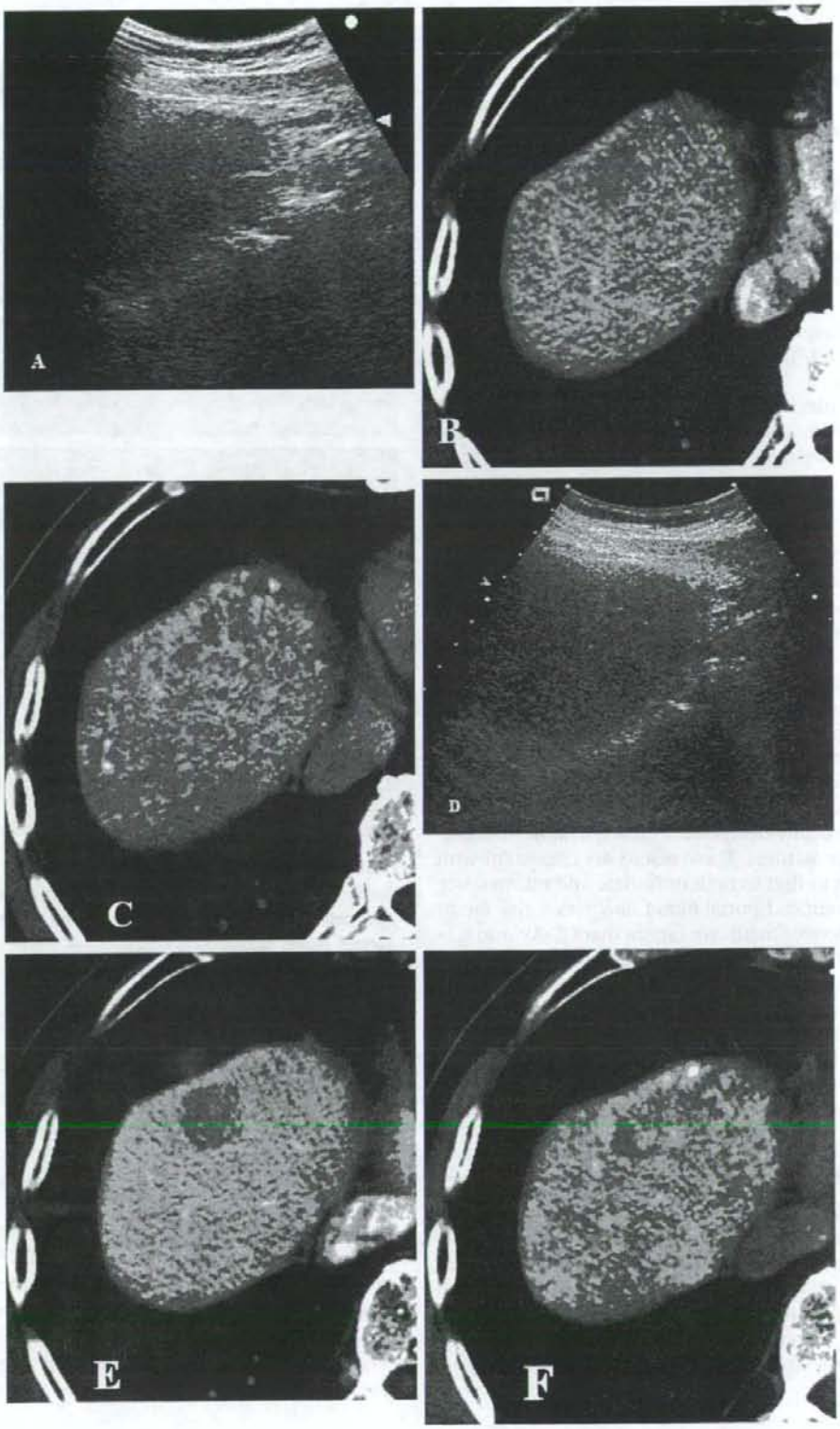

FIGURE 6

aging, and genetic mutation of oncogenes or tumor suppressor genes could occur at higher frequency with aging. We speculate that such alterations may generally increase risk of carcinogenesis with increasing age.

