Table 3 Baseline of hepatitis C virus carriers with 31–40 U/L aminotransferase who received antiviral therapy

	PLT ≥ 150 000/mL (group B-1)	PLT < 150 000/mL (group B-2)	*P*-value
No. patients	141	68	
Age	48.2 ± 11.9	57.9 ± 7.5	<0.001*
Sex (male/female)	80/61	37/31	0.751**
BMI (kg/m^2)	22.9 ± 3.1	22.7 ± 2.6	0.08*
HOMA-IR	3.0 ± 2.0	8.2 ± 9.5	0.8.8*
Genotype: 1/2/others	82/58/1	30/38/0	0.095**
Viral load: low/high	64/77	35/33	0.542**
Histology			
F stage (0/1/2/3/4)	17/91/31/2/0	4/30/26/6/2	<0.001**
Grade (0–1/2,3)	116/25	50/18	0.114**
Fatty change† 0–1/2–4	111/30	50/18	0.10**
Iron load‡ 0/1–4	67/12	30/7	0.762**
Ferritin (ng/mL)	114.4 ± 116.1	127.2 ± 167.8	0.869*
PLT count (/μL)	21.5 ± 4.9	12.2 ± 2.1	<0.001*
Hyaluronate (ng/mL)	46.9 ± 35.4	100.7 ± 0.98.1	<0.001*
Administration of IFN (weeks)	26.1 ± 11.9	27.7 ± 11.4	0.983*
Effects of therapy			
SVR/non-SVR	64/77	35/33	0.409**

**P*-values were calculated by Mann–Whitney-*U*-test. **Fisher-exact-test. †0: no fatty change, 1: ≤10%, 2: 11–33%, 3: 34–66%, 4: ≥67% of hepatocyte; ‡no stain by 400×, 1: few stains by 250×, 2: stains by 100×, 3: stains by 25×, 4: stains by 10×. In group B, there were significant differences in age ($P < 0.001$), distribution of F stage ($P < 0.001$), PLT count ($P < 0.001$), and hyaluronic acid ($P < 0.001$) between B-1 and B-2. Frequency of F2–4 was 23.4% in B-1and 50.0% in B-2, respectively. Values are expressed as mean ± SD.
BMI, body mass index; HOMA-IR, homeostasis model assessment-insulin resistance; IFN, interferon; PLT, platelet counts; SVR, sustained viral responders.

Demographic, clinical, and histological features of 129 HCV carriers with PNALT

The demographic and clinical features of the 129 HCV carriers with PNALT who were followed up for 7.2 years are shown in Table 4. Normal liver histology was noted in 17 patients, 102 showed minimal to mild CH, and 10 had moderate CH. Steatosis was seen in 7% and iron loading was noted in 12%.[18]

Of the 78 patients followed longer than 7 years (mean follow-up period; 10.4 ± 3.1 years), 11 (14%) had continuously normal ALT (G-1), 43 (55%) showed a transient elevation of ALT (G-2), and 24 (31%) changed to CH with continuously elevated ALT (G-3).

Thirty-nine patients received repeated liver biopsies (2–4 times). Of the 39 patients, six were in G-1, 17 were in G-2, and 16 were in G-3. The intervals between the first biopsy and the last biopsy in these three groups were 7.1, 7.8, and 7.2 years, respectively. The progression of the F stage was noted in two of six in G-1, six of 17 in G-2, and seven of 16 in G-3. The median rates of fibrosis progression per year for these three groups were 0.05, 0.05, and 0.08 fibrosis unit. HCC was not detected in any patients during the follow-up periods.

Guidelines for the antiviral therapy of HCV carriers with normal serum ALT focused on the inhibition of the development of HCC

Considering the risk of progression to liver cirrhosis and the development of HCC, as well as the expected efficacy and various side-effects of antiviral therapy, an algorithm is needed for the management of HCV carriers with normal serum ALT. The progression rate of liver fibrosis stage was 0.05/year in HCV carriers with PNALT. The annual incidence of HCC in CH-C patients has been reported to be 0.5% at stages F0–F1, 1–2% at stage F2, 3–5% at stage F3, and 7% at stage F4.[4]

In principle, follow up without antiviral treatment is recommended for HCV carriers with PNALT (ALT ≤30 U/L) and PLT counts ≥150 000/μL, particularly in older patients (i.e. >65 years old), because over 90% show normal or minimal liver damage with good prognoses. However, antiviral therapy is not contraindicated for such patients since roughly 40% are infected with HCV genotype 2,[18] which suggests a high rate of SVR to the therapy with PEG-IFN/Riba.

As for the indication of antiviral therapy for HCV carriers with normal serum ALT (≤40 U/L), the PLT

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Table 4 Characteristics of 129 HCV carriers with persistently normal ALT who received liver biopsy

	$n = 129$	Follow up over 5 years ($n = 78$)
Follow-up period (years)	7.2 ± 3.2	10.4 ± 3.1
Age (years)	48 (21–77)	45 (29–71)
Male ($n = 24$)	49.8 ± 16.4	42.3 ± 14.9
Female ($n = 105$)	47.2 ± 12.5	46.6 ± 11.6
Sex (male/female)	24/105	10/68
ALT (U/L)	8–30	9–30
Male ($n = 24$)	22.5 ± 5.7	21.1 ± 5.4
Female ($n = 105$)	21.6 ± 4.8	22.3 ± 5.1
PLT ($\times 10^4$/mL)	15–31	15–31
Ferritin (ng/mL)	5–225	5–225
Male ($n = 24$)	76.2 ± 53.5	84.6 ± 59.2
Female ($n = 105$)	60.0 ± 43.3	66.6 ± 52.5
HCV genotype	G1 ($n = 58$), G2 ($n = 45$) Mixed and unclassified ($n = 16$)	
BMI (kg/m^2)	16–27	16–27
Male	22.2 ± 1.7	21.9 ± 1.9
Female	21.3 ± 2.2	21.0 ± 2.4

Values are expressed as mean ± SD.
ALT, alanine aminotransferase; BMI, body mass index; HCV, hepatitis C virus; PLT, platelet.

count is a good indicator for discriminating as to whether or not they have minimal to mild fibrosis or moderate to advanced fibrosis. Serum hyaluronate levels were significantly higher in HCV carriers with 31–40 U/L ALT having less than 150 000/μL PLT (Table 3). Advanced hepatic F stage, an elevated ALT level, old age (>65 years old), and sex (male) are important risk factors for the development of HCC.[6,18,30] We advocated an algorithm for such patients (Fig. 1) taking into consideration the risk of the progression to cirrhosis and the development of HCC. Therapy with PEG-IFN/Riba is the first-line treatment; therapy for 48 weeks is recommended for genotype 1 patients with high viral load and 12–24 weeks therapy for genotypes 2 and 1 with low viral load.

DISCUSSION

OUR PREVIOUS STUDY in 129 HCV carriers with PNALT demonstrated a predominance of females, higher frequency of genotype 2, minimal to mild liver histology, and very slow progression of hepatic fibrosis.[18] However, over 30% of these patients advanced to CH-C with elevated ALT levels during the 7-year follow up.

