Table 2. SNPs Associated With the Presence of HCC

Symbol	dbSNP ID	*P* Value: First Screening	*P* Value: Second Screening	*P* Value: Total
Genotype frequency				
CD6	rs2074223	.037	.146	.630
CD74	rs2288817	.025	.813	.171
CRHR2	rs2267716	.008	.160	.003
FKBP6	rs2237285	.005	.842	.170
GDF9	rs39830	.002	.550	.033
GFRA1	**hCV1250702***	**.036**	**.008**	**<.001**
GPR37	rs2299904	.012	.012	.645
MMP1	rs5854	.027	.505	.028
NTSR1	rs2273075	.003	.100	.001
PDGFRB	rs2240780	.018	.532	.565
TNFRSF6	rs2296604	.042	.756	.127
Allele frequency				
A2M	rs1805657	.047	.654	.412
CD74	rs2288817	.029	.818	.183
CRHR2	**rs2267716**	**.035**	**.048**	**.005**
FKBP6	rs2237285	.004	.674	.095
GDF9	rs39830	.001	.668	.067
GRO1	rs4074	.046	.603	.040
IGSF4	rs2275997	.026	.877	.182
IL17R	rs2241044	.013	.904	.093
PDGFRB	rs2240780	.009	.297	.338
SCYB14	**rs2237062**	**.048**	**.005**	**.001**
SELP	rs6128	.021	.305	.42
TBXAS1	rs2267684	.029	.515	.284
TNFRSF6	rs2031613	.031	.210	.870
TNFSF6	rs859668	.046	.100	.006
TRAF1	rs2239657	.025	.229	.502
At-risk alleles				
ACVR2	rs2288190	.024	.417	.033
ATRN	rs2295675	.046	1.000	.156
CD6	rs2074223	.048	.454	.393
CD74	rs2288817	.025	.813	.171
CRHR2	rs2267716	.004	.129	.002
FKBP6	rs2237279	.048	.309	.026
FKBP6	rs2237285	.006	.873	.078
GDF9	rs39830	.000	1.000	.014
GFRA1	**hCV1250702***	**.021**	**.002**	**<.001**
GPR37	rs2299904	.043	.330	.613
GRO1	rs4074	.050	.757	.082
IGSF4	rs2275997	.037	1.000	.200
IL17R	rs2241044	.030	.766	.285
IL18R1	rs2287033	.037	.367	.525
IL1RL1	rs1041973	.036	.650	.340
IL4	rs2227284	.029	.565	.388
LTA	rs2239704	.043	.855	.136
LTA4H	rs2268516	.027	.721	.033
MMP1	rs5854	.041	.620	.059
PAFAH2	rs2275102	.05	.768	.291
PDGFRB	rs2240780	.017	.325	.346
SCYB14	**rs2237062**	**.038**	**.005**	**<.001**
SELP	rs6128	.032	.128	.725
TBXAS1	rs2267684	.034	.882	.171
TNFRSF6	rs2296604	.025	1.000	.118
TNFRSF6	rs2031613	.046	.195	1.000
VIPR1	rs2278215	.046	.768	.242

NOTE. SNPs significantly associated with the presence of HCC in both first and second screening are indicated in boldface.

*Celera SNP ID.

whereas serum albumin level, prothrombin time, and platelet count were lower than in the group of patients without HCC.

Of the 31 SNPs screened, only 3 SNPs in 3 genes were associated with HCC in both the initial and secondary screenings. These were: an intron SNP [hCV1250702 of Celera SNP (Celera Genomics, Rockville, MD), C__1250702_10 of Applied Biosystems] of GDNF family receptor alpha 1 (GFRA1) associated by genotype frequency and at-risk allele analyses; an intron SNP (rs2267716 of dbDNP [http://www.ncbi.nlm.nih.gov/SNP/index.html], IMS-JST021253 of JSNP, C__2570970_1_ of Applied Biosystems) of corticotropin-releasing hormone receptor 2 (CRHR2) associated by allele frequency analysis; and an intron SNP (rs2237062 of dbDNP, IMS-JST017563 of JSNP) of SCYB14 associated by allele frequency and at-risk allele analyses (Table 2).

Association of Polymorphisms of GFRA1, CRHR2, and SCYB14 with HCC in Patients With HCV. The characteristics of all 376 subjects are shown in Table 1. There was no significant difference in alcohol abuse, HCV genotype, viral load, or serum ALT level between the groups of patients with and without HCC. In the group of patients with HCC, patient age, the proportion of male patients, the proportion of patients with cirrhosis, serum total bilirubin level, and serum AFP level were higher, whereas serum albumin level, prothrombin time, and platelet count were lower than in the group of patients without HCC.

The distributions of genotypes, alleles, and at-risk alleles with regard to the presence of HCC are shown in Table 3. The genotype frequencies and allele frequencies of GFRA1, CRHR2, and SCYB14 were significantly different between the groups of patients with and without HCC. The at-risk alleles of GFRA1 and SCYB14 were also associated with the presence of HCC.

Interestingly, a combination of 2 gene genotypes increased the odds ratio up to 8.23 (SCYB14 + GFRA1), 12.6 (SCYB14 + CRHR2), and 21.5 (GFRA1 + CRHR2), respectively. Moreover, a combination of 3 gene genotypes increased the odds ratio up to 12.3.

Factors Associated With the Presence of HCC in Patients With HCV. The following factors were significantly associated with the presence of HCC according to univariate analyses: GFRA1 genotype ($P < .001$), CRHR2 genotype ($P = .003$), SCYB14 genotype ($P = .002$), age older than 60 years ($P < .001$), male sex ($P < .001$), the presence of cirrhosis ($P < .001$), platelet count $<12.5 \times 10^4/\mu L$ ($P < .001$), albumin <3.9 g/dL ($P < .001$), total bilirubin >0.7 mg/dL ($P < .001$), prothrombin time <70% ($P < .001$), and serum AFP >20 μg/L

Table 3. Association of SCYB14, GFRA1, and CRHR2 Polymorphisms With HCC

Polymorphisms	Patients With HCV: Without HCC n = 206	With HCC n = 170	Odds Ratio (95% CI) With vs. Without	P
SCYB14 genotype				.002
C/C	123 (61%)	72 (42%)	1.00	
C/G	67 (33%)	81 (48%)	2.07 (1.39-3.07)	
G/G	12 (6%)	17 (10%)	2.42 (1.50-3.92)	
SCYB14 allele				.001
C	313 (77%)	225 (66%)	1.00	
G	91 (23%)	115 (34%)	1.76 (1.29-2.39)	
SCYB14 at-risk allele				<.001
C/C	123 (61%)	72 (42%)	1.00	
G/G + G/C	79 (39%)	98 (58%)	2.12 (1.41-3.19)	
GFRA1 genotype				<.001
G/G	92 (45%)	42 (25%)	1.00	
G/C	80 (39%)	93 (55%)	2.55 (1.53-4.24)	
C/C	34 (16%)	33 (20%)	2.13 (1.41-3.21)	
GFRA1 allele				.002
G	264 (64%)	177 (53%)	1.00	
C	148 (36%)	159 (47%)	1.60 (1.24-2.07)	
GFRA1 at-risk allele				<.001
G/G	92 (45%)	42 (25%)	1.00	
C/C + G/C	114 (55%)	126 (75%)	2.42 (1.50-3.92)	
CRHR2 genotype				.003
A/A	139 (67%)	98 (58%)	1.00	
A/G	63 (31%)	55 (33%)	1.24 (1.10-1.39)	
G/G	4 (2%)	16 (9%)	5.67 (2.20-14.61)	
CRHR2 allele				.005
A	341 (83%)	251 (74%)	1.00	
G	71 (17%)	87 (26%)	1.66 (1.26-2.20)	

($P < .001$). To evaluate the effects of the polymorphisms in 3 SNPs on the presence of HCC, a stepwise multivariate logistic regression analysis was performed using these 11 variables. Nine variables (GFRA1 genotype, CRHR2 genotype, SCYB14 genotype, age >60 years, male sex, albumin <3.9 mg/dL, total bilirubin >0.7 mg/dL, prothrombin time <70%, and AFP >20 μg/L) were included in the final model with odds ratios of 2.54 (G/G vs. G/C), 9.81 (A/A vs. G/G), 3.13 (C/C vs. G/G), 2.65, 2.27, 3.08, 2.05, 3.04, and 3.62, respectively (Table 4).

Haplotype Analysis. Haplotype analyses of the 3 genes shown to be significant factors by the secondary screening (SCYB14, GFRA1, and CRHR2) were performed in a total of 376 patients by assaying 3 SNPs in SCYB14, 12 SNPs in GFRA1, and 3 SNPs in CRHR2. Five haplotype blocks were built in 3 genes, and 2 haplotype blocks in 2 genes (SCYB14 and GFRA1) were found to be significantly associated with HCC.

The haplotype block in SCYB14 consisted of 3 SNPs (rs1148364, rs2237062, and rs1016666 of dbSNP) (Fig. 1, Table 5). For 3 subjects of 376, the diplotype configuration could not be determined, and 6 haplotypes were missing. The overall P value generated by the FASTEHPLUS T5 test was .001. Haplotype CCT was a significant protective factor for HCC ($P = .013$, OR = 0.68, 95% CI = 0.49-0.92), and haplotype GGC was a significant risk factor for HCC ($P < .001$, OR = 1.82, 95% CI = 1.32-2.53).

Table 4. Factors Associated With the Presence of HCC in Multivariate Analysis

Factor and Category	P Value	Odds Ratio	95% CI
GFRA1 genotype			
G/G		1.00	
G/C	.004	2.54	1.34-4.84
C/C	.180	1.76	0.77-4.00
CRHR2 genotype			
A/A			
A/G	.869	1.05	0.57-1.95
G/G	.015	9.81	1.55-62.07
SCYB14 genotype			
C/C			
C/G	.016	2.11	1.15-3.86
G/G	.043	3.13	1.04-9.45
Male sex	.010	2.27	1.22-4.22
Age > 60 years	.002	2.65	1.43-4.93
Albumin < 3.9 g/dL	<.001	3.08	1.71-5.55
TB > 0.7 mg/dL	.015	2.05	1.15-3.67
PT < 70%	.001	3.04	1.55-5.96
AFP > 20 ng/mL	<.001	3.62	1.99-6.58

Abbreviations: TB, total bilirubin; PT, prothrombin time; AFP, alpha fetoprotein.