With respect to tumor diameter, it is intuitive that larger nodules could represent a more advanced stage in multistep carcinogenesis, and, thus, would progress to HCC more frequently than smaller ones. There is a problem of lead-time bias at this point. However, in our study, 133 of the 154 (86.4%) patients had undergone US screening before the first detection of hepatic nodules, and, thus, lead-time bias should have been minimized.

In multivariate analysis, histologic diagnosis and decrease of portal flow in CT-AP were independent factors for prediction of malignant transformation. In our study, we classified liver histology into three groups; HGDN, LGDN, and RN. According to this classification, as predicted, the progression rate of HCC from HGDN was significantly high, and the annual HCC development rate exceeded 30% in the first 2 years. The regression coefficient of HGDN in multivariate analysis was as much as 16.8 compared with RN. Therefore, we can conclude that HGDN was a true precancerous lesion of HCC.

Because of radiologic innovations, the relation between tumor progression and vascular supply of hepatic tumors is well documented. Hayashi et al.[29] followed up dysplastic nodules detected on CT-HA and CT-AP and described how portal blood flow in the nodule gradually decreased with acquisition of malignant tumor features. These results are consistent with our results in that in both univariate and multivariate analyses, reduced portal blood flow was a risk factor for HCC development. We realize that CT-AP and CT-HA are rather invasive methods and cannot easily be conducted repeatedly. Dysplastic nodules or RNs are usually isovascular or hypovascular.[29] In fact, in our patients, the arterial phase on dynamic CT was isoattenuating in 26 and low-attenuating in the remaining 9, and all 35 nodules were low-attenuating at portal venous phase and/or the equilibrium phase. Although detection of the nodule on dynamic CT was significant for predicting HCC transformation, as determined by univariate analysis, this was not significant by multivariate analysis. This could mean that CT-AP is superior to dynamic CT in detecting reduced portal blood flow and, hence, a better predictor of progression to HCC.

With respect to evaluation of tumor arterial blood flow, although we reviewed the results of CT-HA in our patients, we could not determine the relation between arterial blood flow and tumor progression. Hepatic arterial flow decreased in dysplastic nodules and in early stage HCC, and then it increased as lesions progressed to classic HCC.[29] Such a two-phase arterial

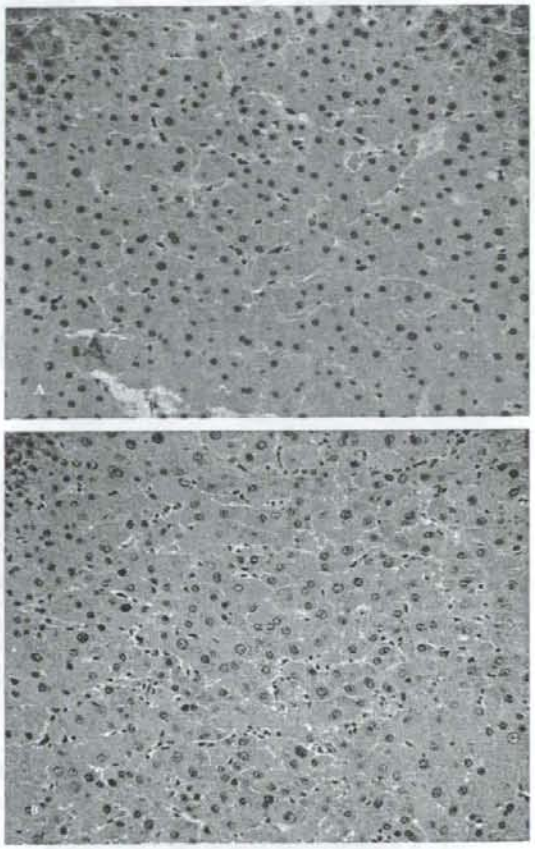

FIGURE 7. **(A)** The first histopathologic diagnosis based on examination of US-guided fine needle aspiration biopsy of the nodule was high-grade dysplastic nodule. **(B)** The nodule was subsequently resected surgically, and the specimen showed histopathologic features of well differentiated HCC. H&E staining in both panels; original magnification ×100.

FIGURE 6. **(A)** A 16-mm diameter hyperechoic nodule was found on US during the course of cirrhosis. **(B)** A vague and slightly low-attenuating area was detected on both CT-AP. **(C)** The nodule showed slightly hypoattenuating area on CT-HA. **(D)** Two years later, the nodule increased in size to 20 mm in diameter on US. **(E)** Further detailed image diagnosis was performed and a relatively well bordered low-attenuating area was detected on CT-AP. **(F)** In addition, a slightly high-attenuating rim appeared in the low-attenuating area on CT-HA.

flow change may have complicated our statistical analysis.

