 000 people die from this disease (www.who.int/whosis/).

Risk factors for the development of primary HCC include viral infection such as hepatitis B virus (HBV) and hepatitis C virus (HCV), alcohol consumption, aflatoxin and immune-related hepatitis.[1] Regarding risk factors for recurrence, several studies have suggested male sex, presence of cirrhosis, high α-fetoprotein (AFP), large tumor foci, multiplicity of tumors, pathologically high-grade atypia of tumor cells and presence of portal venous invasion of tumor.[2–6] Recently, several epidemiological studies have revealed a close association between diabetes mellitus (DM) and HCC. Wideroff *et al.*[7] described the standardized incidence ratios in Denmark for primary liver cancer in subjects with DM compared with the general population as 4.0 (95% confidence interval [CI] = 3.5–4.6) and 2.1 (95% CI = 1.6–2.7) for men and women, respectively. El-Serag *et al.*[8] reported that DM increased the risk of chronic non-alcoholic liver disease and HCC in male patients without concomitant

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liver disease in the USA. Furthermore, patients with chronic hepatitis and cirrhosis tend to experience complications with DM or to show insulin resistance.[9] This is particularly the case for patients with HCV infection and non-alcoholic fatty liver disease (NAFLD), including its most severe form, non-alcoholic steatohepatitis (NASH), which can lead directly to HCC.[10–12] The HCV core protein induced insulin resistance by increasing tumor necrosis factor-α which disrupts tyrosine phosphorylation of insulin receptor substrate-1.[13] Thus, DM including insulin resistance seems to be closely associated with various liver diseases that can lead to HCC, although the impact of insulin resistance on the recurrence of HCC has not been evaluated.

In this study, to identify the impact of insulin resistance on recurrence after initial curative treatment for HCC, we designed a prospective, case series analysis to examine recurrence-free survival in consecutive patients with stage I HCC, stratified by Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) level, which is commonly used for measuring insulin resistance.[14,15] In particular, we focused on HCV-positive patients who were treated with radiofrequency ablation (RFA).

METHODS

Patients

FROM JANUARY 2006 to December 2007, 226 primary HCC patients underwent initial treatment at our institutions, and 199 of them were followed to the end of this study (April 2008). Among them, we had 37 consecutive patients with stage I HCC that met all the criteria: a single tumor of 2 cm or less diameter, with no vascular invasion, no lymph-node invasion and no distant metastasis.[16]

Hepatocellular carcinoma nodules were detected by imaging modalities including abdominal ultrasonography, dynamic computed tomography (CT), dynamic magnetic resonance imaging (MRI) and abdominal arteriography. Diagnosis of HCC was made from a typical hypervascular tumor stain on angiography and typical dynamic-study findings of enhanced staining in the early phase and attenuation in the delayed phase. Etiologies for HCC were HCV in 33 patients, HBV in two and others in two.

Treatment, follow up and determination of recurrence

Three patients were treated with surgical resection, six with transarterial chemoembolization (TACE) and 28 with RFA. Among them, we only recruited those who were positive for HCV and treated with RFA ($n = 24$). Therapeutic effect was judged to be curative using dynamic CT or MRI with total disappearance of imaging characteristics of HCC as described above.

Patients were thereafter followed on an out-patient basis using serum tumor markers such as AFP and protein induced by vitamin K absence or antagonists II (PIVKA-II) every month, and by abdominal ultrasound, dynamic CT scan or dynamic MRI every 3 months. Recurrent HCC was diagnosed using the imaging modalities described earlier as the appearance of another lesion different from the primary one. The follow-up period was defined as the interval from the date of initial treatment until the date of diagnosis of recurrence, or until April 2008 if HCC did not recur. We defined the local tumor progression at the initial HCC site as censored.

Statistical analysis

Baseline characteristics were compared using the Student's *t*-test for continuous variables or χ^2-test for categorical variables. Recurrence-free survival was estimated using the Kaplan–Meier method, and differences between curves were examined by log–rank test. There were 13 possible predictors for recurrence of HCC after the initial curative treatment: sex, age, body mass index (BMI), Child–Pugh classification, serum albumin level, total bilirubin level, alanine aminotransferase (ALT) activity, platelet count, prothrombin time, HOMA-IR (defined as fasting plasma glucose [mg/dL] × fasting immunoreactive insulin [μU/mL] / 405), hemoglobin A_{1c} (HbA_{1c}), and serum tumor markers (AFP and PIVKA-II). The parameters, that proved to be significant by log–rank test, were then subjected to the univariate and multivariate analyses using the Cox proportional hazards model. Statistical significance was declared if the *P*-value was 0.05 or less. In addition, we employed the quantitative insulin sensitivity check index (QUICKI), which directly correlates with the glucose clamp method,[17] to supplement the evaluation of insulin resistance by HOMA-IR: QUICKI = 1 / (log [immunoreactive insulin] + log [fasting plasma glucose]).

RESULTS

Patients' baseline characteristics and laboratory data

THE BASELINE CHARACTERISTICS and laboratory data of 24 patients (15 men and nine women, median age 73 years) are shown in Table 1. Twenty

Table 1 Baseline demographic and clinical characteristics

		Normal range
Sex (male/female)	15/9	
Age (years)	73 (61–82)	
BMI	22.3 (19.5–33.5)	
Child–Pugh classification (A/B/C)	20/4/0	
Follow-up period (days)	365 (60–770)	
ALB (g/dL)	3.75 (2.4–4.4)	3.9–4.9
ALT (IU/L)	48.5 (21–98)	7–40
T-Bil (mg/dL)	0.96 (0.6–2.1)	0.2–1.2
PLT ($\times 10^4/\mu L$)	8.85 (4.1–21)	14.1–32.7
PT (%)	74 (56–118)	70–120
FPG (mg/dL)	107 (75–155)	70–110
FIRI (μg/dL)	10.8 (2.78–32.2)	2–10
HOMA-IR	2.96 (0.76–7.39)	<1.6
HbA1c (%)	5.2 (3.7–7.2)	<5.6
AFP (ng/dL)	26.7 (2.2–203)	<20
PIVKA-II (mAU/mL)	24 (9–127)	<40

Values are median (range).
AFP, α-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

patients were classified into Child–Pugh class A, four patients into class B and none into class C. The median follow-up period was 365 days (range 60–770 days), and no patient died during the study.

Possible risk factors for recurrence of HCC

No local tumor progression was diagnosed in this study period. Seven patients experienced the defined recurrence in the liver, but no one showed distant metastasis. One-year recurrence-free survival in total patients was 64%; Figure 1(a,b) shows Kaplan–Meier curves for recurrence-free survival according to HOMA-IR level (≤2.3 and >2.3), which produced significant difference ($P = 0.0252$). Serum albumin level (≥3.3 and <3.3 g/dL; $P = 0.0004$) was also a significant variable (Fig. 1c).