The haplotype block in GFRA1 consisted of 3 SNPs (rs953920 of dbDNP, hCV1250702 of Celera SNP, and rs2270181 of dbSNP) (Fig. 2, Table 6). For 17 subjects of 376, the diplotype configuration could not be determined, and 34 haplotypes were missing. The overall P value generated by the FASTEHPLUS T5 test was .003. Haplotype GCG was a significant risk factor for HCC ($P = .007$, OR = 1.74, 95% CI = 1.18-2.58).

SCYB14 mRNA Expression in Cancerous and Noncancerous Live Tissues. SCYB14 cDNA was quantified and normalized using GAPDH cDNA as an endogenous expression control. The expression of SCYB14 mRNA in noncancerous liver tissue was significantly higher than that in cancerous liver tissue in all 3 cases (Fig. 3).

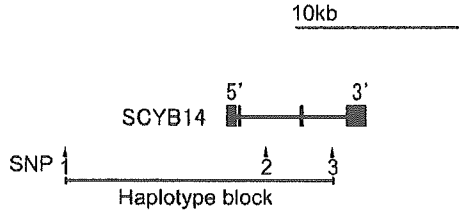

Fig. 1. SCYB14 haplotype block map. Solid boxes represent exons of the SCYB14 gene. The haplotype block in SCYB14 consisted of 3 SNPs (SNP1, rs1148364; SNP2, rs2237062; and SNP3, rs1016666).

Table 5. Haplotypes in Haplotype Block of SCYB14 (rs1148364-rs2237062-rs1016666)

Haplotype No.	Haplotype	Haplotype Count HCC+	Haplotype Count HCC−	Fisher's P Value	Odds Ratio	95% CI
1	CCT	209	285	.013	0.68	0.49-0.92*
2	GGC	114	88	<.001	1.82	1.32-2.53†
3	GCC	14	29	.084	0.56	0.29-1.07
4	CCC	2	2	1.000	1.20	0.17-8.53
5	CGC	0	2	.503	0.24	0.01-4.97
6	GGT	1	0	.456	3.59	0.15-88.47
0	Undetermined	0	6			

NOTE. Overall *P* value (FASTEHPLUS T5; 20,000 permutations): .0014.
*Significantly protective.
†Significantly at risk.

Table 6. Haplotypes in Haplotype Block of GFRA1 (rs953920-hCV1250702-rs2270181)

Haplotype No.	Haplotype	Haplotype Count HCC+	Haplotype Count HCC−	Fisher's P Value	Odds Ratio	95% CI
1	AGA	154	210	.133	0.79	0.59-1.06
2	ACG	82	85	.249	1.23	0.87-1.75
3	GCG	69	53	.007	1.74	1.18-2.58*
4	GGA	13	30	.057	0.51	0.26-0.99
5	AGG	4	9	.402	0.54	0.16-1.75
6	GGG	0	1	1.000	0.40	0.02-0.97
7	ACA	0	4	.131	0.13	0.01-2.50
0	Undetermined	18	20			

NOTE. Overall *P* value (FASTEHPLUS T5, 20000 permutations): .0029.
*Significantly at risk.

Discussion

In this study, the presumptive genetic markers for susceptibility to hepatocarcinogenesis were identified in patients with chronic HCV infection. Three SNPs in 3 different genes were identified as being associated with HCC.

A dual-step screening process for HCC susceptibility genes was adopted to reduce the possibility of a problem with multiple testing.[23] Although 31 SNPs were identified in the initial screening for HCC susceptibility SNPs, only 3 SNPs were finally shown to be associated with HCC after the secondary screening. The other 28 SNPs were removed, probably because they were falsely associated with HCC in the initial screening. The results should be interpreted cautiously, however, because of the possibility that falsely associated SNPs could have been picked up and SNPs that are actually associated with HCC could have been missed. A larger population should be tested for the positive SNPs to confirm the results of this work.

In addition, SNPs associated with HCC development may not be the causative variation of the disease but may be merely genetic markers that are in linkage disequilibrium with other causative variations. All of the 3 SNPs associated with HCC in this study were intron SNPs with functions yet to be elucidated. MicroRNAs recently have been reported to be translated from introns and regulate the mRNA translation of genes associated with development.[24] Thus these 3 SNPs may have a regulatory function. To further determine whether these 3 SNPs are linked with other causative variations, we performed haplotype analyses of these 3 genes and identified 2 haplotype blocks associated with HCC.

Despite the limitations of this study, the fact that 3 genes have been identified as being associated with HCC will allow the generation of new hypotheses for future studies. The association of these 3 genes with HCC is a novel observation. SCYB14, alternatively known as CXCL14 (C-X-C motif chemokine ligand 14) or BRAK (breast and kidney-expressed chemokine), belongs to the cytokine gene family and encodes secreted proteins involved in immunoregulatory and inflammatory processes.

Interestingly, the expression of SCYB14 mRNA was high in noncancerous liver tissue but was very low in cancerous tissue in hepatitis C patients with HCC. SCYB14 is moderately or highly expressed in normal liver tissue but is expressed at very low levels in human HCC cell lines, including HepG2, HLE, Huh6, Huh7, and PLC/PRF/5, according to the RefExA reference database for gene expression analysis from the Laboratory for System Biology and Medicine at RCAST, the University of

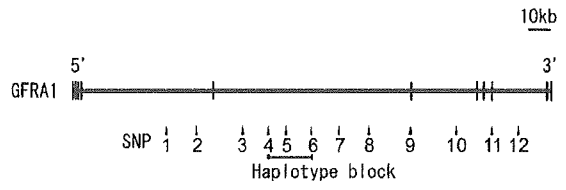

Fig. 2. GFRA1 haplotype block map. Solid boxes represent exons of the GFRA1 gene. The haplotype block in GFRA1 consisted of 3 SNPs (SNP4, rs953920; SNP5, hCV1250702; and SNP6, rs2270181).

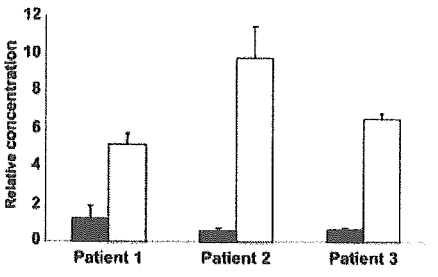

Fig. 3. Relative concentration of SCYB14 in cancerous and noncancerous liver tissues of 3 hepatitis C patients with HCC. Solid bar represents relative concentration of SCYB14 in cancerous liver tissue. Open bar represents relative concentration of SCYB14 in noncancerous liver tissue. Results are expressed as mean ± SD of 3 experiments.

Tokyo (Tokyo, Japan) (http://www.lsbm.org/site_e/database/index.html). In fact, SCYB14 has been reported to be ubiquitously expressed in normal tissue extracts but is absent from a variety of tumor cell lines.[25] Moreover, a previous study with human prostate epithelial cells showed that the expression of SCYB14 is upregulated during senescence, which creates a barrier inhibiting the acquisition of an immortal phenotype, but is downregulated in immortalized cells.[26] These results suggest that SCYB14 may play an important role in carcinogenesis.

CRHR2 was 1 of the 3 genes associated with HCC in our study. CRHR2 mRNA is expressed in liver, although, according to RefExA, its expression level is comparatively low and is similar between normal liver tissue and HCC cell lines. A microarray analysis in our laboratory using the Human 3.8I Glass Array (Clontech Laboratories, Palo Alto, CA) showed that 21 of 27 liver tissue samples obtained by liver biopsy from patients with chronic hepatitis had positive signals for CRHR2 (unpublished data). Although mice deficient for CRHR2 display anxiety-like behavior, are hypersensitive to stress, and have impaired cardiovascular function, the function of CRHR2 in liver is still unknown.[27,28] Urocortin, a corticotropin-related peptide, is thought be the endogenous ligand for CRHR2. Interestingly, the intracisternal injection of urocortin has been reported to exacerbate acute liver injury through the sympathetic nervous system.[29] In addition, urocortin has been shown to be expressed and to exert an anti-inflammatory effect in human gastric mucosa.[30] These data suggest that urocortin may contribute to inflammation, which ultimately increases the risk for developing HCC.[31]

GFRA1 encodes a receptor for glial cell line–derived neutrophic factor (GDNF) and neuturin. GFRA1 is a candidate gene for Hirschsprung disease.[32] Recently, it has been reported that a -193C to G sequence variant in the 5′-untranslated region of GFRA1 was found in 15% of cases with sporadic medullary thyroid cancer, suggesting an association of this variant with carcinogenesis.[33]

Despite the limitations of a cross-sectional study, our analyses showed a prominent effect of 3 gene polymorphisms on the risk of developing HCC. Because most HCV-related HCCs arise from a background of cirrhosis, these 3 SNPs might have association with cirrhosis. In fact, among 3 SNPs, GFRA1 genotype was also associated with the presence of cirrhosis ($P = .002$), although its association was weaker than that with HCC ($P < .001$).

In conclusion, given that many patients are referred to our hospital for the treatment of HCC, our study population may be biased toward patients with HCC. Our multivariate model, however, included most of the previously reported risk factors for HCC plus the polymorphisms of the 3 genes. This implies that our results can be generalized to the Japanese population. The uncertainty of the odds ratios, owing to the study design, should be resolved in a subsequent controlled trial. The genotype of these SNPs may serve as a marker that can be used to identify a subgroup of Japanese patients with chronic HCV infection who have higher risk of developing HCC.