Previous studies showed the usefulness of MRI for characterization of hepatic nodular lesions.[32–35] Earls et al.[32] examined thin-section MRIs of explanted liver and reported that MRI depicted 41 of 42 (98%) hepatic nodular lesions, which included dysplastic nodules as well as HCC. Furthermore, Matsui et al.[33] demonstrated that hyperplastic adenomatous nodules were hyperintense on T1-weighted spin-echo imaging and hypointense on T2-weighted spin-echo imaging, and both features were useful for the differentiation of such borderline lesions from HCC in the cirrhotic liver. Furthermore, other groups also indicated the superiority of dynamic contrast-enhanced MRI and ferumoxides-enhanced MRI relative to CT for diagnosis of small HCC.[34,35] We also examined dynamic MRI in 48 patients and ferumoxides MRI in 25 patients. Because the number of these patients was small, we did not include MRI results in our analysis. Choi et al.[34] indicated that ferumoxides-magnetic resonance imaging can be used instead of CT-AP and CT-HA, because both modalities have almost the same sensitivity and high specificity for diagnosis of HCC. Further studies are needed to confirm the usefulness of ferumoxides-MRI for diagnosis of malignant transformation of hepatic nodular lesions.

There are certain limitations in our study. First, tumor biopsy was usually carried out under US guidance, and, therefore, nodules that were not observed on US were not included in this study. In our clinical practice, we sometimes find hepatic nodules that show low attenuation on CT-HA and/or CT-AP, but they are barely observed on US. Tsuchiyma et al.[31] examined repeated CT-HA and reported that 18.8% of small stained spots progressed to HCC during a mean follow-up of 29 months. Indeed, it is technically difficult to confirm such nodules histologically, and detailed investigation is required in the future.

Second, problems of sampling error and sampling variation are always inherent in this kind of examination. Indeed, nine patients in our study progressed to HCC despite histologic diagnosis of RN at first biopsy. In these cases, we cannot exclude sampling errors or sampling variation. However, to minimize such problems, we routinely recorded the scene of the US-guided biopsy on video recorder to confirm that the sample was actually from the nodule. In addition, it is possible that samples obtained by needle biopsy did not reflect the most malignant part of the nodule, particularly when the nodule was heterogeneous. On this account, we usually obtained samples from two or more parts of a nodule to prevent sampling variation. If a nodule showed a heterogeneous US pattern, we obtained samples from each part of the nodule.

We recognized limitations of biopsy diagnosis for predicting HCC progression, as nine patients whose initial histologic diagnosis was RN later developed HCC. For this reason, it is necessary to include imaging diagnostic techniques such as CT-HA or CT-AP to predict liver cancer development.

Our present results allow us to conclude that dysplastic nodules, in particular HGDN, are true precancerous lesions of HCC. Hemodynamic changes in these nodules predicted progression to HCC; however, angio-computed tomography is an invasive examination, and repeated studies are not feasible. Recently, it was reported that HCC and borderline lesions, like dysplastic or regenerative nodules, can be discriminated by Levovist contrast-enhanced US.[36,37] Because commercial use of Levovist was not possible until 1999 in our country, we have not included this technique in the current study. We are very interested in whether contrast-enhanced harmonic US is useful in predicting HCC development from hepatic nodular lesions, as this examination is less invasive than CT-HA or CT-AP and does not require hospitalization.

On the basis of changes observed during follow-up, our results indicated that enlargement of tumor diameter was the most important factor suggesting malignant transformation. Changes in US pattern also indicated HCC development. Indeed, low-echoic foci appeared in the center of hyperechoic nodules preceding HCC diagnosis in four patients who developed HCC. This finding is consistent with multistep carcinogenesis in HCC.