The Cox proportional hazards model was used to analyze risk factors for recurrence of stage I HCC after the curative RFA, using the 13 variables described earlier (Table 2). HOMA-IR level (odds ratio [OR] = 1.66, 95% CI = 1.01–2.72, $P = 0.0420$), and serum albumin level (OR = 0.08, 95% CI = 0.01–0.45, $P = 0.0036$) were identified as significant risk factors by univariate analysis. Multivariate analysis identified albumin (OR = 0.01,

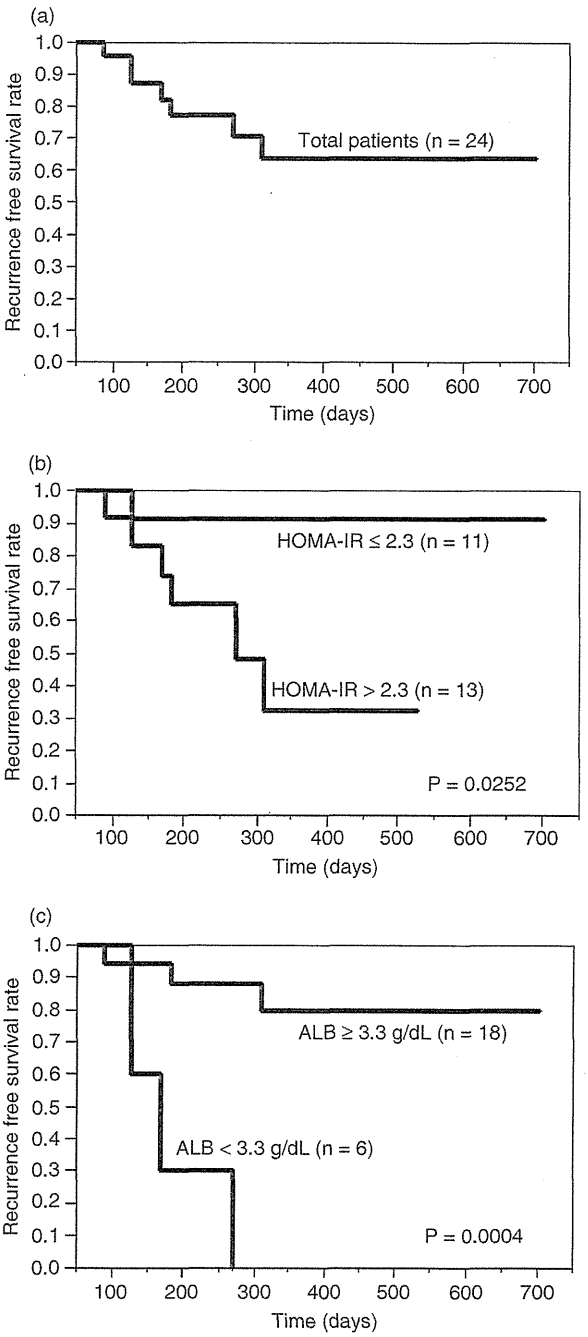

Figure 1 Kaplan–Meier curves for recurrence-free survival in (a) total patients and in subgroups divided according to (b) Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) level or (c) serum albumin level. ALB, albumin.

Table 2 Univariate analyses of possible risk factors for recurrence of hepatocellular carcinoma by Cox proportional hazards model

	95% CI			
	OR	Lower	Upper	*P*-value
Men (vs women)	1.20	0.25	8.41	0.8242
Age (years)	1.06	0.93	1.23	0.3451
BMI	0.90	0.59	1.22	0.6036
Child B (vs A)	4.81	0.60	31.3	0.1253
ALB (g/dL)	0.08	0.01	0.45	0.0036
T-Bil (mg/dL)	2.75	0.27	19.7	0.3603
ALT (IU/L)	0.99	0.95	1.02	0.6923
PLT ($\times 10^4/\mu L$)	0.86	0.65	1.05	0.1770
PT (%)	0.95	0.87	1.01	0.1617
HOMA-IR	1.66	1.01	2.72	0.0420
HbA1c (%)	0.69	0.27	1.53	0.3850
AFP (ng/dL)	1.00	0.99	1.02	0.1242
PIVKA-II (mAU/mL)	1.00	0.96	1.03	0.6172

OR is shown with a unit increase in continuous variables.
AFP, α-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; CI, confidence interval; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; OR, odds ratio; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

95% CI = 0.0002–0.15, $P = 0.0001$) and HOMA-IR level (OR = 3.85, 95% CI = 1.57–14.2, $P = 0.0015$) as significant independent risk factors for recurrence.

Table 3 shows the patients' baseline characteristics and laboratory data divided according to HOMA-IR level (≤2.3 and >2.3). No significant differences were noted between the two subgroups except fasting plasma glucose and fasting immunoreactive insulin. Two patients in the HOMA-IR 2.3 or less subgroup took oral hypoglycemic drugs, sulfonylurea derivatives and voglibose. Three patients in the HOMA-IR more than 2.3 subgroup took oral hypoglycemic drugs; two took sulfonylurea derivatives and one took pioglitazone. No patient received insulin treatment.

We supplementally analyzed the data by excluding the patients under treatment with these oral hypoglycemics and also the patients with fasting plasma glucose above 140 mg/dL, in order to avoid possible unreliability in HOMA-IR evaluation. In nine patients, each remaining in HOMA-IR of 2.3 or less and HOMA-IR of more than 2.3, serum albumin (OR = 0.02, 95% CI = 0.0002–0.40, $P = 0.0060$) and HOMA-IR (OR = 3.49, 95% CI = 1.45–13.8, $P = 0.0033$) were still significant.

In a similar manner, evaluation of insulin sensitivity by QUICKI gave the results that lower QUICKI (≤0.33,

Table 3 Baseline demographic and clinical characteristics of patients classified according to HOMA-IR level

	HOMA-IR ≤2.3 ($n = 11$)	HOMA-IR >2.3 ($n = 13$)	*P*-value
Sex (male/female)	7/4	8/5	0.9157
Age (years)	70 (61–82)	74 (63–80)	0.1846
BMI	23.55 (19.5–33.5)	22.1 (19.5–25.1)	0.1219
Follow-up period (days)	393 (155–701)	337 (60–770)	0.2785
Child–Pugh classification (A/B)	10/1	10/3	0.3483
ALB (g/dL)	3.9 (2.4–4.4)	3.4 (2.7–4.4)	0.3304
ALT (IU/L)	40 (21–98)	53 (25–80)	0.6103
T-Bil (mg/dL)	0.8 (0.7–2.1)	1.0 (0.6–1.7)	0.6655
PLT ($\times 10^4/\mu L$)	8.5 (4.1–21)	8.9 (4.9–13.9)	0.5766
PT (%)	72 (56–95.5)	74 (58–118)	0.9953
FPG (mg/dL)	90 (75–119)	109 (86–155)	0.0151
FIRI (μg/dL)	7.98 (2.78–10.8)	14.1 (7.86–32.2)	0.0005
HOMA-IR	1.79 (0.76–2.27)	3.76 (2.91–7.39)	<0.0001
HbA1c (%)	5.05 (3.7–7.2)	5.3 (4.1–6.8)	0.6848
AFP (ng/dL)	23.2 (2.2–153.2)	28 (8–203)	0.7339
PIVKA-II (mAU/mL)	21 (9–127)	28 (9–67)	0.8071
Presence of oral hypoglycemic drugs (yes/no)	2/9	3/10	0.7678
Presence of insulin treatment (yes/no)	0/11	0/13	1.0000

Values are median (range).
AFP, α-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; BMI, body mass index; FIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; PIVKA-II, protein induced by vitamin K absence or antagonists II; PLT, platelets; PT, prothrombin time; T-Bil, total bilirubin.

i.e. impaired insulin sensitivity) was associated significantly with the increased risk of HCC recurrence (OR = 7.97, 95% CI = 1.32–152, $P = 0.0213$).