There are many reports concerning the natural course of liver fibrosis in CH-C patients, including those who are HCV carriers with normal serum ALT.[19,31–39] More than half of CH-C patients show progression of F stage from F1 to F2–4 within 10 years, and it was reported that the progression of liver fibrosis in HCV carriers with normal serum ALT was more rapid than was observed in the present study.[23] The main reason for the discrepancy between the report by Puoti *et al.*[23] and our results might be due to the definitions used for the normal range of serum ALT. In our previous study, the patients were HCV carriers with PNALT (ALT ≤ 30 U/L) and PLT counts ≥150 000/μL. On the other hand, the patients in the study by Puoti *et al.* had ALT levels ≤40 U/L, irrespective of PLT counts, in which cirrhotic patients might be included.[23] However, recent studies have demonstrated that normal ALT levels are less than 30 U/L[24] or 25 U/L in men[40] and less than 19 U/L[24] or 22 U/L in women.[40]

The present study demonstrated that the different distribution of hepatic F stage became remarkable when the A and B groups were divided into two subgroups according to their PLT counts. In HCV carriers with ALT levels ≤30 U/L, the frequency of stages F2–3 was 16.2% among those with PLT counts ≥150 000/μL; however, the frequency of stages F2–3 was 49.0% in those with PLT counts <150 000/μL. Conversely, in HCV carriers with ALT levels between 31 and 40 U/L, the frequency of stages F2–4 was 23.4% among those with PLT counts ≥150 000/μL and 50.0% in those with PLT counts <150 000/μL. The PLT count is a useful marker in dis-

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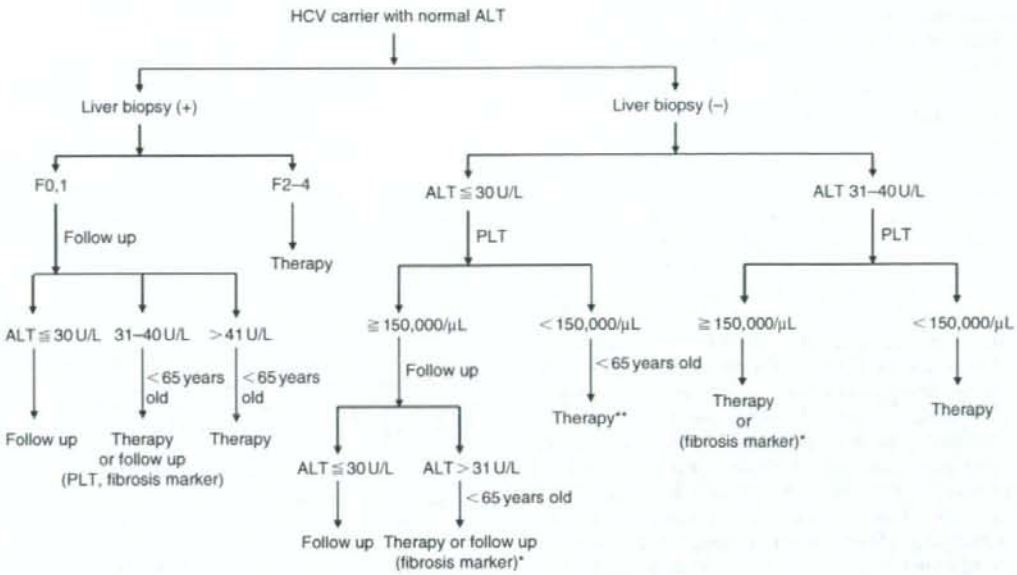

Figure 1 Algorithm for the management of hepatitis C virus (HCV) carriers with normal serum aminotransferase (ALT, ≤40 U/L) focused on the inhibition of the development of hepatocellular carcinoma. In patients who underwent liver biopsy, F0 and F1 patients younger than 65 years are candidates for antiviral therapy, especially those with genotype 2 after the elevation of serum ALT levels. In patients who did not undergo liver biopsy, ALT and platelet (PLT) levels are good indicators for determining candidates for antiviral therapy. Older patients (>65 years) and/or patients having uncontrolled hypertension, diabetes mellitus, or anemia should not be treated with pegylated interferon and ribavirin. Combination therapy with pegylated interferon and ribavirin for 48 weeks is recommended for patients with genotype 1 and high viral load, and 12–24 weeks therapy is suggested for patients with genotype 2 and genotype 1 with low viral load. * **Serum fibrosis markers, such as hyaluronate, might be useful to decide whether patients are candidates for antiviral therapy or not.

criminating between stages F0–1 and F2–4 F in HCV carriers with normal serum ALT (≤40 U/L). In the present study, the mean PLT count in F2 and F3 patients was 16.9 ± 5.3 ($\times 10^4/\mu L$) and 15.9 ± 4.6 ($\times 10^4/\mu L$), respectively. The distribution of the F stage was not significantly different between patients with PLT counts $\geq 15 \times 10^4/\mu L$ versus $< 15 \times 10^4/\mu L$ and $\geq 17 \times 10^4/\mu L$ versus $< 17 \times 10^4/\mu L$.

The SVR rate for genotype 1 patients with high viral load treated with either IFN monotherapy or IFN/Riba were 12.5% and 37.7%, respectively. In genotype 2 patients with high viral load, the SVR rate in the present study was better than the data of Japanese CH-C patients with elevated ALT levels in our previous paper.[6] It was not reasonable to compare the SVR rates between HCV carriers with normal serum ALT and CH-C with elevated ALT in the present study, because the total dosage of IFN and the duration of treatment were significantly different.

The annual incidence of HCC is correlated with the progression of liver fibrosis, that is, the stage of liver disease.[2–4,6] Sustained low serum ALT levels are also associated with a lower incidence of HCC.[2,6,41] PEG-IFN/Riba therapy is expensive and induces various side-effects. The present results indicate that most HCV carriers with normal serum ALT (≤40 U/L) and PLT counts ≥150 000/μL have minimal to mild liver damage, indicating a low risk for the progression to cirrhosis and the development of HCC. This was more remarkable in patients with ALT levels ≤30 U/L and PLT counts ≥150 000/μL. However, nearly half of the patients with PLT count <150 000/μL have F2 or F3 F stages, indicating a certain risk for the progression to cirrhosis and the development of HCC. Fibrosis

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progression is associated with age, baseline and follow-up ALT levels, inflammatory activity and steatosis in the initial liver biopsy, and alcohol consumption.[42] The present results indicate that most HCV carriers with PNALT have a good prognosis and a low risk of developing HCC.

Liver biopsy is a useful procedure for identifying the stage of liver fibrosis; however, it is invasive and may sometimes cause complications.[43,44] The error rate of predicting the F stage with this procedure can be estimated to be as high as 20%.[45] Recently introduced biochemical markers, such as FibroTest,[46] and FibroScan,[47–49] are excellent procedures for identifying liver fibrosis stage in CH-C patients.[50] The combined use of FibroScan and FibroTest is useful for accurately estimating moderate to severe liver fibrosis in most patients with CH-C, but not in F0 and F1 patients.[51]

Recently, Alberti proposed an individualized management algorithm for HCV carriers with PNALT with or without liver biopsy in which HCV genotype, patient age, motivation to receive antiviral therapy, and factors influencing side-effects were included.[52] The algorithm using a combination of serum ALT levels and PLT counts in the present study is simple, but it is useful because it focuses mainly on the inhibition of the progression to cirrhosis and the development of HCC.