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Clinical Studies

Prediction of the ablated area by the spread of microbubbles during radiofrequency ablation of hepatocellular carcinoma

Nouso K, Shiraga K, Uematsu S, Okamoto R, Harada R, Takayama S, Kawai W, Kimura S, Ueki T, Okano N, Nakagawa M, Mizuno M, Araki Y, Shiratori Y. Prediction of the ablated area by the spread of microbubbles during radiofrequency ablation of hepatocellular carcinoma.
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Abstract: *Background/Aim:* Radiofrequency ablation (RFA) is effective for the treatment of hepatocellular carcinoma (HCC). To prevent the ablation of adjacent organs and vessels, the spread of microbubbles generated by heating during RFA was observed by ultrasonography (US) and used to predict the ablated area; however, several reports documented that discrepancies existed between the spread of microbubbles and the ablated area. *Patients and Methods:* The spread of microbubbles during RFA was observed by US in 24 patients with HCC and the areas were compared with the defect of enhancement in contrast enhanced (CE)-US, using Levovist in the same plane. *Results:* During the ablation, the posterior margin was obscure but the border could be visualized 5 min after the ablation. The size of the area of hyperechogenicity 5 min after ablation and that of the defect observed by CE-US was found to correlate ($r^2 = 0.91$, $P < 0.0001$). The shape of the hyperechogenicity corresponded well to the defect area, even in cases showing irregular spread of the microbubbles. *Conclusion:* The observation of microbubbles during RFA can predict the ablated area and might be useful to prevent the unfavorable ablation of adjacent organs and vessels.

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Key words: contrast enhanced – hepatocellular carcinoma – microbubbles – radiofrequency ablation – ultrasonography

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Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide (1). Over the last two decades, several methods have been developed for HCC treatment, and the prognosis of the patients is improving. Local ablation therapies, including percutaneous ethanol injection therapy (PEIT), microwave coagulation therapy (MCT) and radiofrequency ablation (RFA) therapy, and surgical resection achieve a high rate of complete response in patients with HCC at a relatively early stage. Transcatheter arterial embolization (TAE) is effective especially for the treatment of multiple tumors, and liver transplantation is another tactic for the treatment of HCC (1–4).

RFA can physically ablate a relatively large area in a single session and the therapeutic effect of RFA is stronger than that of PEIT and MCT (3, 5–7). Pain and fever are frequently observed and bleeding in the abdominal or chest cavity is not very rare (0.8%). Abdominal infection, biliary stricture, and visceral damage are also reported. In addition, death caused by RFA procedure has been reported. Damage to the adjacent organs is another complication that may put patients under risk. This complication can be avoided if the real-time observation of the ablated area is possible during the procedure.

The appearance of microbubbles, which indicate that the temperature of the area is above boiling point, is observed by ultrasonography (US) during RFA. To know the ablated area, the spread of the bubbles is used as a practical marker of the treated area; however,

discrepancies between the spread of microbubbles and the ablated area was reported (5, 8). Few reports that precisely compare the spread of the microbubbles and the actual ablated area have been published.

Contrast enhanced (CE)-US using Levovist is useful for evaluating the characteristics of liver tumors (9–14). This method is also applied to evaluate the therapeutic effect of RFA or chemoembolization of HCC, and its ability to detect residual tumor is equal to that of CE-computed tomography (12, 13, 15, 16). In this report, we observed the spread of microbubbles during the treatment of HCC by RFA, evaluated the ablated area by CE-US using Levovist in the same plane, and compared the area to know whether the observation of microbubbles during RFA is useful to monitor the ablated area.

Patients and methods

Patients

Consecutive HCC patients admitted to the Hiroshima City Hospital between March 2003 and October 2004, who met the entry criteria, and agreed to participate in the trial, were enrolled. Entry criteria of this study was as follows: (a) the patients had HCC less than 25 mm in diameter and the nodules were treated by US-guided RFA with single puncture; (b) the depth from the skin to the distal side of the nodule was less than 10 cm; (c) the nodule was not adjacent to the extra-hepatic organs or large vessels so that the nodules could be ablated fully without interruption; (d) RFA was performed without artificial pleural effusion and the ablated area could be observed by US at the same plane after the RFA; (e) the RFA needle was kept at the same place throughout the ablation. Among 91 patients treated by RFA during the period, 24 patients met the criteria and were analyzed in this study. Thirteen patients (54%) were men, and the mean patient age was 68 years (range: 41–82). Mean tumor size was 17 mm (range: 10–25). This study was approved by the institutional review board, informed consent were obtained from all patients, and were in accordance with the Helsinki Declaration of 1975.

Treatment of HCC

RFA was performed under US guidance using 17-gauge cooled-tip RF electrode (Radionics, Burlington, MA). When the diameter of HCC was smaller than 1.5 cm, a 2-cm-long exposed metallic tip was used ($n = 8$). A 3-cm-long exposed tip was used in the rest of the cases ($n = 16$). After penetrating the tumor with the electrode, the generator was set at the impedance mode and the output was increased stepwise from 60 W for a 3-cm-exposed tip (40 W for a 2-cm-exposed tip) until it reached maximum power or was impeded out (20 W increase per minute). After it was impeded out, the power was decreased by 20 W, and the ablation was continued for 12 min (6 min for a 2-cm-exposed tip).

Observation of microbubbles

The spread of microbubbles was observed by US for 10 min after the completion of RFA. At least two pictures were taken from the same puncture plane at 0, 5, and 10 min after the ablation and stored on a magneto-optical (MO) disk.

CE-US

To evaluate the ablated area, CE-US was performed 3–5 days after the RFA. The patients were positioned similarly and the ablated area was observed at the same plane. The ultrasound system, Aplio™ (Toshiba Medical systems, Tokyo, Japan), equipped with advanced dynamic flow (ADF) was used for CE-US. After observing the nodule with harmonic B mode using a 5 MHz convex array probe, ADF was performed (gain level, 85 dB). The conditions were as follows: color gain = 40 dB; pulse-repetition frequency (PRF) = 3.9 kHz; mechanical index (M.I.) = 1.3–1.6; and frame rate = 1.7–2.5. The Levovist solution (300 mg/ml, Nihon Schering, Osaka, Japan) was prepared according to the manufacturer's instructions and 7 ml of the solution was injected through a 20-gauge canula into the left antecubital vein at a speed of 1 ml/s. Two vascular images (early arterial phase and late vascular phase) and one liver parenchymal image were observed with ADF and the pictures were stored on MO and videotape.

Imaging analysis

The length of the long axis (corresponding to the puncture line) and the short axis (perpendicular to the long axis) of the hyperechogenic area, and the defect of the Levovist signals at late vascular phase or parenchymal phase were measured in a blinded fashion by two independent doctors. The data were obtained from at least two pictures and maximum length was obtained as representative data and then the means of the two doctors' data were calculated. When the difference of the length measured by the two doctors exceeded 3 mm (4.4% of the cases), they re-measured the length until the difference reached no more than 3 mm.

Statistics

To evaluate the difference of the lengths between the microbubbles and the defect of Levovist, the paired t-test was used. Correlation between the spread of microbubbles and the areas of defect by CE-US (length of the long axis and short axis) was evaluated by linear regression test. All statistical analyses were performed using the SAS package, version 8, or the JMP software, version 5.1 (SAS Institute, Inc., Cary, NC). P-values smaller than 0.05 were considered significant.

Results

Spread of microbubbles during RFA

During energy deposition of RFA, hyperechogenicity was observed around the electrode tip and gradually increased to cover the tumor. At the same time, the drainage of microbubbles was observed in hepatic veins and portal veins. The spread of microbubbles reached a maximum at the end of RFA. At that time, the anterior margin of microbubbles was clearly observed whereas the posterior margin was equivocal because of irregular reflections of ultrasound by the generated bubbles. After completing the ablation, the posterior border became visible gradually and the border could be determined clearly 5 min after the end of the ablation (Fig. 1). The bubbles gradually disappeared. However, the shape of the hyperechogenic area remained constant 5 min after the ablation in all cases. Ten minutes after the ablation, the border became irregular because of the movement of microbubbles to the surrounding area and the size could not be measured in two cases because the hyperechogenicity was too sparse. Thus, in the following studies, we adopted the pictures taken 5 min after the ablation as representative of the spread of microbubbles, because the border of bubbles was clear at that time and the size of the area was constant for at least 5 min (Fig. 2).

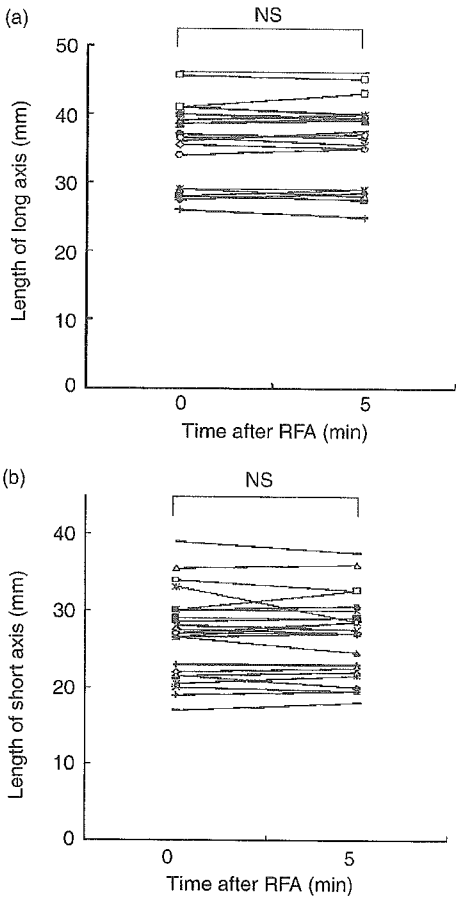

Fig. 2. The size of hyperechogenicity after radiofrequency ablation (RFA). The lengths of the long axis (a) and the short axis (b) of the hyperechogenic area did not changed for at least 5 min after the ablation.

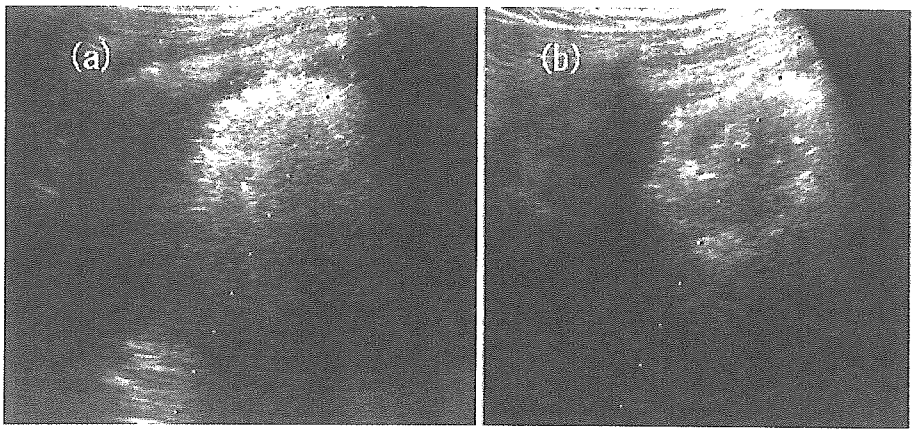

Fig. 1. The findings of ultrasonography during radiofrequency ablation. The posterior margin of the ablated area was obscure while the anterior margin and lateral margins were clear during the ablation (a). The posterior margin became visible gradually and the border could be visualized 5 min after the ablation (b).