DISCUSSION

SEVERAL EPIDEMIOLOGICAL STUDIES have revealed the association of DM with cancer incidence and cancer mortality for various organs such as the liver, biliary tract, pancreas, endometrium, kidney, colon, bladder and breast.[7,18–21] The mechanism by which insulin acts as a carcinogenic factor is currently a focus of interests. First, insulin functions as a growth factor by phosphorylating insulin receptor substrate 1 and activating the downstream mitogen-activated protein kinase cascade, which affects cellular proliferation.[22,23] Second, hyperinsulinemia increases peripheral lipolysis and hepatic accumulation of free fatty acids, and the excess β-oxidation in mitochondria and microsome leads to the production of reactive oxygen species[24,25] that play a significant role in carcinogenesis.[26,27] Adipocyte-secreted cytokines (adipokines) such as tumor necrosis factor-α and interleukin-6 also play a significant role in both insulin residence and carcinogenesis.[28,29] Thus, these factors could cooperatively induce the insulin resistance and carcinogenesis.

We demonstrated in the present study that a higher HOMA-IR level increases the risk of early recurrence after initial curative RFA of stage I HCC in HCV-positive patients. This finding basically agrees with previous studies[7,8,18] that suggested an association between insulin resistance and carcinogenesis as described above, but HbA_{1c} level did not predict recurrence (Table 2). This might be explained by the clinical relevance that HbA_{1c} level in patients with liver cirrhosis is often underestimated because of anemia. Therefore, the results of the present study suggest that the role of hyperinsulinemia is more important than that of hyperglycemia as reflected by HbA_{1c} in the recurrence of HCC. We therefore should pay attention to levels not only of glucose and HbA_{1c} but also of insulin when we follow patients who are at risk for HCC.

Interventional modalities to improve insulin-resistance could be a key to prevent the primary or recurrent HCC in patients complicated with such metabolic disorders. For instance, metformin and thiazolidine derivatives could be potential candidates for this purpose.[30,31] Oral branched-chain amino acid (BCAA) granules might be a candidate for preventing HCC recurrence in DM cases because, in addition to improving hypoalbuminemia,[32,33] this agent improves insulin resistance without stimulating insulin secretion.[34] Improvements of insulin resistance and glucose tolerance by BCAA have been reported in clinical trials.[35,36] Furthermore, Muto *et al.*[37] described that oral supplementation with BCAA granules inhibited liver carcinogenesis in HCV-positive liver cirrhosis with DM and obesity. Such effect of BCAA is also supported in experimental models.[38,39] These reports,[32–39] together with our present findings (Table 2 and Fig. 1b), suggest that insulin resistance is a significant risk factor for early recurrence of HCC and thus might be a critical target to prevent the recurrence and development of second primary HCC.

A limitation of this study is that the therapeutic effect of the primary HCC was judged as curative by imaging diagnosis but not by surgical pathology. Although the recurrent HCC developed apart from the primary tumor, we could not totally differentiate the recurrent lesion and a second primary HCC. A higher recurrence rate in this study (Fig. 1a) might be explained by this fact. Advanced medical imagings such as positron emission tomography would help solving such limitations in future study of this kind. Furthermore, the basic question, if de novo, namely, the first or second primary liver carcinogenesis is regulated by insulin resistance, should be addressed by recruiting HCC-free cirrhotics. However, such study requires a larger sample size and a longer observation period. Instead, we focused on the recurrent HCC, including possible second primary tumors, which develop at a 2–3-fold higher incidence than the first primary one.

Other study limitations are the short observation period, the small number of recruited patients and also the small number of detected events. Such a sample size essentially raises the possibility of β-error, including the absence of the statistical power of AFP and PIVKA-II for predicting the recurrence (Table 2). Previous reports agree that these tumor markers are risk factors of HCC recurrence,[2,6] although some criticisms remain.[4,5] In addition, we should state that the small number of events particularly restricts the reliability of multivariate analysis, while the calculation itself was possible in our study.

In conclusion, we presented for the first time that insulin resistance is significantly associated with the early recurrence of stage I HCC after curative RFA in HCV-positive patients. Increased HOMA-IR, which sensitively reflects insulin resistance, might be a useful biomarker for prediction of high-risk patients who cause early recurrence of HCC.

ACKNOWLEDGMENTS

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Effect of Aging on Risk for Hepatocellular Carcinoma in Chronic Hepatitis C Virus Infection

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An increase in the aging population is an impending problem. A large cohort study was carried out to determine the influence of aging and other factors on hepatocarcinogenesis in patients treated with interferon. Biopsy-proven 2547 chronic hepatitis C patients registered at our referral center since 1992 were included. Of these, 2166 were treated with interferon-based therapy. Incidences of hepatocellular carcinoma (HCC) associated with interferon were analyzed by Kaplan-Meier and person-years methods for an average follow-up of 7.5 years. Factors associated with HCC risk were determined by Cox proportional hazard analysis. HCC developed in 177 interferon-treated patients. The risk for HCC depended on age at primary biopsy and increased more than 15-fold after 65 years of age. Even when stratified by stage of fibrosis, the cumulative and annual incidences of HCC were significantly higher in older patients than in younger patients ($P < 0.001$) at the same stage of fibrosis, except for cirrhosis. Progression of fibrosis over time was significantly accelerated in older patients. The impact of viral eradication on HCC prevention was less significant in older patients than in younger patients. Multivariate analysis confirmed that age, gender, liver fibrosis, liver steatosis, total cholesterol level, fasting blood sugar level, baseline and postinterferon alpha-fetoprotein level, and virological response to interferon were independent risk factors associated with HCC. Aging was the strongest risk factor for a nonvirological response to interferon-based antiviral therapy. *Conclusion:* Elderly patients are at a higher risk for HCC. Hepatitis C viral eradication had a smaller effect on hepatocarcinogenesis in older patients. Patients should therefore be identified at an earlier age and treatment should be initiated. (HEPATOLOGY 2010;52:518-527)

Primary liver cancer is the third most common cause of cancer mortality worldwide,[1] and hepatocellular carcinoma (HCC) is one of the most frequent primary liver cancers.[2,3] Infection with hepatitis C virus (HCV) is a common cause of chronic hepatitis, which progresses to HCC in many patients.[4] The prevalence of older patients has been increasing in Japan, and this is an impending problem in other countries where viral spread has occurred more recently.[5] The number of Americans older than 65 years is expected to double by the year 2030.[6] In Western Europe, people older than 65 years already constitute 15%-18% of the population[7]; thus, aging patient who is chronically infected with HCV is

Abbreviations: AFP, alpha-fetoprotein; HBc, hepatitis B core; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NASH, nonalcoholic steatohepatitis; SVR, sustained virological response.

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one of the most important issues confronted by physicians.

Viral eradication with interferon-based therapy for chronic hepatitis C has been shown to prevent HCC by studies conducted in Japan and Italy.[8-11] However, this finding is controversial according to another study conducted in Europe and Canada,[12] in which viral eradication did not significantly reduce the risk for HCC in 479 consecutively treated patients. The likelihood of development of HCC among interferon-treated patients is difficult to determine because of the paucity of adequate long-term cohort studies. Moreover, in patients who are treated with interferon the effect of certain factors, including aging, on the risk for HCC remains unclear. Furthermore, the benefit of viral eradication with interferon-based therapy, including pegylated interferon and ribavirin combination therapy, in older patients remains unknown. To further clarify this, we conducted a large-scale, long-term cohort study and analyzed the influence of aging and other host and virological factors in patients treated with interferon.

Patients and Methods

Patients. Consecutive patients (n = 2547) chronically infected with HCV who underwent liver biopsy between 1992 and January 2008 at our referral center were enrolled. Of these, 2166 patients were treated with interferon-based antiviral therapy, whereas 381 patients did not receive interferon treatment (Fig. 1). All patients had histologically proven chronic hepatitis or cirrhosis. HCV infection was proven in all patients by identification of HCV RNA. Patients with a history of HCC, autoimmune hepatitis, or primary biliary cirrhosis were excluded. We also excluded patients who had a history of excessive alcohol consumption (50 g/day) and confirmed alcohol abstinence during follow-up. No patient was positive for hepatitis B surface antigen or antihuman immunodeficiency virus antibody. Written informed consent was obtained from all patients. The study was approved by the Ethical Committee of Musashino Red Cross Hospital in accordance with the Declaration of Helsinki.