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Virological Response in Patients with Hepatitis C Virus Genotype 1b and a High Viral Load

Impact of Peginterferon-α-2a plus Ribavirin Dose Reductions and Host-Related Factors

Gotaro Yamada,[1] *Shiro Iino,*[2] *Tadao Okuno,*[3] *Masao Omata,*[4] *Kendo Kiyosawa,*[5] *Hiromitsu Kumada,*[6] *Norio Hayashi*[7] and *Takahiro Sakai*[8]

1 Kawasaki Medical School, Kawasaki Hospital, Okayama, Japan
2 Kiyokawa Hospital, Tokyo, Japan
3 Akashi Municipal Hospital, Hyogo, Japan
4 University of Tokyo, Tokyo, Japan
5 Nagano Red Cross Hospital, Nagano, Japan
6 Toranomon Hospital, Tokyo, Japan
7 Osaka University, Osaka, Japan
8 The Japanese Red Cross Musashino Junior College of Nursing, Tokyo, Japan

Abstract

Background and objective: In Japan the prevalence of the hepatitis C virus (HCV) antibody is highest in the elderly population. Therefore, it is important for elderly patients to undergo interferon (IFN) therapy. In patients with HCV genotype 1b and a high viral load, the sustained virological response (SVR) rate is lower in older compared with younger patients receiving combination antiviral therapy. In addition, inadequate adherence to combination therapy is often seen in elderly patients, and is associated with reduced response rates. The aim of this retrospective analysis was to evaluate the effects of host-related factors (i.e. sex, age, baseline HCV RNA level, bodyweight and fibrosis stage) and peginterferon (PEG IFN)-α-2a plus ribavirin dose reductions on SVR rates.

Methods: A total of 192 treatment-naive patients with a HCV genotype 1b infection and a high viral load were included in the analysis. Patients had been enrolled into a phase III trial of 48 weeks of treatment with PEG IFN-α-2a plus ribavirin or PEG IFN-α-2a plus placebo. All patients were evaluated for effect of drug exposure on SVR. In addition, the impact of host-related factors or dose reductions on SVR was assessed.

Results: Approximately 30% of patients were considered elderly (≥60 years of age). The overall SVR rate was significantly higher in patients treated with combination therapy versus monotherapy (59.4% vs 24.0%, $p < 0.001$). Attainment of an SVR following combination therapy was not influenced by any factor evaluated in the analysis, although elderly males were associated with decreased SVR rates. Younger age (odds ratio [OR] 1.081; 95% CI 1.125, 1.034; $p = 0.0009$), lower baseline HCV RNA levels (OR 1.003; 95% CI 1.006, 1.001;

p = 0.006) and a severe fibrosis stage (F3/4) [OR 6.194; 95% CI 1.037, 37.000; p = 0.0455] significantly increased the likelihood of achieving an SVR with monotherapy. In the combination therapy group, patients maintaining a full dosage schedule of PEG IFN-α-2a and ribavirin and those requiring dose reductions of either study drug had similar SVR rates (64.5% vs 61.9%). However, the SVR rate was reduced to 33.3% among patients who discontinued combination therapy. Three out of the 31 patients who received the full dosage schedule were elderly patients. In addition, of the 15 patients who discontinued combination therapy, three were <50 years of age and six were ≥60 years of age. The SVR rate was reduced in patients with cumulative PEG IFN-α-2a and ribavirin doses of <60%; the majority of these patients were elderly.

Conclusion: The attainment of an SVR following PEG IFN-α-2a plus ribavirin combination therapy was not influenced by any of the host-related factors evaluated in this analysis, although elderly males were associated with a decreased SVR rate. Younger age, male sex and lower baseline HCV RNA levels significantly increased the likelihood of achieving an SVR with monotherapy. In addition, dose reductions appeared to have a negative impact on SVR in elderly patients. Therefore, it is important to minimize PEG IFN-α-2a and ribavirin dose reductions by effectively managing treatment-related adverse events in elderly patients.

Introduction

In Japan, the prevalence of the hepatitis C virus (HCV) antibody is highest in the elderly population. In a recent analysis,[1] the average age of HCV-positive patients in Japan was found to be greater than that of US patients by approximately 20 years. Results of the analysis suggested that the introduction of HCV into the Japanese population occurred >100 years ago, followed by wide dissemination in the 1930s and 1940s. In contrast, HCV was introduced into the US 100 years ago, followed by wide dissemination in the 1960s. This extended period of exposure to HCV was the likely reason for the considerably higher prevalence of hepatocellular carcinoma in Japan.

To date, it is unclear if genetic and/or environmental factors have an influence on the incidence of hepatocellular carcinoma in Japan. The duration of HCV infection appears to be an important factor for the development of hepatocellular carcinoma, although the age of patients with post-transfusion HCV has been reported to be a significant factor, regardless of the duration of exposure to HCV.[2] Therefore, it appears to be important for elderly patients to undergo interferon (IFN) therapy in the absence of serious complications such as uncontrolled hypertension or insulin-dependent diabetes mellitus.

Combination therapy with peginterferon (PEG IFN)-α-2a plus ribavirin was found to be more effective than PEG IFN-α-2a monotherapy in Japanese patients with HCV genotype 1b.[3] However, a recent study showed that sustained virological response (SVR) rates were lower in older (≥40 years of age) compared with younger patients with HCV genotype 1b and a high viral load.[4] In addition, inadequate adherence to combination therapy with IFN-α-2b and ribavirin was independently associated with increasing patient age and a reduction in SVR response rates.[5] There are insufficient numbers of clinical trials evaluating the use of PEG IFN plus ribavirin in elderly patients, and an effective dose and treatment period has not been established.

The aim of this retrospective analysis was to investigate the effects of host-related factors (i.e. sex, age, baseline HCV RNA level, bodyweight and fibrosis stage) and PEG IFN-α-2a plus ribavirin

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dose reductions on SVR rates in patients with a difficult-to-treat form of chronic hepatitis C.

Patients and Methods

We retrospectively analysed data from a phase III, randomized, double-blind clinical trial conducted at 43 Japanese centres between June 2002 and September 2004.[3]

Patients

A total of 192 treatment-naive patients were included in the analysis. Inclusion criteria were Japanese adults aged ≥20 years with an HCV genotype 1b infection, a serum HCV RNA level of $\geq 1 \times 10^5$ IU/mL, an elevated serum alanine aminotransferase (ALT) level of ≥45 IU/L within 6 months of screening, and chronic hepatitis C confirmed by liver biopsy. Patients were excluded if they had neutropenia (<1500 neutrophils/mm^3), leucopenia (<3000 cells/mm^3), thrombocytopenia (<90 000 platelets/mm^3), anaemia (haemoglobin <12 g/dL), a hepatitis B virus co-infection, decompensated liver disease, organ transplant, a creatinine clearance <50 mL/min, poorly controlled psychiatric disease, poorly controlled diabetes, malignant neoplastic disease, severe cardiac or chronic pulmonary disease, immunologically mediated disease, or retinopathy.

Study Design

Patients were randomized according to a 1 : 1 ratio to 48 weeks of treatment with subcutaneous PEG IFN-α-2a (Pegasys®, Roche, Tokyo, Japan)[1] 180 μg/week in combination with either twice daily oral ribavirin tablets (Copegus®, Roche, Basle, Switzerland) or placebo, followed by 24 weeks of untreated follow-up. The ribavirin dosage was 600, 800 or 1000 mg/day in patients with a bodyweight of ≤60, 60–80 or >80 kg, respectively; these dosages were based on the currently used dosages of ribavirin in Japan. Patients were stratified according to HCV RNA level.

Virological Methods

Qualitative and quantitative serum HCV RNA assessments were conducted using the Cobas Amplicor HCV Test PCR assay (version 2.0; limit of detection 50 IU/mL) and the Cobas Amplicor HCV Monitor Test (version 2.0; limit of quantitation, 500 IU/mL), respectively. HCV genotyping was performed according to the method described by Okamoto et al.[6] The presence of serum anti-HCV antibodies was not assessed.