CE-US

Vessels are gradually enhanced approximately 15 s after starting the injection of Levovist. From the beginning of the enhancement at the early arterial phase to the end of the late vascular phase (approximately 50 s following injection), the enhancement was not observed around the needle tract. At the parenchymal phase (5 min following injection), the defect of the enhancement was observed in the same area (Fig. 3). Pictures of the parenchymal phase were used for further analysis.

Comparison of microbubbles and CE-US

The shapes were well matched between the spread of microbubbles and the defect at CE-US (Fig. 4a). The correlation was observed even in cases showing irregular spread of the microbubbles (Fig. 4b). The lengths of the long axis and the short axis closely correlated between the defect at CE-US and the spread of microbubbles [Fig. 5, $y = -1.18+1.05x$ (mm), $r^2 = 0.91$, $P<0.0001$].

Discussion

The increase in echogenicity during ablation is a well-known phenomenon and spread of hyperechonenicity was used to estimate the boundaries of the ablated area (5, 7, 8). There are two possible explanations for the hyperechogenicity generated around the needle tip. First, the tissue in the area was heated to boiling point and the tissue and water vapor microbubbles were formed, meaning that the area was ablated. Second, the cells were not heated enough to generate microbubbles, but microvessels in the area filled with microbubbles flew out from the ablated area and stayed because of the hyperemia induced by the ablation (5). We observed the kinetics of microbubbles and demonstrated that the size of the area did not change for at least 5 min in all cases. In addition, CE-US revealed that blood flow was interrupted, and the englobe function of Kupffer cells that can be identified by Levovist imaging was damaged in the same area for at least 2 days. These results indicated that the area of hyperechogenicity matched the ablated area.

In this study, we found that the posterior margin could be visualized well within 5 min after the ablation, and the area well correlated with the ablated area that was defined by CE-US. However, some reports found that the area of hyperechogenicity seen on US did not correlate well and cannot be used to predict the ablated area (8, 17). The major reason for the disagreement seems to be based on the invisibleness of the posterior margin of the hyperechogenicity by irregular reflections of ultrasound by the bubbles during the ablation. We also could not observe the posterior margin during the ablation; however, the anterior border and bilateral borders were clearly observed during the ablation and the border did not change for 5 min, which well correlated with the ablated area analyzed by CE-US. Therefore, operators can predict the areas of ablation during RFA by changing the placement of the US-probe and observing the spread of microbubbles from different directions, although it is sometimes impossible to observe the tumor from other sites because of physical limitations. By observing the area of hyperechogenicity carefully, the damage to the adjacent vessels and organs could be reduced.

The observation of microbubbles may have another merit to predict the complication. We observed sector-shaped hyperechogenicity toward the liver surface from the tumor during the ablation. The area turned out to be the place

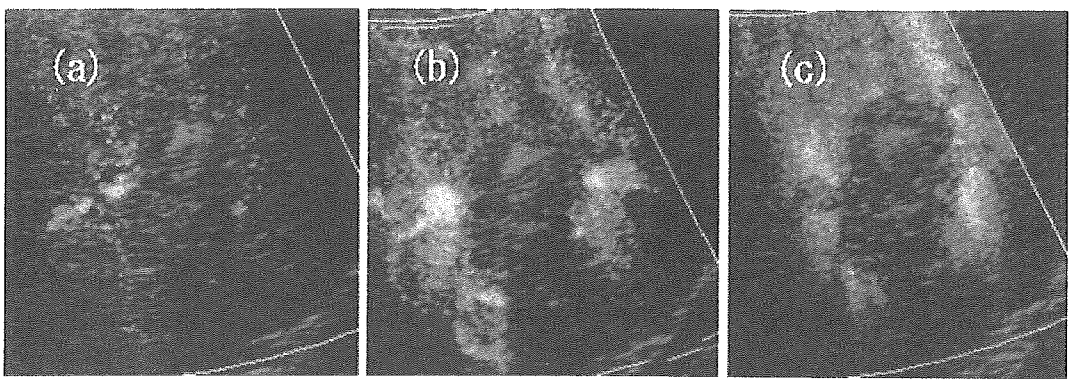

Fig. 3. Contrast enhanced-ultrasonography (CE-US) by Levovist. No enhancement was observed in the ablated area in CE-US using Levovist. Note that the size of the unenhanced area did not change from the arterial phase to the liver parenchymal phase. (a) early arterial phase, (b) late vascular phase, (c) liver parenchymal phase (Kupffer phase).

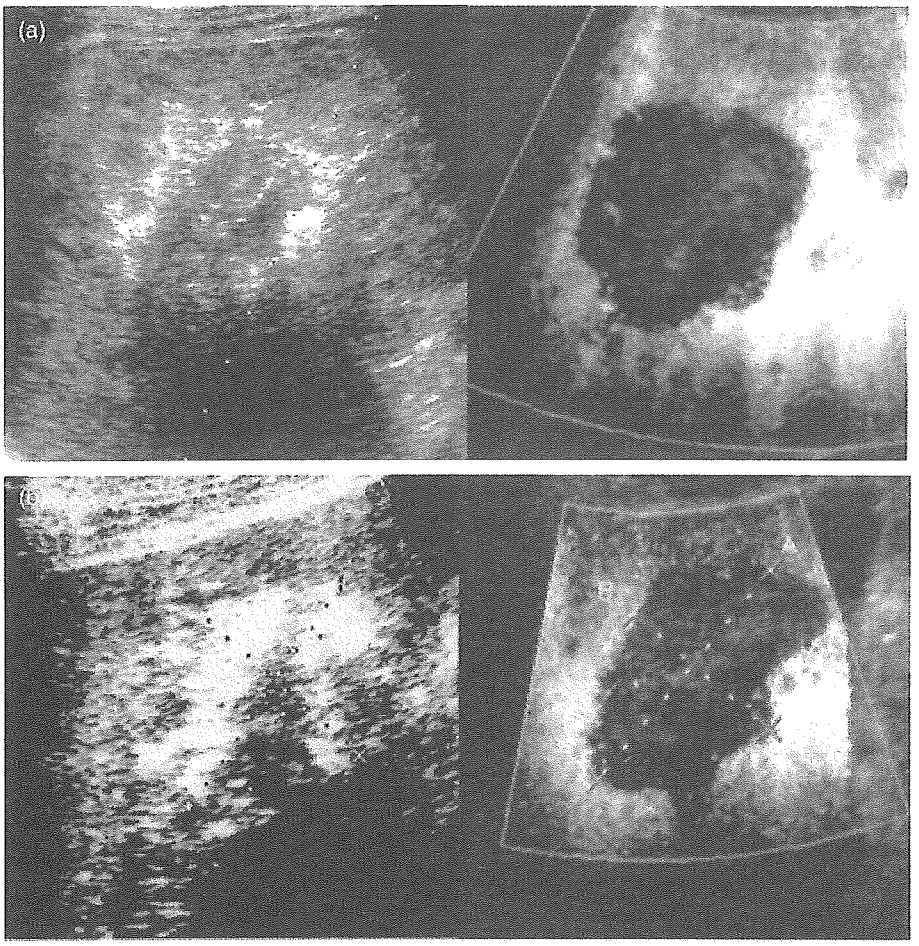

Fig. 4. The images of hyperechogenicity 5 min after the ablation and contrast enhanced-ultrasonography (CE-US). The area of hyperechogenicity matched well with the defect of CE-US in cases of both oval-shaped ablation (a) and irregular-shaped ablation (b).

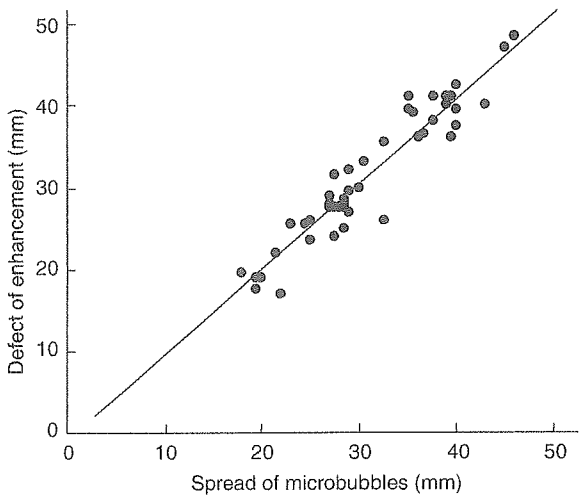

Fig. 5. Correlation between the hyperechogenic area and defect of the enhancement by contrast enhanced-ultrasonography (CE-US). The lengths of the long and the short axis of the hyperechogenic area linearly correlated with those of CE-US [$y = -1.18+1.05x$ (mm), $r^2 = 0.91$, $P<0.0001$].

of hepatic infarction by CT. The observation of the drainage of microbubbles during ablation, and the appearance of sector-shaped weak hyperechogenicity is not rare; however, the gradual increase in microbubble accumulation might be a sign of infarction.

In conclusion, an overall observation of the microbubbles at and around the tumor is useful to know real-time ablation status during the procedure and consequently may help to decrease the complications of RFA.

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ARTICLE

Antiviral Therapy for Cirrhotic Hepatitis C: Association with Reduced Hepatocellular Carcinoma Development and Improved Survival

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Background: Although cirrhosis is a major risk factor for development of hepatocellular carcinoma, no definitive prospective analyses have assessed the long-term efficacy of antiviral therapy in cirrhotic patients.

Objective: To elucidate the role of antiviral therapy in the suppression of liver tumors and survival over a long-term follow-up period.

Design: Prospective cohort study.

Setting: 25 clinical centers.

Patients: 345 patients with chronic hepatitis C and cirrhosis enrolled in previous trials.