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Prediction model of hepatocarcinogenesis for patients with hepatitis C virus-related cirrhosis. Validation with internal and external cohorts

Kenji Ikeda[1,*], Yasuji Arase[1], Satoshi Saitoh[1], Masahiro Kobayashi[1], Takashi Someya[1], Tetsuya Hosaka[1], Norio Akuta[1], Yoshiyuki Suzuki[1], Fumitaka Suzuki[1], Hitomi Sezaki[1], Hiromitsu Kumada[1], Akihisa Tanaka[2], Hideharu Harada[2]

[1]*Department of Gastroenterology, Okinaka Memorial Institute for Medical Research, Toranomon Hospital, Toranomon 2-2-2, Minato-ku, Tokyo 105-8470, Japan*

[2]*Department of Gastroenterology, Tokyo National Hospital, National Hospital Organization, Tokyo, Japan*

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***Background/Aims*: To estimate hepatocarcinogenesis rates in patients with hepatitis C virus (HCV)-related cirrhosis, an accurate prediction table was created.**

***Methods*: A total of 183 patients between 1974 and 1990 were assessed for carcinogenesis rate and risk factors. Predicted carcinogenesis rates were validated using a cohort from the same hospital between 1991 and 2003 ($n=302$) and an external cohort from Tokyo National Hospital between 1975 and 2002 ($n=205$).**

***Results*: The carcinogenesis rates in the primary cohort were 28.9% at the 5th year and 54.0% at the 10th year. A proportional hazard model identified alpha-fetoprotein (≥ 20 ng/ml, hazard ratio 2.30, 95% confidence interval 1.55–3.42), age (≥ 55 years, 2.02, 95% CI 1.32–3.08), gender (male, 1.58, 95% CI 1.05–2.38), and platelet count ($<$ 100,000 counts/mm^3, 1.54, 95% CI 1.04–2.28) as independently associated with carcinogenesis. When carcinogenesis rates were simulated in 16 conditions according to four binary variables, the 5th- and 10th-year rates varied from 9 to 64%, and 21–93%, respectively. Actual carcinogenesis rates in the internal and external validation cohorts were similar to those of the simulated curves.**

***Conclusions*: Simulated carcinogenesis rates were applicable to patients with HCV-related cirrhosis. Since, hepatocarcinogenesis rates markedly varied among patients depending on background features, we should consider stratifying them for cancer screening and cancer prevention programs.**

***Keywords*: Cirrhosis; Hepatocellular carcinoma; Carcinogenesis; Hepatitis C virus; Simulation; Proportional hazard model; Validation; Prediction**

1. Introduction

There is increasing evidence that chronic hepatitis C virus (HCV) infection is closely associated with the occurrence of hepatocellular carcinoma (HCC) [1–4]. The incidence of patients with HCV-related HCC has increased recently in several parts of the world [5–9]. In Japan, blood transfusion and parenteral drug use became prevalent in 1960s, and patients with HCV-related cirrhosis gradually increased around 1980s. Since, an effective and truly curative therapy for a large and advanced HCC still remains limited at best, evaluation and assessment of carcinogenesis in chronic liver disease and detection at an early stage of HCC are of great importance. Reports of HCC development rates in HCV-cirrhosis differ [10–13], probably due to

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* Corresponding author. Tel.: +81 44 877 5111; fax: +81 44 860 1623.
E-mail address: ikedakenji@tora.email.ne.jp (K. Ikeda).

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differences of patient characteristics in varied study populations. The lack of reliable data as to the natural history of cirrhosis makes it difficult to evaluate the exact role and cost-effectiveness of interferon therapy.

Platelet count has been used to predict hepatocarcinogenesis [10,13,14], but its usefulness for distinguishing the HCC appearance rate is based on discrimination between chronic hepatitis and cirrhosis [15–18]. Predicting carcinogenesis solely on the basis of platelet count is less valuable in a cohort of patients with cirrhosis, because the liver disease has already advanced to a certain stage with a uniformly low platelet count. When a cohort of patients with HCV-related cirrhosis is analyzed by platelet count, it is usually not possible to discriminate between a super-high-risk group for carcinogenesis and a relatively low-risk group. The availability of a general model that can accurately predict the HCC development rate in HCV-related disease based on readily available data would be helpful in planning the treatment of these patients. Moreover, such a model could be used for the selection and stratification of patients for clinical trials.

In this study, we tried to develop a prediction model for hepatocarcinogenesis rate, using a large cohort with a long observation period. This model was also validated with two independent patient cohorts for generalization and clinical application.