Histology

Liver biopsies were taken within 12 months of enrolment. An independent pathologist evaluated, graded and staged liver biopsy specimens according to the Ishak modified hepatic activity index and the new European classification.[7,8]

Assessment of Efficacy

The primary efficacy end point of the study was the SVR rate, which was defined as a HCV RNA level of <50 IU/mL after 24 weeks of untreated follow-up.

Statistics

The Cochran-Mantel-Haenszel test was used to compare treatment groups, with a significance level of $p < 0.05$.

Host-related factors associated with an SVR were evaluated using stepwise and multiple logistic-regression models. The following pretreatment factors were considered: sex, age, bodyweight, serum HCV RNA and fibrosis stage (F1/2: mild/moderate; F3/4: severe/cirrhosis). Factors such as the maintenance of a full dosage schedule, the requirement of dose reductions, and treatment discontinuation were also considered.

All patients receiving at least one dose of study drug were included in the efficacy analysis. Patients without follow-up data were considered not to have attained an SVR.

1 The use of trade names is for product identification purposes only and does not imply endorsement.

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Results

Patient Demographics

A total of 192 patients were randomized to treatment and patient characteristics were similar at baseline in the two treatment groups (table I). Approximately 30% of patients were considered elderly (≥60 years of age).

Virological Response

The overall SVR rate was significantly higher in patients who received combination therapy with PEG IFN-α-2a plus ribavirin (57/96 patients; 59.4%; 95% CI 48.9, 69.3) versus PEG IFN-α-2a monotherapy (23/96 patients; 24.0%; 95% CI 15.8, 33.7), resulting in an odds ratio (OR) of 4.65 (95% CI 2.49, 8.69; $p < 0.001$).

Factors Associated with Sustained Virological Response (SVR)

Following combination therapy, the attainment of an SVR was not influenced by any pretreatment factor (including fibrosis stage, age, sex, HCV RNA level and bodyweight) evaluated in this analysis (table II). In the combination therapy group, the SVR rate tended to be higher in younger males (<50 years of age) versus males aged ≥60 years (figure 1).

Multiple logistic-regression analyses found no significant correlations between host-related factors

Table I. Baseline characteristics of study patients

Characteristic	Peginterferon-α-2a + placebo (n = 96)	Peginterferon-α-2a + ribavirin (n = 96)
Sex [no. (%)]		
Male	59 (61.5)	61 (63.5)
Female	37 (38.5)	35 (36.5)
Age (y)		
Mean	50.8	52.1
Range	20–73	20–69
Age groups (y) [no. (%)]		
≤29	7 (7.3)	4 (4.2)
30 to 39	13 (13.5)	9 (9.4)
40 to 49	22 (22.9)	22 (22.9)
50 to 59	24 (25.0)	36 (37.5)
60 to 69	27 (28.1)	25 (26.0)
≥70	3 (3.1)	
Weight (kg) [mean]	61.9	62.9
ALT activity (IU/L) [mean]	101.0	100.9
Serum HCV RNA (IU/mL) [no. (%)]		
1 to $<5 \times 10^5$	26 (27.1)	21 (21.9)
5 to $<8.5 \times 10^5$	27 (28.1)	32 (33.3)
$\geq 8.5 \times 10^5$	43 (44.8)	43 (44.8)
Fibrosis staging[a,b] [no. (%)]		
F1	23 (24.0)	18 (18.8)
F2	58 (60.4)	60 (62.5)
F3	15 (15.6)	16 (16.7)
F4		1 (1.0)

a F1 = mild, F2 = moderate, F3 = severe, F4 = cirrhosis.

b One patient was not classified in the combination therapy group.

ALT = alanine aminotransferase; **HCV** = hepatitis C virus.

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Table II. Sustained virological response (SVR) at the end of follow-up

Variable	No. of patients achieving SVR (%)	
	peginterferon-α-2a + placebo	peginterferon-α-2a + ribavirin
Overall	23 (24.0)	57 (59.4)
Sex		
Male	18 (30.5)	39 (63.9)
Female	5 (13.5)	18 (51.4)
Age (y)		
≤29	5 (71.4)	2 (50.0)
30 to 39	5 (38.5)	7 (77.8)
40 to 49	4 (18.2)	16 (72.7)
50 to 59	4 (16.7)	20 (55.6)
60 to 69	5 (18.5)	12 (48.0)
≥70	0 (0.0)	
Weight (kg)		
<50	5 (41.7)	4 (66.7)
50 to <60	4 (12.5)	15 (45.5)
60 to <70	5 (17.2)	22 (66.7)
70 to <80	6 (33.3)	10 (62.5)
≥80	3 (60.0)	6 (75.0)
Serum HCV RNA (IU/mL)		
1 to <5 × 10^5	10 (38.5)	12 (57.1)
5 to <8.5 × 10^5	6 (22.2)	21 (65.6)
≥8.5 × 10^5	7 (16.3)	24 (55.8)
ALT activity (IU/L)		
<50	2 (10.5)	13 (76.5)
50 to <100	11 (23.4)	25 (62.5)
100 to <200	6 (27.3)	17 (51.5)
≥200	4 (50.0)	2 (33.3)
Fibrosis staging[a]		
F1	5 (21.7)	10 (55.6)
F2	13 (22.4)	38 (63.3)
F3	5 (33.3)	7 (43.8)
F4		1 (100.0)

a F1 = mild, F2 = moderate, F3 = severe, F4 = cirrhosis.
ALT = alanine aminotransferase; HCV = hepatitis C virus.

and the achievement of an SVR with combination therapy.

In contrast, in patients receiving monotherapy, lower baseline HCV RNA levels (OR 1.003; 95% CI 1.006, 1.001; p = 0.006), younger age (OR 1.081; 95% CI 1.125, 1.034; p = 0.0009) and a severe fibrosis stage (OR 6.194; 95% CI 1.037, 37.000; p = 0.0455) significantly increased the likelihood of achieving an SVR.

Effect of Medication Adherence on SVR

In the combination therapy group, patients maintaining a full dosage schedule of PEG IFN-α-2a and ribavirin and those requiring dose reductions of either study drug had similar SVR rates (figure 2). However, the SVR rate was reduced to 33.3% among patients who discontinued combination therapy. Only 3 out of the 31 patients who received the full dosage schedule were ≥60 years of age; the majority of elderly patients failed to complete the

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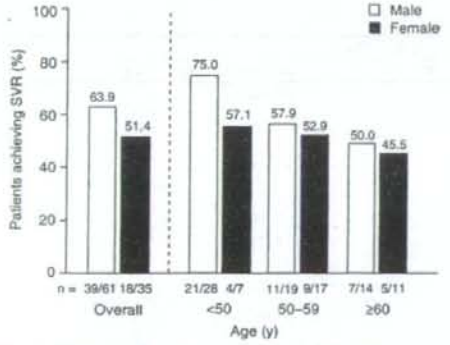

Fig. 1. Effect of patient age on sustained virological response (SVR).

full dosage schedule as a result of adverse events. Similarly, of the 15 patients who discontinued combination therapy, three were <50 years of age and six were ≥60 years old.

The SVR rate was reduced in patients receiving <60% of the cumulative PEG IFN-α-2a and ribavirin planned total doses (figure 3). Dose reductions negatively affected the SVR rate in elderly patients who had received <60% of the cumulative PEG IFN-α-2a and ribavirin doses, which was achieved by 0/10 (0%) and 3/13 (23%) patients who were ≥50 years of age, and by 0/6 (0%) and 2/7 (28.6%) patients who were ≥60 years of age, respectively.