Intervention: 271 patients received 6 to 9 million U of interferon 3 times weekly for 26 to 88 weeks; 74 received no treatment.

Measurements: Blood tests and abdominal ultrasonography were done regularly to detect hepatocellular carcinoma.

Results: Hepatocellular carcinoma was detected in 119 patients during a 6.8-year follow-up: 84 (31%) in the interferon-treated group and 35 (47%) in the untreated group. Cumulative incidence of hepatocellular carcinoma among interferon-treated patients was significantly lower than in untreated patients (Cox model: age-adjusted hazard ratio, 0.65 [95% CI, 0.43 to 0.97]; $P = 0.03$), especially sustained virologic responders. A total of 69 patients died during follow-up: 45 (17%) in the treated group and 24 (32%) in the untreated group. Interferon-treated patients had a better chance of survival than the untreated group (Cox model: age-adjusted hazard ratio, 0.54 [CI, 0.33 to 0.89]; $P = 0.02$). This was especially evident in sustained virologic responders.

Limitation: This was not a randomized, controlled study. Patients enrolled in the control group had declined to receive interferon treatment even though they were eligible for treatment.

Conclusion: Interferon therapy for cirrhotic patients with chronic hepatitis C, especially those in whom the infection had been cured, inhibited the development of hepatocellular carcinoma and improved survival.

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*For members of the Tokyo-Chiba Hepatitis Research Group, see the Appendix, available at www.annals.org.

Chronic hepatitis C is a common disease that progresses slowly to cirrhosis and eventually may lead to hepatocellular carcinoma (1–5). The annual incidence of hepatocellular carcinoma and mortality rate were 1.4% to 3.3% and 1.9% to 5.5%, respectively, in retrospective series of white patients with hepatitis C virus (HCV)–related compensated cirrhosis (4–9); in Japan, the annual incidence of hepatocellular carcinoma was 5% to 7% (10–12). Risk factors for hepatocellular carcinoma include age older than 50 to 60 years, male sex, advanced fibrosis stage, high histologic activity score, and high alanine aminotransferase (ALT) levels (4–14).

Several retrospective studies have shown inhibition of hepatocellular carcinoma development after interferon therapy (11–14). This inhibitory effect was seen in patients with moderate fibrosis for whom antiviral therapy was effective (11–14). However, the inhibitory effect in patients with liver cirrhosis was not statistically significant (4, 7, 8, 15), possibly because of the low efficacy of interferon therapy in cirrhotic patients (15–17). Other retrospective (6, 9) and prospective (18) studies that had small patient samples indicated that interferon therapy reduced the development of hepatocellular carcinoma.

Because cirrhosis is a major risk factor for hepatocellular carcinoma, a prospective study is needed to determine whether interferon therapy benefits cirrhotic patients. We previously performed 2 prospective studies on the efficacy of interferon treatment in cirrhotic patients (19, 20). During enrollment, many cirrhotic patients who fulfilled the inclusion criteria were enrolled as controls to clarify the long-term effect of interferon therapy on development of liver tumors. We conducted a 7-year study on the inhibition of hepatocellular carcinoma development in the previous cohorts of our multicenter, prospective study.

METHODS

Study Sample

Enrollment of Patients with Compensated Liver Cirrhosis

Designs for the 2 protocols, "Interferon alfa-2a prospective trial for cirrhotic patients—modification of treat-

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Appendix
Conversion of figures and tables into slides

Context

Few studies address long-term outcomes of antiviral therapy for patients with chronic hepatitis C and cirrhosis.

Contribution

This prospective study of adults with chronic hepatitis C and cirrhosis compares outcomes between 74 patients who declined treatment and 271 patients treated with thrice-weekly interferon injections for 26 to 88 weeks. Median follow-up was 6.8 years. Fewer treated patients developed hepatocellular cancer (31% vs. 47% of untreated patients) or died (17% vs. 32%).

Cautions

Because the study was not a randomized, controlled trial, prognostic factors other than interferon might have contributed to the differences between groups.

–The Editors

ment duration by monitoring HCV RNA in the serum" (19) and "Natural interferon trial for cirrhotic patients—modification of interferon treatment duration according to pretreatment viral load" (20) were finalized on 18 December 1992 and 20 April 1993, respectively. While discussing these 2 protocols, we decided to extend the prospective studies after the initial trial to examine the effect of antiviral therapy on the inhibition of hepatocellular carcinoma development and patient survival as secondary end points.

Inclusion Criteria

Our previous reports (19, 20) describe in more detail the diagnosis of chronic hepatitis C with cirrhosis and the inclusion criteria for the 2 trials (19, 20). The diagnostic criteria were elevated serum ALT levels for more than 6 months, positivity for anti-HCV antibody by the phytohemagglutinin assay (Dinabbot, Tokyo, Japan) or enzyme-linked immunosorbent assay (Ortho Diagnostic Systems, Tokyo, Japan), and abnormal histologic findings on liver biopsy specimens. The presence of HCV RNA was tested by reverse transcriptase polymerase chain reaction (RT-PCR) (detection limit, 10^2 copies/mL), and the serum HCV RNA level was measured by competitive RT-PCR according to the method of Kato and colleagues (21). The HCV genotype was established by using the method of Okamoto and colleagues (22). Liver biopsy was done in all patients within 12 months before enrollment, and specimens were evaluated according to the criteria of Desmet and colleagues (23). Inclusion criteria were based on liver histologic characteristics indicating fibrotic stage F4, positivity for HCV RNA by RT-PCR, platelet count greater than 50×10^9 cells/L, leukocyte count greater than 3×10^9 cells/L, and Child–Pugh A classification.

We excluded patients who had liver cirrhosis caused by hepatitis B, autoimmune hepatitis, primary biliary cirrhosis, or drug-induced liver disease. Before enrollment, patients had abdominal ultrasonography, dynamic computed tomography (CT), or magnetic resonance imaging (MRI); we excluded patients who were found to have hepatocellular carcinoma.

Antiviral Therapy

A total of 157 patients received 9 million units (MU) of interferon-α2a (Nippon Roche KK, Tokyo, Japan) by intramuscular injection 3 times a week for 32 to 88 weeks; duration of therapy was based on serum HCV RNA status during therapy (19). The mean and median duration of therapy were 44 and 48 weeks, respectively, and the mean and median dose of interferon were 1011 and 936 MU (range, 42 to 2378 MU), respectively. A total of 114 patients received 9 or 6 MU of natural interferon-α (Sumitomo Pharmaceutical Co., Osaka, Japan) by subcutaneous injection 3 times a week for 6 months (patients with low viral load) or 12 months (patients with high viral load) (20). The mean and median duration of therapy were 33 and 26 weeks, respectively, and the mean and median dose of interferon were 688 MU and 564 MU (range, 18 to 1404 MU), respectively.

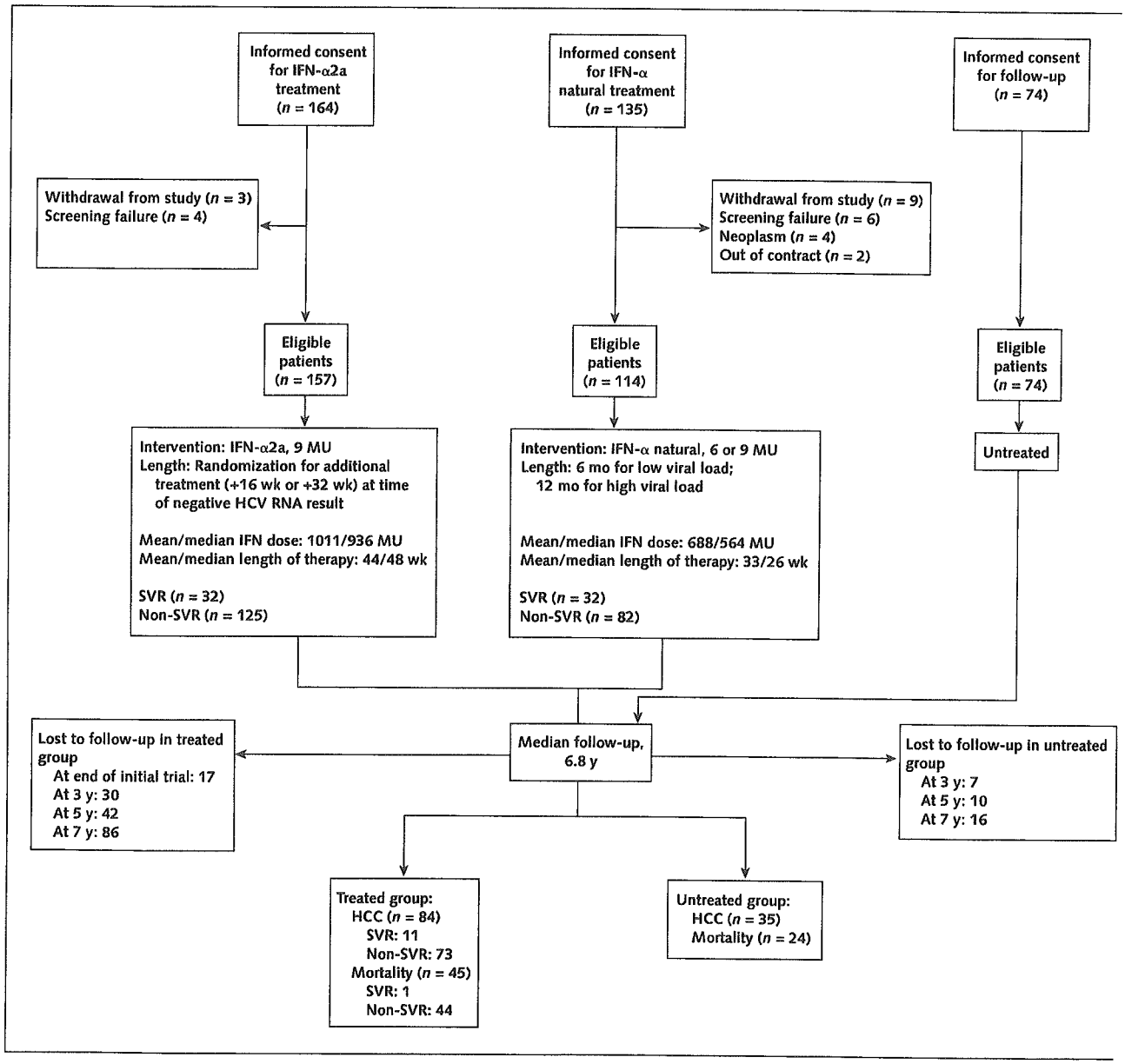
For the 2 trials combined, the mean duration of treatment was 39 weeks (range, 1 to 88 weeks) and the mean dose of interferon was 875 MU (range, 18 to 2376 MU). Eighty-eight percent of the patients took more than 80% of the drug during 80% of the scheduled treatment period.