2. Patients and methods

2.1. Study population

Among 457 consecutive patients diagnosed with liver cirrhosis between 1974 and 1990 at Toranomon Hospital, Tokyo, 258 patients had positive anti-HCV antibody (second-generation anti-HCV, enzyme-linked immunosorbent assay, Dainabot, Japan), positive HCV-RNA, and negative hepatitis B surface antigen (HBsAg, radioimmunoassay, Dainabot, Tokyo, Japan). Among them, 75 patients met either of the following exclusion criteria: (1) possible association with HCC, (2) association of hemochromatosis, autoimmune liver disease, primary biliary cirrhosis, alpha-1-antitrypsin deficiency, or Wilson disease, (3) daily drinking habit of 75 g or more, (4) alpha-fetoprotein (AFP) of 400 ng/ml or higher, (5) advanced and decompensated stage of cirrhosis with encephalopathy and refractory ascites, or (6) a short follow-up period of 6 months or less. We excluded those patients with Child–Pugh [19] stage C, because of substantial difference in carcinogenesis [20,21]. Consequently, 183 patients were retrospectively analyzed for HCC appearance rate.

2.2. Background and laboratory data

Table 1 summarizes the profiles and data of the 183 patients at the time of diagnosis. The group consisted of 92 men and 91 women aged from 28 to 80 (median, 55 years). The diagnosis of cirrhosis was made by peritoneoscopy, biopsy or both in 118 patients, and by clinical symptoms with ultrasonographic findings in 55 patients. When the ultrasonography (US) showed a typical irregular-surfaced liver with coarse internal architecture in addition to overt ascites or esophageal varices demonstrated by fiberscopic examination, we regarded the disease as cirrhosis. Although 12.7% of patients (23/181) showed normal aminotransferases at the time of the diagnosis of cirrhosis, all of those patients had been followed up as having chronic hepatitis with fluctuated aminotransferases.

Table 1
Patient profiles and laboratory data at the time of diagnosis of cirrhosis (primary cohort of Toranomon Hospital between 1974 and 1990, $n = 183$)

Demography and backgrounds		
Total number	183	
Sex (M/F)	92/91	
Age, median (range)	55 (28–80)	
Diagnostic method		
Peritoneoscopy and/or biopsy	118 (64.5%)	
Clinical (ultrasonography plus varices or ascites)	65 (35.5%)	
History of blood transfusion	82 (44.8%)	
Diabetes mellitus	23 (12.6%)	
Previous medical history of chronic hepatitis	34 (18.6%)	
Interferon therapy during observation	24 (12.0%)	
Refractory ascites and/or encephalopathy	0	
Hepatitis B surface antigen, positive	0 (100%)	
Anti-hepatitis C virus, positive	183 (100%)	
Hepatitis C virus RNA, positive	183 (100%)	
Child–Pugh score A	136 (74.3%)	
Child–Pugh score B	47 (25.7%)	
Observation period (year) median (range)	10.5 (0.5–26.0)	
Laboratory data	Median (range)	Valid data
Albumin (normal, 3.9–5.1 g/dl)	3.9 (2.5–5.1)	183
Bilirubin (normal, 0.3–1.1 mg/dl)	1.1 (0.4–4.4)	183
Aspartic transaminase (normal, ≤38 IU/L[a])	69 (17–372)	181
Alanine transaminase (normal, ≤50 IU/L[a])	56 (9–282)	181
Platelet (normal, 149–315×1000^3/mm^3)	95 (33–213)	183
ICG R15[b] (normal, ≤10%)	27 (6–81)	173
Prothrombin time (normal, ≥70%)	79 (54–100)	183
Gamma-globulin (normal, <1.5 g/dl)	1.9 (1.0–3.5)	174
Alpha-fetoprotein (normal, <5 mg/L)	16.5 (3–256)	166
HCV genotype[c]		
1b	107 (69.9%)	153
2a/2b	39 (25.5%)	
Combined/others	7 (4.6%)	
Not examined	30	

[a] Numbers of normal aspartic and alanine transaminases were 25 (13.8%) and 69 (38.1%), respectively. Both transaminases were normal at the time of the diagnosis of cirrhosis in 23 patients (12.7%).
[b] ICG R15: indocyanine green retention rate at 15 min.
[c] HCV genotyping was classified according to Simmonds et al. [22].

HCV-RNA measurement and HCV genotyping [22] are analyzed with nested polymerase chain reaction using initial sera stored at −80 °C.