Discussion

Combination therapy with PEG IFN-α-2a plus ribavirin was associated with significantly higher SVR rates compared with PEG IFN-α-2a monotherapy, in treatment-naive patients infected with HCV genotype 1b (61% vs 26%; $p < 0.001$).[3] This outcome is noteworthy, because individuals with HCV genotype 1 infections are considered to be relatively difficult to treat.[9]

Previously, there were no data on the association between sex or age and virological response following treatment with PEG IFN-α-2b plus ribavirin.[10] Our data indicate that the attainment of an SVR following combination therapy was not influenced by any of the pretreatment host-related factors (including age, sex, HCV RNA level, fibrosis stage and bodyweight) evaluated in this retrospective analysis, although younger males (<50 years) appeared to have a higher SVR rate compared with males aged ≥60 years (75% vs 50%). Younger age, lower baseline HCV RNA levels and a severe fibrosis stage significantly increased the likelihood of achieving an SVR with monotherapy. In contrast, a previous study[11] showed that a histological activity index score of >10 and a lack of cirrhosis or bridging fibrosis were independent factors associated with SVR attainment among patients treated with monotherapy, which suggests that severe fibrosis staging negatively impacts the SVR rate. In our study, a

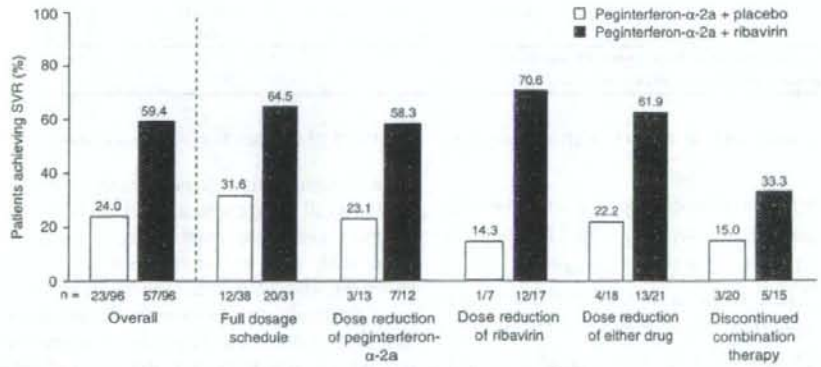

Fig. 2. Effects of dose reduction and discontinuation on sustained virological response (SVR).

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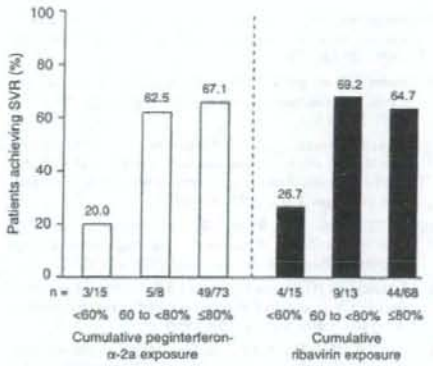

Fig. 3. Effects of peginterferon-α-2a and ribavirin exposure on sustained virological response (SVR). The cumulative exposure of patients to the study drug(s) was expressed as a percentage of the planned total dose.

severe fibrosis stage was reported in only 15.6% of patients. As a result, the small proportion of patients with severe fibrosis staging may have influenced the outcome of the current analysis.

Anaemia is a common adverse effect that can occur soon after the initiation of treatment with PEG IFN plus ribavirin for HCV infections. This complication can negatively impact patient quality of life, and is the most common reason for dose reductions and the temporary or permanent discontinuation of ribavirin. Such dose modifications have been shown to reduce the efficacy of treatment.[12] In general, females were predicted to have a higher likelihood of becoming anaemic than male patients.[13] In addition, the dose reduction rate of PEG IFN-α-2a and ribavirin is higher in elderly patients, which negatively impacts the achievement of an SVR.[5]

In a recent pooled analysis[14] of two phase III trials of 48 weeks of treatment with PEG IFN-α-2a plus ribavirin, the SVR rate was significantly reduced ($p = 0.0006$) in patients with a cumulative ribavirin dose of <60%. Prolonged periods of dose reduction, temporary interruptions or premature cessation of ribavirin were also associated with decreased SVR rates.

Previous studies have not assessed the impact of reducing the dose of PEG IFN independent of ribavirin, or differentiated between dose reduction, or interrupting or prematurely discontinuing treatment. An analysis of the HALT-C (Hepatitis C Antiviral Long-term Treatment against Cirrhosis) trial[15] investigated the impact of PEG IFN-α-2a and ribavirin dose reductions during the retreatment of patients infected with chronic HCV genotype 1 who did not respond to standard IFN with or without ribavirin treatment. A decrease in the cumulative dose of PEG IFN-α-2a received during the first 20 weeks of treatment (lead-in phase), from full dose (≥98%) to ≤60%, reduced the SVR rate from 17% to 5%. In contrast, reducing the dose of ribavirin from full dose to ≤60% did not affect the SVR rate as long as ribavirin administration was not interrupted for more than seven consecutive days. However, the premature discontinuation of ribavirin, even with full-dose PEG IFN-α-2a, reduced the SVR rate to 3%. This suggests that sufficient dosage during the early stages of therapy is required to achieve a high SVR rate with combination therapy. In our study, the SVR rate was also reduced in patients who received cumulative PEG IFN-α-2a and ribavirin doses of <60%, which was further decreased in patients who discontinued combination therapy. Therefore, it is important to alter the way adverse events of PEG IFN-α-2a and ribavirin therapy are managed to minimize the number of patients needing to reduce doses or discontinue therapy.

Conclusion

The attainment of an SVR following PEG IFN-α-2a plus ribavirin combination therapy was not influenced by any of the host-related factors evaluated in this analysis, although males aged ≥60 years tended to have a lower SVR rate. In contrast, younger age, male sex and lower baseline HCV RNA levels significantly increased the likelihood of achieving SVR with monotherapy. Dose reductions had a negative impact on SVR in elderly patients receiving combination therapy. Therefore, it is important to minimize PEG IFN-α-2a and ribavirin dose reductions by effectively managing treatment-related adverse events in elderly patients.

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Correspondence: Dr *Gotaro Yamada*, Department of Internal Medicine, Kawasaki Medical School, Center for Liver Diseases, Kawasaki Hospital, Okayama City 2-1-80, Okayama, 700-0986, Japan.
E-mail: g.yamada@kawasaki-hp.jp

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ORIGINAL ARTICLE

Gene Expression in Fixed Tissues and Outcome in Hepatocellular Carcinoma

Yujin Hoshida, M.D., Ph.D., Augusto Villanueva, M.D., Masahiro Kobayashi, M.D., Judit Peix, A.S., Derek Y. Chiang, Ph.D., Amy Camargo, B.A., Supriya Gupta, B.S., Jamie Moore, M.A., B.S., Matthew J. Wrobel, M.S., Jim Lerner, B.S., Michael Reich, B.S., Jennifer A. Chan, M.D., Jonathan N. Glickman, M.D., Ph.D., Kenji Ikeda, M.D., Masaji Hashimoto, M.D., Goro Watanabe, M.D., Maria G. Daidone, Ph.D., Sasan Roayaie, M.D., Myron Schwartz, M.D., Swan Thung, M.D., Helga B. Salvesen, M.D., Ph.D., Stacey Gabriel, Ph.D., Vincenzo Mazzaferro, M.D., Jordi Bruix, M.D., Scott L. Friedman, M.D., Hiromitsu Kumada, M.D., Josep M. Llovet, M.D., and Todd R. Golub, M.D.