Patients negative for HCV RNA more than 24 weeks after the completion of interferon therapy were considered to show a sustained virologic response, while patients positive for HCV RNA more than 24 weeks after the completion of interferon therapy were considered to show a nonsustained response.

Study Design

This was not a randomized study. A total of 271 patients received interferon therapy; 74 patients who fulfilled the inclusion criteria for the trials but declined to receive interferon therapy instead received periodic medical screenings at outpatient clinics in each institute, provided informed consent, and were enrolled in the untreated group (**Figure 1**). Thus, the sample size for this study was set at 345 as of April 1996. We established a 5-year follow-up study to obtain statistical significance with a power of 80% on the assumption that the efficacy of interferon therapy would be 25% and the incidence of hepatocellular carcinoma development among responders would be reduced to a risk ratio of 0.3 compared to untreated patients or nonsustained responders based on the preliminary data from the retrospective cohort study (11). In April 2001, we extended the length of the mean follow-up period from 5 years to 7 years because the incidence of hepatocellular carcinoma development in the interferon group was higher than initially anticipated.

Figure 1. Flow diagram of the trial.



HCC = hepatocellular carcinoma; IFN = interferon; Non-SVR = nonsustained virologic response; SVR = sustained virologic response.

Approval

The ethics committee of each participating institution approved the study. Informed consent was obtained from each patient according to the Helsinki Declaration. Previously participating physicians at 6 institutes had resigned before this follow-up study began, and the new chief physicians did not resubmit this protocol to the ethical committee of each institute. Thus, we did not follow the patients enrolled at these institutes (n = 17). We considered these patients to be censored participants who did not go on to participate in the subsequent follow-up study.

Patient Follow-up

We followed patients by performing blood tests and measuring biochemical variables every 1 to 2 months. Abdominal ultrasonography was done every 3 to 6 months. Patients did not receive any additional antiviral therapy thereafter because the Japanese National Health Insurance plan did not approve interferon treatment for cirrhotic patients. If a patient relocated during follow-up, data from medical examinations at the nearest outpatient clinic were collected from a private physician or by fax or telephone. Patients without data from a medical consultation were

Table 1. **Baseline Clinical Characteristics at Time of Enrollment in Initial Trials***

Characteristic†	Interferon-Treated Patients (n = 271)	Untreated Patients (n = 74)	P Value
Age, y‡	57 (49, 62)	61 (55, 73)	<0.001
Men/women, *n/n*	102/169	35/39	0.17
Blood transfusion (yes/no/unknown), *n/n/n*	138/125/8	28/27/19	>0.2
Albumin level (n = 268, 69), *g/L*‡	40 (37, 42)	40 (37, 42)	>0.2
ALT level (n = 271, 72), *IU/L*‡	97 (57, 142)	75 (53, 106)	0.008
AST level (n = 268, 69), *IU/L*‡	82 (57, 118)	73 (53, 100)	0.08
Platelet count (n = 271, 70), $\times 10^9$ *cells/L*‡	105 (85, 134)	105 (86, 134)	>0.2
HCV RNA level (n = 270, 74), $\times 10^n$ *copies/L*‡	5.8 (4.5, 6.3)	5.5 (4.3, 6.8)	>0.2
HCV genotype, *n (%)*			
Genotype 1b	199 (73.4)	49 (66.2)	>0.2
Genotype other than 1§	67 (24.7)	23 (31.1)	
Unknown genotype	5 (1.8)	2 (2.7)	

* ALT = alanine aminotransferase; AST = aspartate aminotransferase; HCV = hepatitis C virus.
† In first column, the n values in parentheses refer to the number of patients in the interferon-treated and untreated groups, respectively, if some data were missing.
‡ Values are the median (25th, 75th percentile).
§ Consists of genotypes 2a and 2b; no patients in this study had genotype 4.

contacted by letter or telephone and advised to receive a medical check-up at the closest outpatient clinic. The median follow-up period from the time of initial enrollment was 6.8 years (range, 0.04 to 10.4 years).

Detection of Hepatocellular Carcinoma

If a suspicious hepatocellular lesion was detected by ultrasonography, the patient had dynamic CT or MRI, along with arteriography. Board-certificated radiologists at each institute diagnosed hepatocellular carcinoma on the basis of typical patterns, such as early-phase hyperattenuation area and late-phase hypoattenuation on dynamic CT or MRI. At times, board-certified pathologists who were unaware of patients' clinical data confirmed the diagnosis using ultrasonography-guided tumor biopsy.

Treatment of Hepatocellular Carcinoma

If the liver tumor consisted of fewer than 3 nodules that were less than 3 cm in diameter, patients received percutaneous ethanol injection therapy, microwave coagulation therapy, radiofrequency ablation therapy, or surgical hepatectomy (24–28). Patients with stage III and IV hepatocellular carcinomas were treated with transarterial chemoembolization or chemotherapy (29).

Patient Survival

We examined patient survival or the causes of death.

Statistical Analysis

We used SAS, version 8.2 (SAS Institute, Inc., Cary, North Carolina), for statistical analysis. A Wilcoxon test or Fisher exact test was used to compare the distribution of variables between the groups. The primary outcomes were hepatocellular carcinoma development, overall survival, and liver-related mortality. The time frame for each outcome was defined as the time from study entry until onset of the event. Kaplan–Meier life tables were constructed for development of hepatocellular carcinoma and patient survival. Each continuous factor except age was transformed into a 2-category variable by using the median as a cutoff point, and age was considered a continuous variable. Statistical analysis was performed by using univariate and multivariate models. Several baseline data were missing (for 8 of 345 patients at most); however, we did not use any imputational method and excluded missing and unclassified data from our analysis. Only the complete data set (334 of 345 patients) was used for multivariate analysis.

Hepatocellular carcinoma development and patient survival were compared between the interferon-treated and untreated groups by using the Cox numeric age-adjusted model and a model stratified by 3 age groups. We used Schoenfeld residuals and (log(-log(survival), log(time)) (LLS plot) to assess the proportional hazards assumption. Since the survival curves for the interferon-treated and untreated patients crossed each other, a piecewise Cox model with hazard ratios changing over time (year <6 and year ≥ 6) was used in an exploratory analysis (30).

Additional analyses (post hoc) were further stratified according to the presence of HCV RNA after interferon treatment. All reported P values are 2-sided, and P values less than 0.05 are considered statistically significant.

RESULTS

Baseline Characteristics

In the initial studies, 271 patients received interferon therapy and 74 were untreated. **Table 1** shows patient demographic characteristics at the time of enrollment. The only characteristics that differed between the treated and untreated groups were age ($P < 0.001$) and serum ALT level ($P = 0.008$). Of the 271 interferon-treated patients, 64 showed a sustained virologic response and 207 showed a nonsustained response. Sustained virologic response in the interferon-α2a trial and the natural interferon-α trial occurred in 32 (20%) and 32 (28%) patients, respectively. The end-of-treatment response rate and the sustained re-

sponse rate for HCV genotype 1 were 35% (70 of 199 patients) and 15% (30 of 199 patients), respectively; the respective rates for genotypes other than 1 were 64% (46 of 72 patients) and 47% (34 of 72 patients).

Seventeen patients (4.9%) dropped out at the end of the preceding trials; at 3, 5, and 7 years, 30, 42, and 86 patients from the interferon-treated group and 7, 10, and 16 patients from the untreated group dropped out, respectively (**Figure 1**).

Development of Hepatocellular Carcinoma

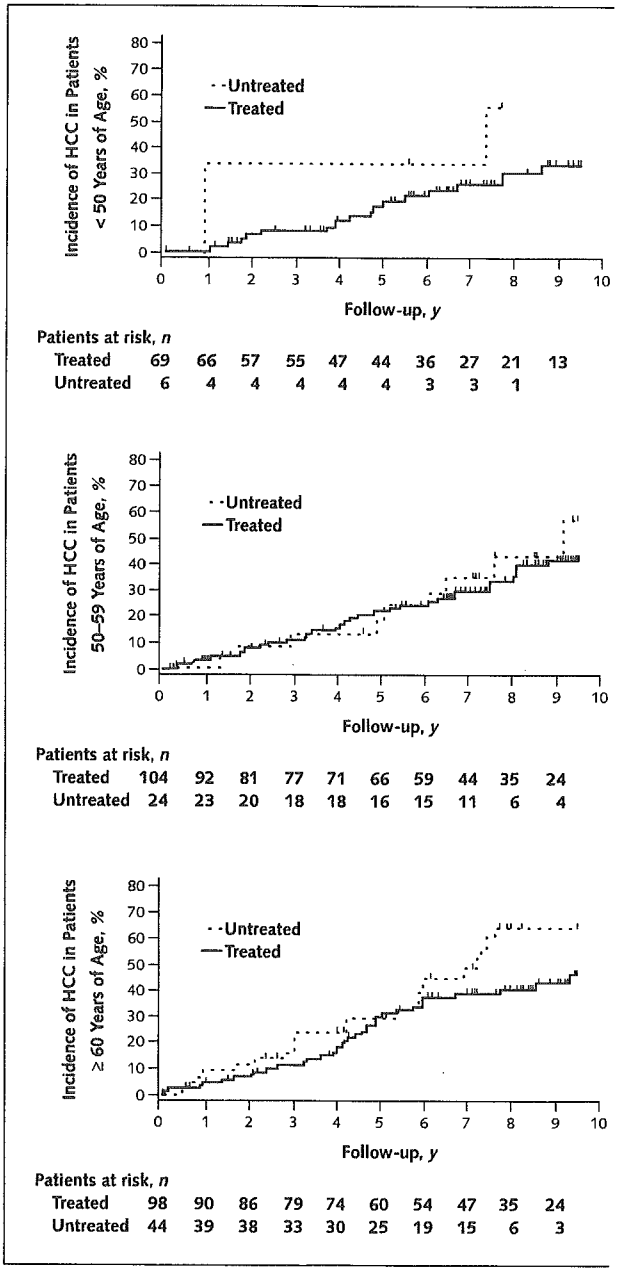
Hepatocellular carcinoma was detected in 119 patients during follow-up: 84 (31.0%) of the 271 interferon-treated patients and 35 (47.3%) of the 74 untreated patients. Of the 64 patients with a sustained virologic response and 207 with a nonsustained response, hepatocellular carcinoma developed in 11 (17.2%) and 73 (35.3%) patients, respectively. The median tumor nodule diameter was 2 cm (range, 1 to 10 cm). The median number of tumors was 1 (range, 1 to 6); 75 patients (62%) had 1 nodule, and 44 patients had 2 or more nodules. The size and number of tumors did not differ between the interferon-treated and untreated groups. All patients with hepatocellular carcinoma had dynamic CT or MRI and arteriography, as well as abdominal ultrasonography. One hundred thirteen patients (95%) showed a typical pattern of hyperattenuation in the early phase and hypoattenuation in the late phase on dynamic CT or MRI. Tumor blush was confirmed in these patients. Six patients were found to have atypical features (hypoattenuation in the early phase and late phase) on dynamic CT. Hepatocellular carcinoma was histologically confirmed for 83 patients (70%), including the 6 patients who had atypical features on dynamic CT. Hepatocellular carcinoma in the remaining 36 patients was diagnosed by the typical pattern on dynamic CT or MRI, and these tumors were characterized by larger size (median diameter, 3 cm), multiple tumor nodules (≥3 nodules in 12 patients), and high serum α-fetoprotein (AFP) levels (≥100 ng/mL in 13 patients).