Histological Evaluation. A liver biopsy specimen was obtained laparoscopically using 13G needles. When laparoscopy was impossible, ultrasound-guided liver biopsy was performed with 15G needles (n = 254). The mean length of the specimen was 18 mm (range 12-40 mm), and the mean number of portal tracts was 17 (range 8-34). Liver biopsy specimens

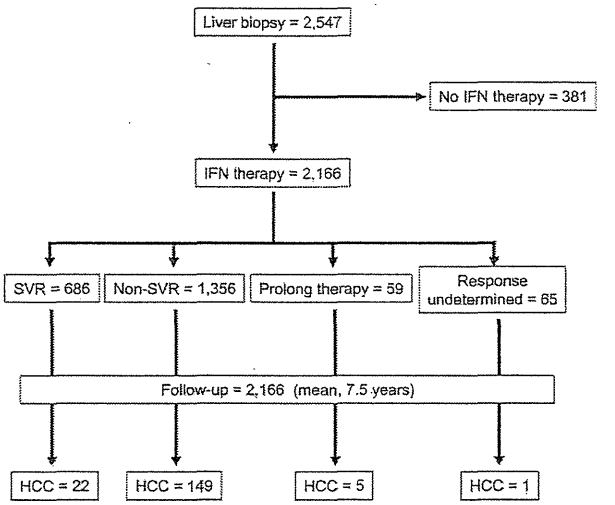


Fig. 1. Clinical outcomes of the patients enrolled in the present study. HCC, hepatocellular carcinoma; SVR, sustained virological response.

were scored by board-certified pathologists for stage of fibrosis and grade of inflammatory activity according to the classification of Desmet et al.[13] Additional macroscopic pathological information was obtained from laparoscopic findings. The percentage of steatosis was quantified by determining the average proportion of hepatocytes affected by steatosis. In this study, superimposed nonalcoholic steatohepatitis (NASH) was defined as a central pattern of colocalization of hepatic steatosis and hepatocyte ballooning with pericellular/perisinusoidal fibrosis or Mallory hyaline.

Interferon Treatment. Among the 2166 patients treated with interferon-based antiviral therapy, 1062 patients received interferon-alpha or beta monotherapy either for 24 weeks (n = 1003) or for 2 to 5 years (n = 59); 386 patients received interferon-alpha and ribavirin combination therapy for 24 weeks; 306 received pegylated interferon-alpha monotherapy for 48 weeks; and 412 received pegylated interferon-alpha and ribavirin combination therapy for 48 weeks. All interferon treatment was initiated within 48 weeks after liver biopsy.

Definitions of Response to Interferon Therapy. A patient negative for serum HCV RNA after the first 6 months of completion of interferon-based therapy was defined as a sustained viral responder. HCV RNA was determined by the qualitative Amplicor or TaqMan HCV assay (Roche Molecular Diagnostics, Tokyo, Japan).

Data Collection and Patient Follow-up. Data on patient characteristics, biochemical data, hematological

data, virological data, histological data, and treatment details were collected at enrollment. Age was determined at primary liver biopsy. Patients were examined for HCC with abdominal ultrasonography, dynamic computed tomography, and/or magnetic resonance imaging every 3-6 months. Serum alpha-fetoprotein (AFP) levels were measured every 1-2 months. This screening program constitutes the standard of care in Japan. To evaluate the effect of interferon-induced AFP reduction on hepatocarcinogenesis, the average AFP level after interferon treatment was calculated in each patient. HCC diagnosis was confirmed with needle biopsy, surgically resected specimens, or typical radiological findings diagnosed by board-certified radiologists. Figure 1 shows the schema for patient follow-up and clinical outcomes.

The start date of follow-up was the date of primary liver biopsy and the endpoint of follow-up was the development of HCC or the latest medical attendance until January 2009. The mean follow-up period was 7.5 years (range 0.5-17 years). The factors associated with development of HCC were retrospectively analyzed.

Change in Fibrosis Staging Over Time. To evaluate change in fibrosis staging over time, 271 patients who had not achieved a sustained virological response (SVR) with interferon therapy underwent a sequential biopsy after the initial biopsy. The interval between the paired biopsies was on average 4.8 years (range 0.7-14 years). The yearly rate of progression of fibrosis was calculated as the change in fibrosis staging divided by the time between paired biopsies.

Statistical Analysis. Categorical data were compared by the chi-square test and Fisher's exact test. Distributions of continuous variables were analyzed with Student's t test or the Mann-Whitney U test for two groups. All tests of significance were two-tailed and a P value of <0.05 was considered statistically significant. The cumulative incidence curve was determined with the Kaplan-Meier method and differences among groups were assessed using the log-rank test. Factors associated with HCC risk and virological response to interferon therapy were determined by the Cox proportional hazard model and logistic regression analysis, respectively. To depict the role of aging in developing risk for HCC, the multivariate Cox proportional hazard model was used after adjusting for stage of liver fibrosis, steatosis, and virological response to interferon. A polynomial regression was used to fit risk ratios for segments of the age distribution. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, IL).

Results

Patient Characteristics. Patient characteristics at the time of enrollment are shown in Table 1. The distribution of stages of liver fibrosis differed between younger and older patients, indicating the need to adjust for stage of liver fibrosis when comparing the two subgroups.

Response to Interferon Therapy. The response to interferon therapy was determined in 2042 (97.2%) of the interferon-treated patients, excluding those who received prolonged interferon treatment at the endpoint. SVR rates are shown in Table 1. The percentage of patients showing SVR was significantly lower in older patients ($\geq$65 years) than in younger patients (<65 years) ($P < 0.001$). Overall response rates to the different types of interferon therapy were as follows: interferon monotherapy, 31.5% (312/992); interferon-alpha and ribavirin combination therapy, 28.6% (108/378); pegylated interferon-alpha monotherapy, 37.9% (108/285); and pegylated interferon-alpha and ribavirin combination therapy, 41.1% (159/387). Response rates in genotype-1 patients (n = 1347) were 20.6% (114/554), 17.9% (29/162), 18.9% (56/297), and 36.8% (123/334), and those in nongenotype-1 patients (n = 565) were 52.2% (163/312), 63.1% (77/122), 65.0% (52/80), and 70.6% (36/51). Overall response rates of interferon and pegylated interferon monotherapy seem to be high because of the high response rates in the nongenotype-1 patients treated with these regimens.

Overall Cumulative Incidence of HCC. During follow-up, HCC developed in 177 interferon-treated patients (Fig. 1). The cumulative incidence of HCC 5, 10, and 15 years after interferon therapy was 4.7%, 11.6%, and 15.5%, respectively. The cumulative incidence in SVR patients was 2.1%, 4.3%, and 4.3%, respectively, which was significantly lower than that in non-SVR patients (5.8%, 14.9%, and 20.2%, respectively; log-rank test, $P < 0.001$).

Effect of Aging on Risk for HCC. The risk ratio determined by multivariate Cox proportional hazards analysis after adjustment for stage of liver fibrosis, degree of liver steatosis, and virological response to interferon demonstrated that the risk for HCC after interferon treatment was age-dependent and increased predominantly when the age at primary liver biopsy was >65 years (Fig. 2A). Hence, we defined older patients as those $\geq$65 years of age at primary liver biopsy and younger patients as those aged <65 years. As shown in Fig. 2B, the cumulative incidence of HCC was significantly higher in older patients than in younger patients (log-rank test, $P < 0.001$).