ABSTRACT

BACKGROUND

It is a challenge to identify patients who, after undergoing potentially curative treatment for hepatocellular carcinoma, are at greatest risk for recurrence. Such high-risk patients could receive novel interventional measures. An obstacle to the development of genome-based predictors of outcome in patients with hepatocellular carcinoma has been the lack of a means to carry out genomewide expression profiling of fixed, as opposed to frozen, tissue.

METHODS

We aimed to demonstrate the feasibility of gene-expression profiling of more than 6000 human genes in formalin-fixed, paraffin-embedded tissues. We applied the method to tissues from 307 patients with hepatocellular carcinoma, from four series of patients, to discover and validate a gene-expression signature associated with survival.

RESULTS

The expression-profiling method for formalin-fixed, paraffin-embedded tissue was highly effective: samples from 90% of the patients yielded data of high quality, including samples that had been archived for more than 24 years. Gene-expression profiles of tumor tissue failed to yield a significant association with survival. In contrast, profiles of the surrounding nontumoral liver tissue were highly correlated with survival in a training set of tissue samples from 82 Japanese patients, and the signature was validated in tissues from an independent group of 225 patients from the United States and Europe ($P=0.04$).

CONCLUSIONS

We have demonstrated the feasibility of genomewide expression profiling of formalin-fixed, paraffin-embedded tissues and have shown that a reproducible gene-expression signature correlated with survival is present in liver tissue adjacent to the tumor in patients with hepatocellular carcinoma.

From the Broad Institute of Massachusetts Institute of Technology and Harvard University, Cambridge, MA (Y.H., D.Y.C., A.C., S.G., J.M., M.J.W., J.L., M.R., J.A.C., S.G., T.R.G.); Howard Hughes Medical Institute (T.R.G.), Dana–Farber Cancer Institute (Y.H., D.Y.C., T.R.G.), and Brigham and Women's Hospital (J.N.G.), Harvard Medical School, Boston; Mount Sinai Liver Cancer Program, Mount Sinai School of Medicine, New York (A.V., J.P., S.R., M.S., S.T., S.L.F., J.M.L.); Toranomon Hospital, Tokyo (M.K., K.I., M.H., G.W., H.K.); National Cancer Institute, Milan (M.G.D., V.M.); Haukeland University Hospital, University of Bergen, Bergen, Norway (H.B.S.); and Barcelona Clinic Liver Cancer Group, Institut d'Investigacions Biomèdiques August Pi i Sunyer Centro de Investigaciónes en Red de Enfermedades Hepáticas y Digestivas Hosptial Clínic Barcelona (J.B., J.M.L.) and Institució Catalana de Recerca i Estudis Avançats (J.M.L.) — both in Barcelona. Address reprint requests to Dr. Golub at the Cancer Program, Broad Institute of Massachusetts Institute of Technology and Harvard University, 7 Cambridge Center, Cambridge, MA 02142, or at golub@broad.mit.edu, or to Dr. Llovet at the Division of Liver Diseases, Box 1123, Mount Sinai School of Medicine, 1425 Madison Ave., New York, NY 10029, or at josep.llovet@mssm.edu.

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In developing countries, hepatocellular carcinoma often comes to medical attention when the tumors are at an advanced stage and curative therapies are of limited benefit. In developed countries, however, at-risk populations of patients (e.g., those who are infected with hepatitis virus and have cirrhosis) are often under close surveillance; as a result, hepatocellular carcinoma is usually detected when the tumors are small and treatment is more likely to be successful.[1,2] Nevertheless, recurrences eventually occur in most patients.[1,2] Studies suggest that chemopreventive strategies may suppress recurrence and prolong survival,[1,3-6] although these findings are still uncertain. It would be ideal to treat only patients at greatest risk for recurrence. Several methods have been used to predict survival among patients with hepatocellular carcinoma, including the enumeration of anatomical and histopathological attributes (e.g., tumor multinodularity and vascular invasion), but these have become less useful as hepatocellular carcinoma is increasingly diagnosed at earlier stages.

A technical challenge facing the use of gene-expression profiling to predict the outcome of hepatocellular carcinoma has been the lack of suitable specimens from patients. Current methods of genomewide expression profiling require frozen tissue for analysis, whereas tissue banks with clinical outcome data generally have formalin-fixed, paraffin-embedded specimens. Even today, the vast majority of specimens are formalin-fixed; the collection of frozen tissues has yet to become routine clinical practice.

We tested a method for genomewide expression profiling of formalin-fixed, paraffin-embedded tissues. We applied the method to the analysis of the clinical outcome of hepatocellular carcinoma.

METHODS

PATIENTS AND SAMPLES

The training set consisted of tissue samples from 106 patients who were consecutively treated with surgery for primary hepatocellular carcinoma between 1990 and 2001 at Toranomon Hospital in Tokyo and for whom data on clinical outcomes (over a median follow-up period of 7.8 years) and formalin-fixed, paraffin-embedded blocks of tumor and adjacent tissue were available (Fig. 1). The validation set included tissue samples from 234 patients with hepatocellular carcinoma who consecutively underwent surgery between 1994 and 2005: 92 patients at the Mount Sinai School of Medicine in New York, 46 at Hospital Clínic Barcelona, and 96 at the National Cancer Institute of Milan (members of the HCC Genomic Consor-

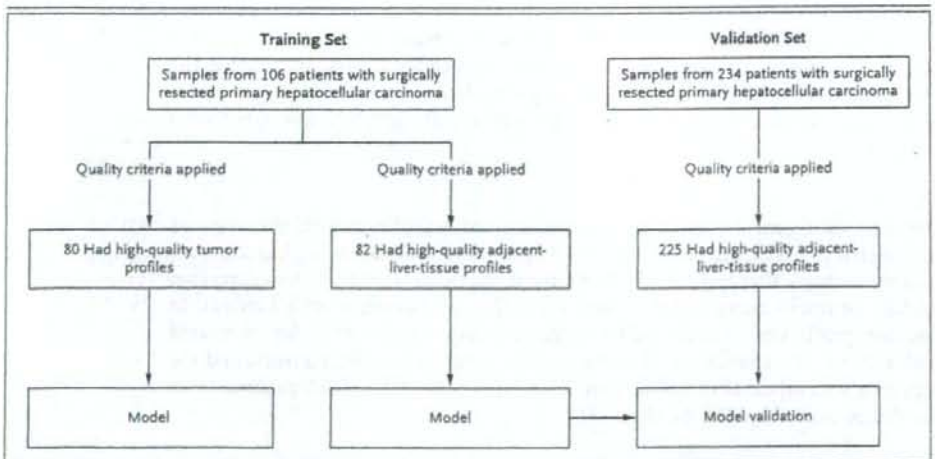

Figure 1. **Study Design.**
In the training set, tumor tissue and liver tissue adjacent to the tumor were profiled separately, and each was used to generate an outcome model. The model based on adjacent liver tissue was validated with the use of an independent validation set.

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tium). Archived formalin-fixed, paraffin-embedded tissues obtained as part of routine clinical care were analyzed, with approval by the local institutional review boards granted on the condition that all samples be made anonymous. Formalin-fixed, paraffin-embedded blocks obtained at the time of resection were cut into three or four sections (each 10 μm thick), macrodissected to isolate tumor and adjacent liver tissue, and subjected to RNA extraction as described in the Supplementary Appendix (available with the full text of this article at www.nejm.org).