Although we had not initially determined how often serum AFP levels would be measured during follow-up, serum AFP was measured in 79 patients at the time of hepatocellular carcinoma diagnosis; 21 patients had an AFP level of at least 100 ng/mL, 15 had a level of 50 to 99 ng/mL, and 43 had a level less than 49 ng/mL.

We analyzed the cumulative incidence of tumor development by using the Kaplan–Meier method (**Figure 2**). The cumulative incidence of hepatocellular carcinoma was lower in interferon-treated patients than in untreated patients, and the hazard ratio for treatment was similar between the models stratified by 3 age groups and the numeric age-adjusted model (age-adjusted hazard ratio, 0.65 [95% CI, 0.43 to 0.97]); $P = 0.03$).

Of the 119 patients with hepatocellular carcinoma, 7 had surgical hepatectomy, 103 had local ablation therapy using percutaneous ethanol injection or radiofrequency,

Figure 2. **Cumulative incidence of hepatocellular carcinoma (HCC) (age-stratified analysis).**



The Kaplan–Meier method was used to gauge the cumulative incidence of hepatocellular carcinoma development in interferon-treated and untreated patients stratified according to age (<50 years, 50 to 59 years, ≥60 years).

and 9 had transarterial chemoembolization. No patients underwent liver transplantation. Of the 84 interferon-treated patients, surgical hepatectomy was performed in 6 (7.1%), local ablation therapy in 72 (85.7%), and transarterial chemoembolization in 6 (7.1%). Of the 35 control patients, 1 (2.9%) had hepatectomy, 31 (88.6) had local ablation, and 3 (8.6%) had a transarterial procedure.

Table 2. **Predictors of Development of Hepatocellular Carcinoma and Death***

Predictor	Hepatocellular Carcinoma				Death			
	Univariate Analysis		Multivariate Analysis		Univariate Analysis		Multivariate Analysis	
	Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)	*P* Value
Age (for every 1-year increment)	1.04 (1.01–1.06)	0.003	1.04 (1.01–1.07)	0.003	1.05 (1.01–1.08)	0.005	1.05 (1.01–1.08)	0.008
Sex (female vs. male)	1.08 (0.75–1.06)	>0.2			0.89 (0.55–1.45)	>0.2		
Albumin level (≥41 vs. ≤40 g/L)	0.62 (0.42–0.90)	0.01	0.66 (0.44–0.98)	0.04	0.58 (0.35–0.97)	0.04	0.69 (0.41–1.17)	0.17
AST level (≥80 vs. ≤79 IU/L)	1.44 (0.99–2.08)	0.05	1.12 (0.65–1.93)	>0.2	1.88 (1.15–3.08)	0.01	2.04 (0.99–4.21)	0.05
ALT level (≥91 vs. ≤90 IU/L)	1.37 (0.96–01.97)	0.09	1.48 (0.87–2.52)	0.15	1.43 (0.88–2.31)	0.15		
Platelet count (≥106 vs. ≤105 × 10^9 cells/L	0.64 (0.44–0.92)	0.02	0.81 (0.54–1.20)	>0.2	0.64 (0.39–1.04)	0.07	0.78 (0.46–1.30)	>0.2
HCV RNA								
Viral load ($10^{5.5}$ vs. ≥$10^{5.5}$ copies/mL)	1.34 (0.93–1.93)	0.12			1.27 (0.79–2.07)	>0.2		
Genotype (non-1 genotype vs. genotype 1)	0.88 (0.57–1.36)	>0.2			0.76 (0.42–1.36)	>0.2		
Interferon treatment (treated vs. untreated; age-adjusted)	0.65 (0.43–0.97)	0.03†	0.61 (0.41–0.93)	0.02	0.54 (0.33–0.89)	0.02†	0.55 (0.33–0.93)	0.02
Trial (natural interferon vs. interferon-α2a)	0.80 (0.55–1.18)	>0.2			0.88 (0.52–1.47)	0.62		
Post hoc analysis								
Total interferon dose (per 100 MU)	0.97 (0.92–1.02)	0.19			0.95 (0.89–1.02)	0.17		
Interferon response								
SVR vs. untreated	0.31 (0.16–0.61)	<0.001†			0.05 (0.006–0.34)	0.003†		
Non-SVR vs. untreated	0.77 (0.51–1.16)	0.21†			0.71 (0.43–1.18)	0.19†		

* ALT = alanine aminotransferase; AST = aspartate aminotransferase; HCV = hepatitis C virus; non-SVR = nonsustained virologic response; SVR = sustained virologic response.
† Age-adjusted analysis.

Treatment methods did not differ between the interferon-treated and untreated groups ($P > 0.2$).

We used a Cox proportional hazards model to analyze the predictors of hepatocellular carcinoma. In the univariate analysis, age, albumin, platelet count, and interferon treatment were significant factors in the inhibition of hepatocellular carcinoma development (**Table 2**); HCV RNA load, HCV genotype, ALT level, the difference of the preceding trial, and total interferon dose were not significant. In a multivariate analysis, age, treatment, and albumin level were significant predictors (**Table 2**).

When we stratified interferon-treated patients according to sustained response state, cumulative incidence of hepatocellular carcinoma among sustained responders was lower than in untreated patients (age-adjusted hazard ratio, 0.31 [CI, 0.16 to 0.61]; $P < 0.001$), although a nonsustained response was not significant compared with untreated controls (age-adjusted hazard ratio, 0.77 [CI, 0.51 to 1.16]; $P > 0.2$).

Mortality and Survival

Causes of Death

Sixty-nine patients died during the follow-up period: 45 patients (17%) from the interferon-treated group and 24 (32%) from the untreated group (**Table 3**). Thirty-two of the interferon-treated patients died of liver-related diseases: 25 of hepatocellular carcinoma, 6 of liver failure, and 1 of varix rupture. Of the 13 remaining interferon-treated patients, 2 died of pancreatic cancer, 4 of cerebral hemorrhage, 3 of pneumonia or pulmonary interstitial fibrosis, 1 of heart disease, 1 of peritonitis, 1 of suicide, and 1 of unknown causes. Of the 24 untreated patients, 19 died of liver-related diseases: 11 of hepatocellular carcinoma and 8 of liver failure. The other 5 patients died of neoplasms in the stomach and biliary tract, cerebral hemorrhage, heart failure, and peritonitis, respectively. Deaths from liver disease (hepatocellular carcinoma, hepatic failure, or varix rupture) occurred in none of 64 sustained responders, 32 (15%) of 207 nonsustained responders, and 19 (26%) of 74 untreated patients ($P < 0.001$ and $P = 0.08$, respectively).

Survival

The median duration of follow-up for the survivors was 8.0 years (25th and 75th percentiles, 5.8 and 9.3 years). We analyzed survival by using the Kaplan–Meier method with an age-adjusted analysis (**Figure 3**). We used a Cox proportional hazards model to analyze predictors. Of the variables tested, only age and treatment were significant predictors in the multivariate analysis (**Table 2**). Interferon-treated patients had a better chance of survival than did the untreated patients (Cox model: age-adjusted hazard ratio, 0.54 [CI, 0.33 to 0.89]; $P = 0.02$). Because the survival curves in the interferon-treated and untreated patients crossed each other, we used a piecewise Cox model in which the hazard ratio changed over time. The age-adjusted hazard ratio for survival in the interferon group

was 1.03 (CI, 0.46 to 2.29; $P > 0.2$) before the 6-year follow-up and 0.32 (CI, 0.16 to 0.62; $P = 0.001$) after the 6-year follow-up. Furthermore, sustained virologic response was associated with a better chance of survival (age-adjusted hazard ratio, 0.05 [CI, 0.006 to 0.34]; $P = 0.003$ compared with the untreated group) than was nonsustained virologic response (age-adjusted hazard ratio, 0.71 [CI, 0.43 to 1.18]; $P = 0.19$ compared with the untreated group).