Table 1. Characteristics of Patients Enrolled in the Present Study

Characteristics	Total	<65 year	≥65 year	*P* Value*
Patients, n	2166	1614	552	
Sex, n (%)				<0.001†
Male	1080 (49.9)	840 (52.0)	240 (43.6)	
Female	1086 (50.1)	774 (48.0)	312 (56.4)	
Age (SD), year	55.4 (12.1)	51.1 (10.8)	68.4 (2.9)	<0.001‡
BMI (SD), kg/m^2	23.3 (3.1)	23.4 (3.0)	23.3 (3.1)	0.9‡
Fibrosis stage, n (%)				<0.001†
F0	27 (1.3)	24 (1.5)	3 (0.5)	
F1	860 (39.7)	704 (43.6)	156 (28.2)	
F2	733 (33.8)	515 (31.9)	218 (39.5)	
F3	444 (20.5)	301 (18.6)	143 (25.9)	
F4	102 (4.7)	70 (4.3)	32 (5.8)	
%Severe steatosis (≥10%)	27.6	27.1	29.3	0.4†
ALT level (SD), IU/L	95 (18)	101 (119)	76 (58)	<0.001‡
HCV load (SD), KIU/mL	880 (1046)	861 (1016)	924 (1116)	0.2‡
HCV genotype, n (%)				<0.001†
1a	7 (0.3)	5 (0.3)	2 (0.4)	
1b	1414 (69.6)	1036 (68.9)	378 (71.3)	
2a	373 (18.3)	273 (18.2)	100 (18.9)	
2b	211 (10.4)	164 (10.9)	47 (8.9)	
Others	28 (1.4)	25 (1.7)	3 (0.6)	
Duration (SD), year	7.5 (4.4)	8.1 (4.4)	5.8 (3.7)	<0.001‡
IFN regimen, n (%)				<0.001†
IFN mono	1062 (49.0)	833 (51.6)	229 (41.5)	
PEG-IFN mono	306 (14.1)	200 (12.4)	106 (19.2)	
IFN + RBV	386 (17.8)	291 (18.0)	95 (17.2)	
PEG-IFN + RBV	412 (19.0)	290 (18.0)	122 (22.1)	
SVR, n (%)	686 (33.6)§	565 (36.6)‖	121 (24.3)¶	<0.001‡

Unless otherwise indicated, data are given as the mean (SD).

ALT, alanine aminotransferase; BMI, body mass index; HCV, hepatitis C virus; IFN, interferon; N/A, not applicable; PEG, pegylated; RBV, ribavirin; SVR, sustained virological response.

*Comparison between <65 years and ≥65 years.

†Chi-squared test.

‡Student t test.

§Virological responses were determined in 2042 patients.

‖Virological responses were determined in 1545 patients.

¶Virological responses were determined in 497 patients.

As shown in Fig. 2C-E, even when stratified by stage of fibrosis the cumulative incidences among patients at stages F0/F1, F2, and F3 were significantly greater in older patients than in younger patients (log-rank test, $P < 0.001$). These differences were not significant among patients with cirrhosis (Fig. 2F, log-rank test, $P = 0.7$).

The annual incidence of HCC after interferon treatment was calculated by the person-years method (Table 2); it increased with the degree of liver fibrosis from 0.2% (F0 or F1) to 4.6% (F4) and was higher among older patients at the same stage of liver fibrosis.

Among the 177 patients with HCC, 92 showed evidence of a single blood transfusion. We analyzed the relationship between duration of infection and age in these 92 patients. A significant and strong negative correlation was found between the interval from blood transfusion to development of HCC and the age of the patients at the time of blood transfusion ($r = -0.74$, $P < 0.001$) (Fig. 3A). The mean duration of chronic infection was 22.0 years in patients who had received blood transfusion at >40 years of age, which was significantly shorter than that in patients who received it at ≤40 years of age (40.6 years, $P < 0.001$).

The presence of cirrhosis at the time of development of HCC, which was defined as having any of the following criteria, was evaluated: (1) histological evidence for cirrhosis, (2) findings of cirrhosis in any radiological study, or (3) presence of marked portal hypertension (i.e., presence of esophagogastric varices). Following this, 142 of the 177 with HCC (80.2%) were diagnosed as having cirrhosis, of which 42 were diagnosed histologically, 69 radiologically, and 31 based on the presence of marked portal hypertension. No significant difference was found in the proportion of patients with cirrhosis between older and younger patients, at the rate of 78.3% (94/120) in older