ANALYSIS OF GENE EXPRESSION

Gene-expression profiling was performed according to the complementary DNA–mediated annealing, selection, extension, and ligation (DASL) assay[7,8] (Illumina), and we selected 6100 transcriptionally informative genes for analysis (see the Supplementary Appendix). (Microarray data are available at www.ncbi.nlm.nih.gov/geo/, accession numbers GSE10143 and GPL5474.) Genes whose expression was associated with disease-specific survival and time to recurrence were selected with the use of the Cox score (see the Supplementary Appendix). The value of the signature was assessed on the basis of overall survival. Late recurrence was defined as tumor recurrence occurring more than 2 years after surgery.[9,10] Outcome association analysis was performed with the use of a nearest-neighbor–based method (see the Supplementary Appendix).

Table 1. **Characteristics of Patients in the Training Set and in the Validation Set, at the Time of Surgery.***

Characteristic	Training Set (N=82)	Validation Set (N=225)	P Value
Age — yr			<0.001
Median	59	66	
Interquartile range	52–64	57–71	
Male sex — no. (%)	64 (78)	173 (77)	0.88
HCV infection — no. (%)	60 (73)	104 (48)	<0.001
HBV infection — no. (%)	17 (21)	62 (29)	0.25
Alcohol use — no. (%)	3 (4)	19 (9)	0.22
Tumor diameter — cm			<0.001
Median	2.2	3.5	
Interquartile range	1.7–3.2	2.3–5.5	
Histopathological grade — no. (%)			
Well differentiated	18 (22)	34 (26)	0.68
Moderately differentiated	49 (60)	80 (60)	
Poorly differentiated	15 (18)	19 (14)	
Vascular invasion — no. (%)	4 (5)	74 (34)	<0.001
BCLC stage — no. (%)			
0	25 (30)	21 (9)	0.64†
A	50 (61)	186 (83)	<0.001‡
B	7 (9)	16 (7)	
Child–Pugh class A — no. (%)	72 (88)	204 (97)	0.52
Alpha-fetoprotein >100 ng/ml — no. (%)	53 (65)	53 (24)	0.14
Median follow-up — yr	7.8	2.2	—

* Some data were not available for all patients. The Barcelona Clinic Liver Cancer staging system (BCLC) ranks hepatocellular carcinoma in five stages, ranging from 0 (very early stage) to D (terminal stage). Histopathological grade was defined according to the International Union against Cancer (UICC) system. The Child–Pugh system classifies the severity of liver disease from A to C, with A representing the best liver function. HBV denotes hepatitis B virus, and HCV hepatitis C virus.

† P=0.64 for the pairwise comparison of stages 0 and A with stage B.

‡ P<0.001 for the multiple comparison of stage 0, stage A, and stage B.

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STATISTICAL ANALYSIS

Functional annotation was performed by means of gene set enrichment analysis (GSEA, www.broad.mit.edu/gsea/).[11] Survival analyses were performed with the use of the log-rank test and Cox regression modeling. Subgroup analysis was performed on data from patients with a longer duration of follow-up (treated no later than 2004) and those with carcinoma classified as stage 0 or stage A according to the Barcelona Clinic Liver Cancer staging system (BCLC), which ranks hepatocellular carcinoma in five stages, ranging from 0 (very early stage) to D (terminal stage).[1,12] The hazard function for tumor recurrence was calculated as previously described.[10,13] All analyses were performed with the use of GenePattern[14] (www.broad.mit.edu/cancer/software/genepattern/) or the R statistical package (www.r-project.org). (See the Supplementary Appendix for details on the statistical analyses and methods of clonality analysis.)

RESULTS

VALIDATION OF THE PROFILING METHOD

We first sought a method that was suitable for gene-expression profiling of formalin-fixed, paraffin-embedded material. An approach has been reported for the analysis of several hundred transcripts based on DASL, a multiplex, locus-specific polymerase-chain-reaction (PCR) assay.[7,8] However, an unbiased discovery of diagnostic signatures requires a genomewide profiling method. Accordingly, we modified the DASL method for probe selection and analysis and performed a bioinformatic meta-analysis to identify 6000 transcripts that captured the majority of variance in gene expression across the human transcriptome (see the Supplementary Appendix). This 6000-gene DASL assay served as a potential tool for genomewide analysis of formalin-fixed, paraffin-embedded tissues. We found the assay to be highly reproducible (R^2>0.96 in replicate experiments), with an overall success rate of 90% among all the specimens, including formalin-fixed, paraffin-embedded tissue blocks collected up to 24 years ago (see the Supplementary Appendix). We found that representing each transcript with one probe only (as opposed to three, as previously reported[7,8]) resulted in little loss of assay performance (Fig. 1 in the Supplementary Appendix).

Figure 2 (facing page). **Survival Signatures and Survival Curves in the Training Set.**

Curves are shown for survival according to the association of the gene signature with survival, based on leave-one-out cross-validation testing (Panel A), and for overall survival according to the level of expression of the 186 signature genes (Panel B); of these, 113 were associated with a good prognosis and 73 with a poor prognosis. Panel C shows the expression pattern of the survival signature (comprising 186 genes). The 20 genes most closely associated with a poor prognosis are listed on the left, and the 20 most closely associated with a good prognosis on the right. Red indicates high expression; blue indicates low expression. Panel D shows representative photomicrographs of sections of liver tissue adjacent to tumor that were profiled in this study; there were no histologic correlates with survival. Staining was with hematoxylin and eosin.

PATIENTS

Table 1 summarizes the clinical characteristics of the patients in the training and validation sets. All patients were treated with curative surgical resection, which was, in some cases, followed by second-line treatments at the time of recurrence.

By design, the training set included tissue samples from a large proportion of patients with very-early-stage hepatocellular carcinoma (BCLC stage 0), because these patients represent the greatest clinical challenge with respect to outcome prediction. Indeed, no clinical variables, either alone or in combination, were associated with survival among these patients (Table 1 in the Supplementary Appendix). Although there were no significant differences between the training set and validation set with respect to the number of patients with advanced-stage carcinoma (BCLC stage B) or the status of liver function, there was heterogeneity between the two sets with respect to certain tumor characteristics, such as diameter and type of viral infection (Table 1). Such heterogeneity may help to ensure that molecular predictors have real-world applicability across heterogeneous populations of patients.