Discussion

Cirrhosis is a major risk factor for hepatocellular carcinoma and death. Antiviral therapy may be the most beneficial form of treatment for patients with chronic hepatitis C and cirrhosis, although this idea remains controversial. Retrospective cohort studies involving many patients with chronic hepatitis C showed that antiviral therapy reduces the risk for hepatocellular carcinoma in patients who have mild to moderate fibrosis (11–14). Nishiguchi and colleagues (18) showed that interferon therapy can reduce the incidence of hepatocellular carcinoma in cirrhotic patients, regardless of interferon response. However, these investigators examined only 90 patients and reported a low rate of response to interferon therapy (sustained response, 16%). Cohort studies by Fattovich (7) and Niederau (8) and their colleagues showed that interferon therapy does not reduce the risk for hepatocellular carcinoma, perhaps because of the lower rate of liver tumor development (1% to 2% annually), low response rate (7% to 12%), and short follow-up period (4 to 5 years). A retrospective study by Serfaty and coworkers (9) showed that interferon treatment induces a favorable outcome for hepatocellular carcinoma development or decompensation; however, their study included only 103 patients. Because of the contradictory findings and various built-in biases of previous studies, we designed a prospective study to clarify the exact role of interferon therapy in reducing the rate of hepatocellular carcinoma over a 7-year observation period.

Our study participants consisted of interferon-treated and untreated patients who were enrolled in our 2 preceding prospective trials. Those trials had been conducted to clarify the efficacy of interferon therapy in patients with chronic hepatitis C and cirrhosis (19, 20). One limitation we encountered was that while untreated patients fulfilled the inclusion criteria for the trials, interferon-treated patients and untreated patients differed with regard to age and serum ALT levels. This difference may be due to greater anxiety in older patients or patients with mildly elevated ALT levels stemming from reports in the popular press in Japan in the 1990s about the high prevalence of adverse effects associated with interferon. Other than age and serum ALT levels, the interferon-treated and untreated groups were very similar and the untreated group was not sicker (**Table 1**). Because only age was favorable to the outcome for interferon-treated patients, we accounted for age in the statistical analyses. However, abnormal ALT, aspartate aminotransferase, and albumin levels did not affect the hazard ratio of interferon use.

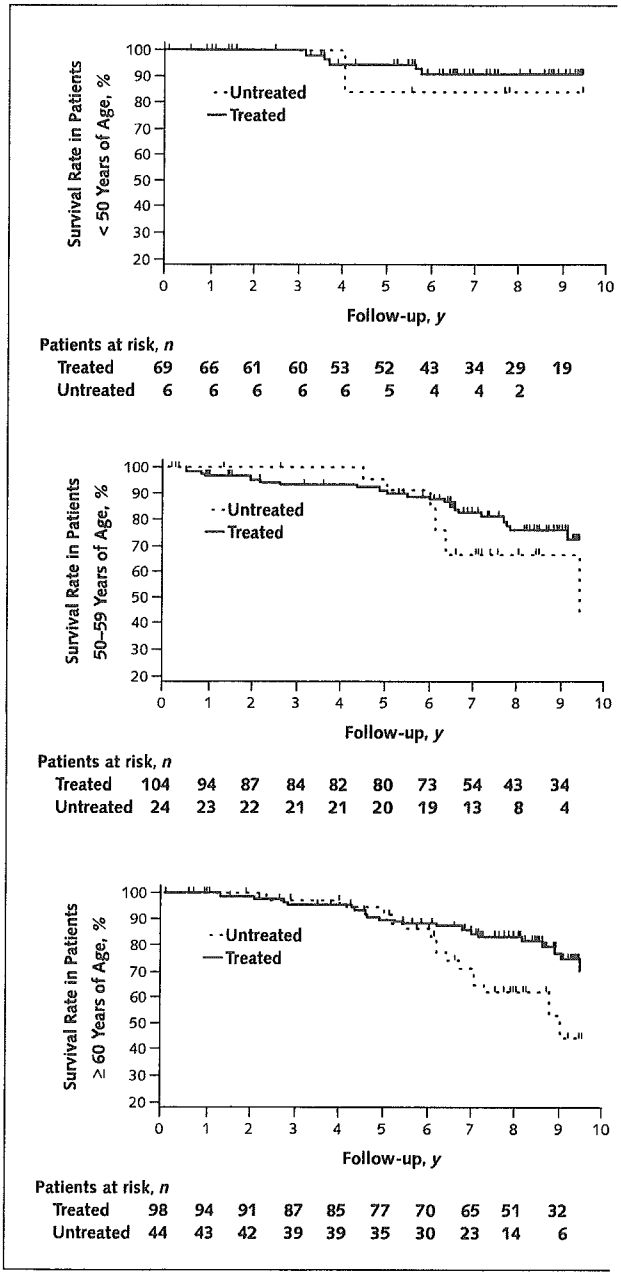
In addition to the preceding limitation, ours was not truly a randomized trial because we did not randomly choose the patients in the untreated groups. Instead, these groups consisted of patients who were offered but declined treatment. This situation introduces a possible bias because not only were the patients older and otherwise different

Table 3. **Causes of Death***

Cause of Death	Interferon-Treated Patients (n = 271)			Untreated Patients (n = 74)
	All	Patients with SVR (n = 64)	Patients with Non-SVR (n = 207)	
Patients who died	45	1	44	24
Liver-related deaths				
Overall, *n (%)*	32 (71)	0 (0)	32 (73)	19 (79)
Hepatocellular carcinoma, *n*	25	0	25	11
Liver failure, *n*	6	0	6	8
GI bleeding, *n*	1	0	1	0
Deaths unrelated to liver disease				
Overall, *n (%)*	13 (29)	1 (100)	12 (27)	5 (21)
Neoplasm, *n*				
Pancreas	2	1	1	0
Stomach	0	0	0	1
Biliary tract	0	0	0	1
Cerebral hemorrhage, *n*	4	0	4	1
Pneumonia, *n*	3	0	3	0
Heart failure, *n*	1	0	1	1
Peritonitis, *n*	1	0	1	1
Other, *n*				
Suicide	1	0	1	0
Unknown	1	0	1	0

* GI = gastrointestinal; Non-SVR = nonsustained virologic response; SVR = sustained virologic response.

Figure 3. **Patient survival (age-stratified analysis).**



The Kaplan–Meier method was used to evaluate survival of interferon-treated and untreated patients according to age (<50 years, 50 to 59 years, ≥60 years).

from the treated group at baseline (**Table 1**), but they may have also differed from the treated patients in other ways. Follow-up rate and treatment tactics for patients with hepatocellular carcinoma did not differ between the 2 groups; thus, we believe that these biases were small.

Despite the contradictory findings in previous studies (6–9, 15, 18) and the built-in biases in this study, our current study clearly showed that the incidence of hepatocellular carcinoma development was significantly reduced in the interferon-treated group compared with the untreated group, even after adjustment for age. Although the difference in tumor development reduction was larger in patients younger than 50 years of age than in those 50 to 59 years of age and those 60 years of age or older, both stratified and numeric age-adjusted analyses showed a significant reduction in the interferon-treated group. Furthermore, the cumulative incidence of hepatocellular carcinoma was markedly decreased, especially in sustained virologic responders when compared with nonresponders and untreated patients. However, transient virologic response during interferon treatment followed by relapse was not associated with the outcome (data not shown). Although baseline serum ALT levels differed between the 2 groups, this variable was not associated with the inhibition of hepatocellular carcinoma. Furthermore, while the patients enrolled in this study also participated in the 2 preceding trials (which used a different treatment schedule), this participation was not associated with tumor inhibition. In this study, sex, total dose of interferon, viral load at baseline, and HCV genotype were not associated with outcome, although these variables are known to be related to interferon response. The present results may be explained by the fact that 72% of the patients in the natural interferon trial had a low viral load and that most of the enrolled patients in the preceding trials were male.

In this study, the annual incidence of hepatocellular carcinoma in untreated patients was 6% to 8%; in interferon-treated patients, the incidence was 4% to 5%. Analysis of interferon response indicated that the annual incidence of tumor development in sustained responders was markedly decreased to 2.5% (3% to 4% in the initial 2 years and 2% thereafter), in contrast to the 5% in nonsustained responders (4% in the initial 2 to 3 years and 5% to 8% thereafter). Furthermore, normalization of serum ALT level (biochemical response) in nonsustained responders did not contribute to the reduction in development of hepatocellular carcinoma (data not shown).

In addition to hepatocellular carcinoma development, we also analyzed mortality rates among patients with chronic hepatitis C and cirrhosis. The survival results turned out to be more important than the incident results because it is theoretically possible that tumors that arise after interferon treatment have a different prognosis or treatment response than those that arise without viral suppression. In the end, only the survival advantage justified the use of preventive treatment. Of the overall deaths in the interferon-treated and untreated groups, liver-related death (hepatocellular carcinoma and liver failure) occurred in 71% and 79%, respectively. Liver failure caused 40% of these deaths in the untreated group and 20% of those in the interferon-treated group.

According to an age-adjusted analysis, survival among interferon-treated patients was significantly better than that among untreated patients. Furthermore, sustained negativ-

ity of HCV RNA after interferon therapy was associated with a reduced risk for death. Treatment tactics for hepatocellular carcinoma in the interferon-treated and untreated groups were similar, and the groups had a similar survival rate after the development of hepatocellular carcinoma ($P = 0.15$; data not shown). In the ad hoc analysis, the untreated group and nonresponders had even greater similarity ($P > 0.2$; data not shown).

This study found a large decline in survival at 6 years. This finding may be related to aspects of the protocol (extension of follow-up period); changes in follow-up procedure for the last 10 years, such as increased ease of CT and MRI use; and a change in tumor treatment from percutaneous ethanol injection therapy to radiofrequency ablation. Thus, the survival data should be considered circumspectly. In the ad hoc analysis, the large decline in survival was due not only to hepatocellular carcinoma but also to liver failure that occurred after 6-year follow-up, especially in untreated patients (data not shown). These data coincide with the results of our latest cohort analysis of 2889 patients with chronic hepatitis C. In that study, patients treated with interferon, especially sustained virologic responders, had improved survival (31).

We conclude that antiviral treatment improved the natural course in cirrhotic patients with HCV infection, especially cirrhotic patients with viral eradication. Because the efficacy of antiviral therapy for patients with chronic hepatitis C, even cirrhotic patients, reportedly increases with combined interferon–ribavirin or pegylated interferon–ribavirin therapies (32–35), combination therapy for cirrhotic patients may lead to a favorable outcome. Trials studying combination therapy in a large number of cirrhotic patients are being conducted in Japan. The survival advantage of interferon treatment may not be as great in western cohorts because the incidence of hepatocellular carcinoma may not be as high in such groups as in Japan. However, with higher rates of sustained virologic response obtained through use of pegylated interferon, the survival advantage may be similar.

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