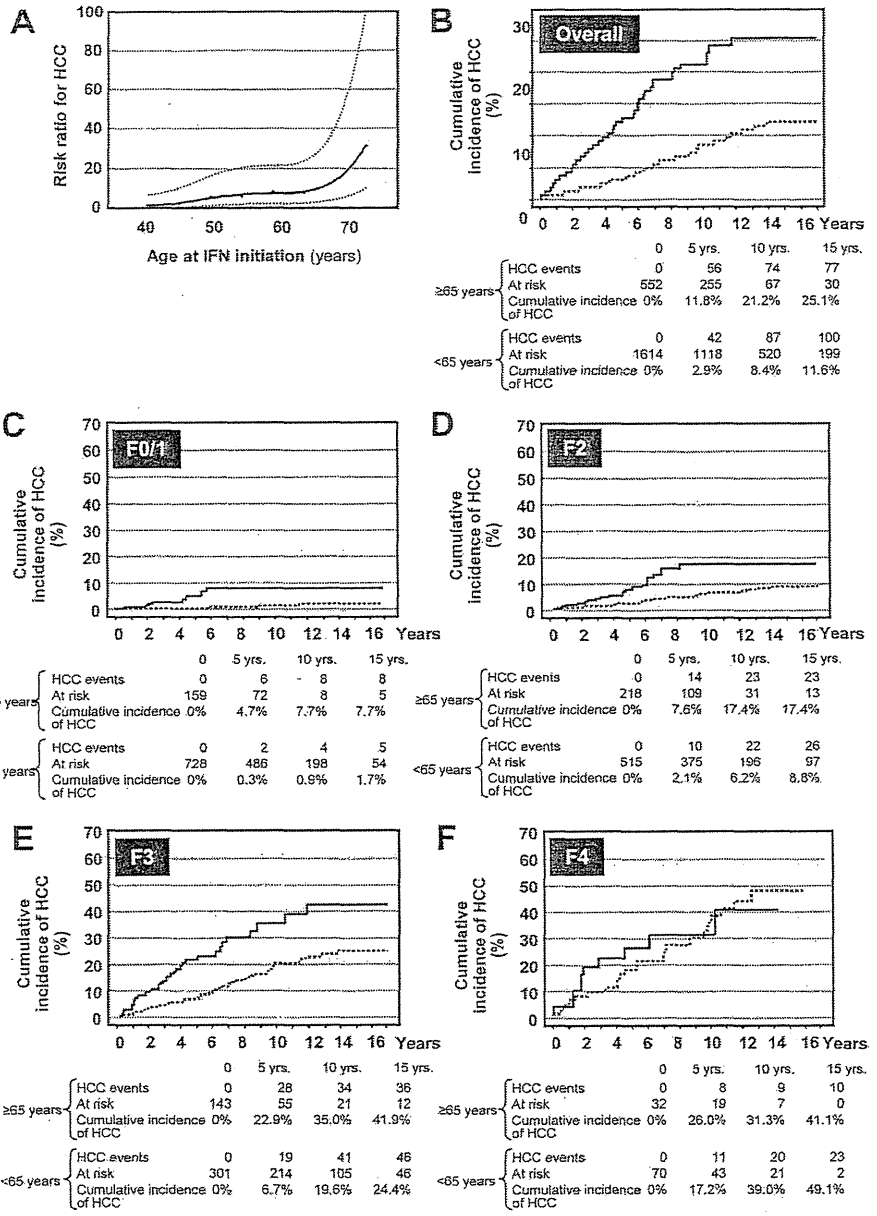


Fig. 2. Effect of aging on the risk for HCC. (A) Risk ratio (solid line) and 95% CI (dotted lines) for the risk of HCC according to age. To show the age-dependent relationship, a multivariate Cox proportional hazard model was used after adjustment for gender, stage of liver fibrosis, body mass index, and virological response to interferon therapy. Curves were fitted using polynomial regression. (B-F) Cumulative incidence of HCC after interferon therapy among younger (<65 years, n = 552, dotted line) and older patients (≥65 years, n = 1614, solid line). (B) Overall data, $P < 0.001$. (C) Patients with stage F0 or F1 liver fibrosis (no or mild fibrosis with portal expansion), $P < 0.001$. (D) Patients with stage F2 liver fibrosis (bridging fibrosis without architectural distortion), $P < 0.001$. (E) Patients with stage F3 liver fibrosis (bridging fibrosis with architectural distortion), $P < 0.001$. (F) Patients with stage F4 liver fibrosis (cirrhosis), $P = 0.7$. All P values were obtained by the log-rank test. The numbers of HCC events and patients at risk at each timepoint are shown below the graphs.

patients and 84.2% (48/57) in younger patients ($P = 0.36$, comparison at the age of HCC development).

Influence of Aging on Progression in Fibrosis Staging Over Time. In 271 patients who underwent paired biopsies, fibrosis staging progressed in 69 patients (25.5%), remained unchanged in 154 (56.8%), and regressed in 48 patients (17.7%). The overall rate of progression of fibrosis in these patients was 0.06 ± 0.02 fibrosis stages per year. Progression of fibrosis over time was significantly accelerated in older patients than in younger patients (0.21 ± 0.10 versus 0.03 ± 0.21 fibrosis stages per year, $P = 0.03$, Mann-Whitney U test) (Fig. 3B).

Effect of Viral Eradication on Risk for HCC in Older Patients. As shown in Fig. 4, the effect of viral eradication on the prevention of HCC was less significant in older patients than in younger patients. The annual incidence was higher among older patients than among younger patients with the same virological response (Table 2).

Influence of Liver Steatosis on Risk for HCC. The cumulative incidence of HCC after interferon therapy was significantly higher in patients with severe steatosis (≥10%) than in those with milder steatosis (at 5, 10, and 15 years: 8.6%, 19.1%, 32.0% versus 1.8%, 4.8%, 7.0%, respectively, log-rank test, $P < 0.001$).

Table 2. Annual Incidence of HCC After IFN Treatment

Factors	Total	<65 Years	≥65 Years
Fibrosis stage			
F0/F1	0.2%	0.1%	0.9%
F2	0.8%	0.6%	1.7%
F3	2.5%	1.8%	4.6%
F4	4.6%	4.4%	5.1%
Total	1.1%	0.8%	2.4%
Degree of liver steatosis			
<10%	0.5%	0.2%	1.4%
≥10%	2.0%	1.8%	3.0%
Virological response			
SVR	0.4%	0.2%	1.3%
Non-SVR	1.4%	1.0%	2.9%

Data were calculated by the person-years method. IFN, interferon; SVR, sustained virological response.

The annual incidence was higher in older patients than in younger patients with the same degree of liver steatosis (Table 2). In patients with severe steatosis (≥10%), superimposed NASH was diagnosed in 6.0% (26/435). Overall, superimposed NASH was significantly associated with hepatocarcinogenesis on univariate analysis (risk ratio, 4.1; 95% confidence interval [CI], 1.8-9.4; $P < 0.001$), but not on multivariate analysis. Superimposed NASH was significantly associated with high body mass index (27.2 ± 4.6 kg/m^2 versus 23.0 ± 3.1 kg/m^2, $P < 0.001$), hyperglycemia (186 ± 67 mg/dL versus 115 ± 39 mg/dL, $P < 0.001$), and advanced fibrosis (F3) (risk ratio, 2.9; 95% CI, 1.4-6.0; $P = 0.005$).

Factors Associated with Hepatocarcinogenesis After Interferon Therapy. Univariate analysis demonstrated factors that increase the risk ratio for the development of HCC (Table 3). Multivariate analysis using Cox proportional hazards regression confirmed that aging was one of the most significant independent factors associated with the development of HCC after interferon therapy. In this analysis, advanced fibrosis, presence of steatosis, male gender, lower total cholesterol level, higher fasting blood sugar level, higher baseline AFP level, insignificant improvement of mean AFP level after interferon therapy, and nonresponse to interferon therapy were also significantly associated with risk for HCC (Table 3).

We identified 22 patients in whom HCC developed even after achieving SVR. Univariate and multivariate logistic regression analyses indicated that both liver steatosis and aging were independently associated with the development of HCC among patients who achieved SVR (n = 686) (Table 4). Anti-HBc was detected in only 4 out of 22 patients and the age distribution was similar among anti-HBc-positive and anti-HBc-negative patients.

Response to Interferon Therapy in Older Patients. Multivariate logistic regression analysis confirmed that aging, female gender, severe liver fibrosis, extremely severe liver steatosis, genotype-1, high HCV load, and nonuse of pegylated interferon and ribavirin were independent risk factors for non-SVR (Supporting Table 1). The odds ratio, determined by multivariate logistic regression analysis after adjustment for these factors, demonstrated that the risk for non-SVR was age-dependent (Supporting Fig. 1). It was also ≈2.5 times higher in patients aged ≥65 years than in those aged <35 years.

In patients with genotype-1b and a high viral load who were treated with pegylated interferon and ribavirin combination therapy, the SVR rate was significantly lower in older patients than in younger patients (<49 years, 59.3%; 50-59 years, 50.5%; 60-65 years, 27.3%; ≥65 years, 25.2%; intention-to-treat analysis). Multivariate logistic regression analysis showed that