PROFILES OF HEPATOCELLULAR CARCINOMA TUMORS

We first investigated whether gene-expression profiles of hepatocellular carcinoma tumors were associated with the clinical outcome. For each of the 106 patients in the training set, tumor-containing portions of the formalin-fixed paraffin-embedded blocks were macrodissected away from

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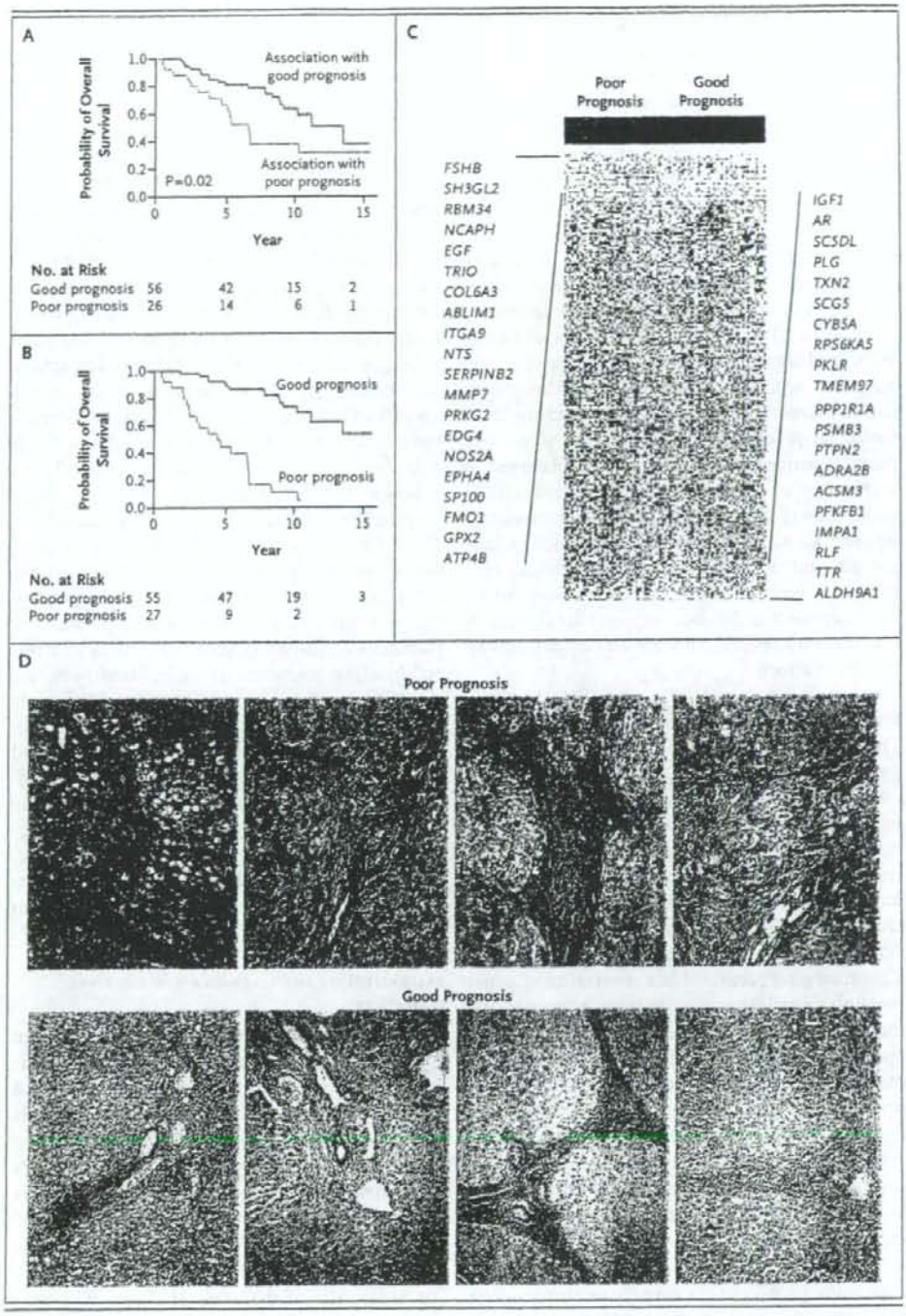

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surrounding liver tissue. Eighty tumors (75%) yielded high-quality gene-expression profiles (see the Supplementary Appendix). Using a leave-one-out cross-validation procedure and a nearest-neighbor–based algorithm, we failed to detect a significant gene-expression correlate of either tumor recurrence (P=0.22) or survival (P=0.70) (Fig. 2A in the Supplementary Appendix). Furthermore, a previously reported signature associated with survival among patients with hepatocellular carcinoma[15] was not associated with survival in our series of patients (P=0.76) (Fig. 2B in the Supplementary Appendix). This failure to identify an outcome-associated signature is unlikely to be due to a technical flaw of the formalin-fixed, paraffin-embedded DASL method, because we observed the same molecular-subclass structure in the formalin-fixed, paraffin-embedded samples as that observed in collections of frozen samples of hepatocellular carcinoma (Fig. 2B and 3B in the Supplementary Appendix). Although this result does not exclude the possibility of tumor-derived expression profiles as predictors of the outcome of hepatocellular carcinoma, the data suggest that at least in this training set, the outcome was largely related to other factors.

SURVIVAL SIGNATURE IN ADJACENT LIVER TISSUE

The lack of association between tumor-derived gene-expression profiles and survival led us to consider the pattern of recurrence of early-stage hepatocellular carcinoma. In contrast to advanced tumors, which tend to recur rapidly after resection, early-stage tumors, which are increasingly diagnosed in modern clinical practice, recur much later, generally more than 2 years after resection[9,10] (Fig. 4 in the Supplementary Appendix). This emerging pattern of late recurrence of hepatocellular carcinoma (due at least in part to the diagnosis of hepatocellular carcinoma at an early stage) has led to the notion that a late recurrence may not be an actual recurrence but rather a second primary tumor in an at-risk liver, presumably due to the carcinogenic effects of cirrhosis.[1,2,9] We therefore hypothesized that the surrounding liver tissue — not the tumor itself — might harbor a gene-expression signature associated with subsequent recurrence.

To test this hypothesis, we assessed the gene-expression profiles of the liver tissue surrounding the resected tumor in the 106 formalin-fixed, paraffin-embedded blocks that constituted the training set. Eighty-two samples (77%) yielded high-quality gene-expression profiles (see the Supplementary Appendix). Using a standard leave-one-out cross-validation procedure, we found the liver signature to be significantly correlated with survival (P=0.02) (Fig. 2A). The aggregate survival-correlated signature contained 186 genes (Fig. 2B and 2C, and Table 2 in the Supplementary Appendix) and was tested in the validation set. Using GSEA, which shows whether a defined set of genes has a significant association with a phenotype of interest, we found the good-prognosis signature to contain genes associated with normal liver function (Tables 2 and 3 in the Supplementary Appendix), including the plasma proteins C4, C5, C8, C9, and F9 and several drug-metabolizing enzymes: the alcohol dehydrogenases ADH5 and ADH6, the aldo–keto-reductases AKR1A1 and AKR1D1, the aldehyde dehydrogenase ALDH9A1, the cytochrome P450 CYP2B6, and hepatic lipase (LIPC). These findings are consistent with the association between impaired liver function and a poor outcome.[1] In addition, the poor-prognosis signature contained gene sets associated with inflammation, including those related to interferon signaling, activation of nuclear factor-κB, and signaling by tumor necrosis factor α. Histologic features of liver inflammation were not found to be associated with the outcome (Fig. 2D, and Table 4 and Fig. 5 in the Supplementary Appendix). Of particular interest, GSEA showed that the downstream targets of interleukin-6 were strongly associated with the poor-prognosis signature, which is consistent with the finding that disruption of interleukin-6 signaling protects mice from chemically induced hepatocellular carcinoma.[16]

VALIDATION OF THE LIVER-DERIVED SURVIVAL SIGNATURE

We next tested the 186-gene survival signature in an independent set of tissue samples from eligible patients at three treatment centers in the United States and Europe. Of the 234 samples in this validation set, 225 (96%) yielded gene-expression profiles of high quality (see the Supplementary Appendix). The survival signature (Fig. 3A) was associated with significant differences in survival among patients (P=0.04) (Fig. 3B), despite the modest duration of follow-up (median, 2.2 years). The separation of the survival curves was even more pronounced when, in a prespecified subgroup analysis, we limited our attention to the 168

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