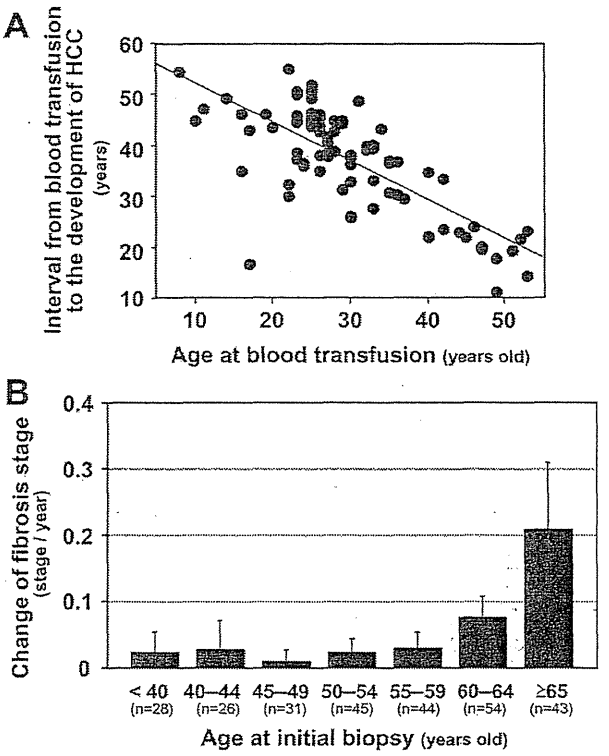
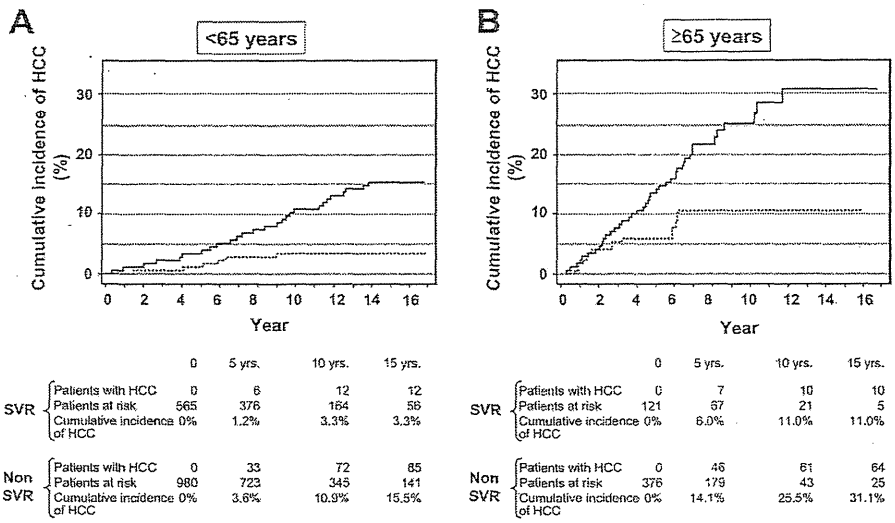


Fig. 3. (A) Relationship between the interval from blood transfusion to development of HCC and the age at blood transfusion (n = 92). A significant and strong negative correlation was observed ($r = -0.74$, $P < 0.001$). (B) Change in fibrosis staging over time. A total of 271 patients who had not achieved SVR by interferon therapy underwent a sequential biopsy after the initial biopsy. The yearly rate of progression of fibrosis was calculated as the change in fibrosis stage divided by the time between the paired biopsies. The yearly rate of progression of fibrosis was significantly higher in older patients (≥65 years) than in younger patients (<65 years) ($P = 0.03$, Mann-Whitney U test).



		0	5 yrs.	10 yrs.	15 yrs.
SVR	Patients with HCC	0	6	12	12
	Patients at risk	565	376	164	56
	Cumulative incidence of HCC	0%	1.2%	3.3%	3.3%
Non SVR	Patients with HCC	0	33	72	85
	Patients at risk	980	723	345	141
	Cumulative incidence of HCC	0%	3.6%	10.9%	15.5%

		0	5 yrs.	10 yrs.	15 yrs.
SVR	Patients with HCC	0	7	10	10
	Patients at risk	121	67	21	5
	Cumulative incidence of HCC	0%	6.0%	11.0%	11.0%
Non SVR	Patients with HCC	0	46	61	64
	Patients at risk	376	179	43	25
	Cumulative incidence of HCC	0%	14.1%	25.5%	31.1%

Fig. 4. Cumulative incidence of HCC after interferon therapy among SVRs (dotted lines) and non-SVRs (solid lines) according to age. (A) Younger patients (<65 years). The cumulative incidence of HCC was significantly higher in SVR than in non-SVR (log-rank test, $P < 0.001$). (B) Older patients (≥65 years). The cumulative incidence of HCC was significantly higher in SVR than in non-SVR (log-rank test, $P = 0.02$). However, the difference between SVR and non-SVR was less in older patients than in younger patients. The number of HCC events and patients at risk at each timepoint are shown below the graphs.

aging was the strongest independent factor contributing to SVR in these patients (data not shown). The odds ratio for the risk of non-SVR was 1.8 for each additional 10 years of age (95% CI, 1.5-2.3, $P < 0.001$).

Discussion

In this large cohort study we demonstrated that aging is significantly associated with the development of HCC in patients treated with interferon. The risk ratio increased predominantly in patients older than 65 years, which was more than 15 times that in patients in their 20s. Aging is becoming the most critical risk factor for the development of HCC. Although liver fibrosis was also an important risk factor, we clearly demonstrated that the risk for hepatocarcinogenesis after interferon treatment was significantly higher in older patients at each stage of liver fibrosis except for cirrhosis. Hence, physicians should be aware that older patients can develop HCC regardless of the stage of fibrosis.

Because the present study included a large cohort, it was difficult to determine the duration of infection in all patients, and this might have affected the risk determination for HCC development. Therefore, we analyzed the relationship between duration of chronic infection and HCC development in patients who underwent a single blood transfusion. We found a significant and strong negative correlation between the interval from blood transfusion to development of HCC and the age of the patients at the time of blood transfusion. Consistent with our results, a previous report with posttransfusion HCV demonstrated that the age of patients, rather than the duration of HCV infection, was more significant for HCC development.[14-16] Therefore, older age and not duration of infection is more likely to influence hepatocarcinogenesis. Moreover, our analysis of sequential biopsy specimens demonstrated that the progression rate of liver fibrosis significantly accelerated in patients aged >65 years. Hence, the progression of fibrosis along with aging may also contribute to the increased risk for hepatocarcinogenesis in older patients.

We further demonstrated that liver steatosis was an independent risk factor for the development of HCC, which was not mentioned in previous reports.[8-11] The presence of steatosis is related to both viral (genotype-3 or HCV core protein) and host metabolic factors.[17,18] In our cohort, most superimposed NASH was associated with host metabolic factors such as high body mass index and hyperglycemia, whereas infection of genotype-3 was only noted in two patients. In vitro experiments have suggested an association between liver steatosis induced by HCV core protein and hepatocarcinogenesis,[19] and have proposed virus-associated steatohepatitis as a new aspect of chronic hepatitis C.[20,21] Because steatosis was likely to be related to hepatocarcinogenesis, patients with chronic hepatitis C, whose liver histology shows superimposed NASH,

Table 3. Factors Associated with HCC After IFN Therapy

	Univariate Analysis		Multivariate Analysis	
Risk Factor Value	Risk Ratio (95% CI)	*P* Value	Risk Ratio (95% CI)	*P* Value
Age (by every 10 year)	2.2 (1.8-2.7)	<0.001	3.0 (1.9-4.8)	<0.001
Sex				
Female	1		1	
Male	1.2 (0.9-1.6)	0.2	2.0 (1.0-3.8)	0.04
BMI (by every 10 kg/m^2)	2.0 (1.2-1.3)	0.005	1.1 (0.4-3.5)	0.8
Fibrosis stage				
F0/F1/F2	1		1	
F3/F4	5.4 (3.9-7.5)	<0.001	2.5 (1.2-4.9)	0.01
Degree of steatosis				
<10%	1		1	
≥10%	4.5 (3.0-6.9)	<0.001	3.5 (1.9-6.4)	<0.001
Esophagogastric varices				
No	1		1	
Yes	3.3 (2.0-5.3)	<0.001	1.6 (0.6-4.4)	0.3
Virological response				
SVR	1		1	
Non-SVR	3.3 (2.1-5.2)	<0.001	2.6 (1.2-5.5)	0.001
Genotype				
Non-1	1		1	
1	1.7 (1.2-2.5)	0.006	1.0 (0.5-2.3)	0.9
Albumin (by every 1 g/dL)	0.2 (0.1-0.3)	<0.001	0.6 (0.2-2.2)	0.3
ALT (by every 100 IU/L)	1.0 (0.9-1.0)	0.8	0.4 (0.1-1.8)	0.6
AST (by every 100 IU/L)	1.2 (1.1-1.3)	0.001	1.1 (0.6-1.8)	0.8
γ-GTP (by every 100 IU/L)	1.3 (1.1-1.6)	0.009	0.6 (0.3-1.6)	0.3
ALP (by every 100 IU/L)	1.3 (1.2-1.5)	<0.001	0.6 (0.3-1.2)	0.2
Total bilirubin (by every 1 mg/dL)	1.6 (1.3-2.1)	<0.001	1.2 (0.6-2.7)	0.6
Total cholesterol (by every 100 mg/dL)	0.3 (0.2-0.6)	<0.001	0.2 (0.1-0.6)	0.006
Triglyceride (by every 100 mg/dL)	0.8 (0.5-1.1)	0.2	0.1 (0.02-1.1)	0.08
Fasting blood sugar (by every 100 mg/dL)	1.8 (1.5-2.2)	<0.001	1.1 (1.0-1.1)	0.04
WBC (by every 100/μL)	0.1 (0.03-0.3)	<0.001	0.1 (0.01-2.2)	0.2
RBC (by every 10^6/μL)	0.5 (0.4-0.7)	<0.001	1.8 (0.7-4.4)	0.2
Platelet counts (by every 10^6/μL)	0.3 (0.2-0.4)	<0.001	0.6 (0.3-1.5)	0.3
Baseline AFP (by every 10 ng/mL)	1.0 (0.9-1.1)	0.2	1.3 (1.0-1.7)	0.04
Post IFN AFP (by every 10 ng/mL)	1.2 (1.1-1.3)	<0.001	1.9 (1.5-2.4)	<0.001
HCV load (by every 100 KIU/mL)	1.0 (0.9-1.0)	0.4	1.0 (1.0-1.1)	0.06
IFN regimen				
IFN monotherapy	1		1	
IFN + RBV (24 W)	1.2 (0.8-1.8)	0.4	1.5 (0.7-3.2)	0.3
PEG-IFN monotherapy (48 W)	1.1 (0.6-1.9)	0.8	1.5 (0.4-5.5)	0.6
PEG-IFN + RBV	0.4 (0.2-0.9)	0.03	1.0 (0.3-3.1)	0.9

Risk ratios for development of HCC were calculated by Cox proportional hazards regression analysis. AFP, alpha fetoprotein; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspirate aminotransferase; BMI, body mass index; γ-GTP, gamma-glutamyltranspeptidase; HCC, hepatocellular carcinoma; IFN, interferon; PEG, pegylated; RBC, red blood cell counts; RBV, ribavirin; SVR, sustained virological response; WBC, white blood cell count.

may be at a higher risk of developing HCC. Further study is necessary to confirm this association in a clinical situation. Because several developed countries are in the midst of a growing obesity epidemic, the risk related to obesity cannot be ignored in patients with chronic hepatitis C who are treated with interferon.

Several retrospective cohort studies have been conducted to evaluate the effect of interferon on the incidence of HCC among patients with chronic hepatitis C.[8-11] Our results, obtained from one of the largest cohort studies, confirm the efficacy of viral eradication in preventing HCC. In one study conducted in a Western population, no statistically significant reduction was found in the development of HCC among patients with SVR compared with those without SVR (adjusted hazard ratio, 0.46; 95% CI, 0.12-1.70; $P = 0.25$).[12] Because relatively few occurrences of HCC were observed in this cohort, and the duration of follow-up was shorter, the differences in HCC development between patients with and without SVR might be less pronounced.

Interestingly, our results demonstrated that the risk for HCC remains even after achieving SVR in older patients, confirming the findings of previous studies conducted with a smaller number of patients.[22,23] The cumulative incidence of HCC during the first 5 years

Table 4. Factors Associated with Development of HCC After Achieving SVR

Risk Factor	Odds Ratio (95% CI)	*P*-value
Univariate analysis		
Age (by every 10 year)	3.2 (1.8-5.5)	<0.001
Sex		
Female	1	
Male	3.0 (1.0-8.8)	0.04
Fibrosis stage		
F0/F1/F2	1	
F3/F4	5.9 (2.5-14.0)	<0.001
Degree of steatosis		
<10%	1	
≥10%	5.5 (2.0-15.2)	0.001
BMI (by every 10 kg/m^2)	3.2 (0.8-12.6)	0.09
ALT (by every 10 IU/L)	0.9 (0.7-1.3)	0.7
AST (by every 10 IU/L)	1.1 (0.9-1.4)	0.3
Genotype		
Non-1	1	
1	1.2 (0.6-3.0)	0.5
HCV load (by every 100 KIU/mL)	0.9 (0.8-1.0)	0.2
IFN regimen		
IFN monotherapy	1	
IFN + RBV (24 W)	0.7 (0.2-2.3)	0.5
PEG-IFN monotherapy (48 W)	0.8 (0.2-3.6)	0.8
PEG-IFN + RBV	0.3 (0.03-2.0)	0.2
Multivariate analysis		
Age (by every 10 year)	2.7 (1.5-5.1)	0.002
Sex		
Female	1	
Male	4.1 (0.9-18.9)	0.06
Fibrosis stage		
F0/F1/F2	1	
F3/F4	2.6 (0.9-7.5)	0.08
Degree of steatosis		
<10%	1	
≥10%	5.6 (1.9-16.5)	0.002

Odds ratios for SVR were calculated by logistic regression analysis.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; HCV, hepatitis C virus; IFN, interferon; HCC, hepatocellular carcinoma; PEG, pegylated; RBV, ribavirin; SVR, sustained virological response.

after completion of interferon therapy was similar between SVR and non-SVR patients in the older age group, and the risk for HCC remained for 9 years after eradication of HCV in our patients. Therefore, HCC patients with SVR who have a risk factor should be screened for at least 5-10 years after the completion of interferon therapy.

It has been reported that coffee consumption has a protective effect against hepatocarcinogenesis[24,25] and liver disease progression in patients with chronic HCV infection.[26] Because we could not review coffee consumption in all the patients and fewer data were available in the previous literature as to whether a habitual change of reducing coffee consumption occurs in older patients, it is unclear whether increased risk for HCC in older patients is an effect of this habitual change in older patients. However, the majority (68%) of Japanese patients who have HCV (n = 1058) drink less than 1 cup of coffee per day, and only 7.6% consume more than 3 cups of coffee per day.[27] Therefore, it is unlikely that a habitual change in older patients affects the increased risk for hepatocarcinogenesis in older patients.

Recently, it was reported that interferon therapy might be less effective in preventing HCC among patients with chronic hepatitis C who are positive for anti-HBc antibody,[28] but this finding is still controversial.[29,30] In the present study, anti-HBc was only detected in 4 of 22 patients in whom HCC developed after viral eradication, and age distribution was similar among anti-HBc-positive and anti-HBc-negative patients. Because no significant difference in mean age was found between anti-HBc-positive and anti-HBc-negative patients in the recent study conducted in Japan,[28] it is unlikely that previous exposure to hepatitis B virus or occult hepatitis B virus infection is responsible for the difference in risk for HCC between younger and elderly patients found in the present study.

In conclusion, aging has become one of the most important risk factors for HCC. Even after stratification by stage of fibrosis, the risk for HCC after antiviral treatment was significantly higher in older patients, and HCV eradication had a smaller effect on HCC-free survival in older patients. Patients with HCV should therefore be identified at an earlier age and antiviral treatment should be initiated. The present results have potentially important clinical implications for physicians that may influence their decisions about the treatment strategy in individual patients.

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