2.3. Follow-up of patients and diagnosis of hepatocellular carcinoma

Patients were followed-up monthly following the diagnosis of cirrhosis by monitoring hematological and biochemical data. Diagnostic imaging by US was taken approximately once a year in each patient. After 1987, imaging procedures with US or computerized tomography (CT) were performed twice or more per year in the majority of patients for early detection of HCC. HCC was diagnosed by typical hypervascular characteristics on angiography. When combined use of imagings could not demonstrate a typical image of HCC (13/107, 12.1%), a fine needle biopsy was obtained for microscopic examination.

Twenty-four patients (13.1%) received interferon during the follow-up period. Since the therapy could affect the natural clinical course of viral hepatitis, they were treated as censored at the time of the initiation of interferon in the analysis. Sixteen (8.7%) cases were lost to follow-up, and median observation period was 10.5 years (range, 7.0–14.9). Those patients lost to follow-up were treated as censored data in the following statistics.

Any death unrelated to liver disease and cirrhosis-related liver failure were also classified as withdrawal and regarded as a censored case.

2.4. Statistical analysis and predictive model for carcinogenesis

The HCC development rate was analyzed using Kaplan–Meier technique [23] and differences in curves were tested using the log-rank test. The independent risk factors associated with the rate of HCC development were studied using stepwise method of non-time-dependent Cox regression analysis [24]. Potential risk factors assessed for liver carcinogenesis included the following 16 variables: age, sex, HCV genotype, association of diabetes mellitus, total alcohol intake (cumulative alcohol intake ≥ 200 kg), family history of liver disease, history of blood transfusion, association of ascites, serum aspartic transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transpeptidase (GGT), globulin, platelet count, AFP, indocyanine green retention rate at 15 min (ICG R15), and Child–Pugh score [19]. Each variable was transformed into categorical data consisting of two simple ordinal numbers (zero or one) for univariate and multivariate analyses. Although, proper transformation of variables were recommended in this kind of study [25], logarithmic transformation was not employed even for variables with non-symmetric distribution, because simple dichotomization also seemed reliable and robust statistically and because the simplicity was considered to bring about eventual clinical usefulness. Although, a cut-off value of 20 ng/ml proved to be an important point in our previous studies about prediction of liver cancer development in cirrhosis [10,26], other threshold values of dichotomizations were chosen from near figures to median values. In running the proportional regression analysis, care was taken to avoid overfitting the model by studying no more than one variable for every 10 events of carcinogenesis. Goodness-of-fit test together with log-minus–log plot was performed to confirm the proportionality assumption in the model. Since, missing data was not replaced, reduced numbers of cases were use in multivariate analysis. A P-value of less than 0.05 was considered to be significant.

The prognostic model was generated using Cox's regression procedure from the database of the 183 cirrhotic patients in Toranomon Hospital from 1974 to 1990. Using a final model for prediction of HCC appearance, carcinogenesis rate was predicted by substituting the corresponding ordinal numbers (zero or one) for every significant covariate in a given condition of the patients. Simulated carcinogenesis rates were computed for each state consisting of all statistically significant variables.

An internal and external cohorts of patients with HCV-positive cirrhosis verified the predicted carcinogenesis rates and curves: a cohort of 302 patients with HCV-cirrhosis diagnosed at Toranomon Hospital between 1991 and 2003 (internal validation group), and a cohort of 205 patients diagnosed at Tokyo National Hospital, Tokyo, Japan, between 1975 and 2002 (external validation group). The actual survival rates were calculated by the Kaplan–Meier technique in each risk group from the two validation cohorts, and evaluated by log-rank test according to the procedures of Christensen et al. [27].

Data analysis was performed with SAS version 9.1.3 software (SAS Institute, Inc., NC, USA).

The Human Ethics Review Committee of Toranomon Hospital approved the study protocol.

3. Results

3.1. Rate of hepatocellular carcinogenesis and risk factors

During the observation period, 107 (58.5%) out of 183 patients with HCV-related cirrhosis developed HCC. The cumulative HCC appearance rates of all patients were 15.0% at the end of the 3rd year, 28.9% at the 5th year, 37.8% at the 7th year, and 54.0% at the 10th year. Crude HCC development curve was drawn together with those of internal and external validation cohorts (Fig. 1).

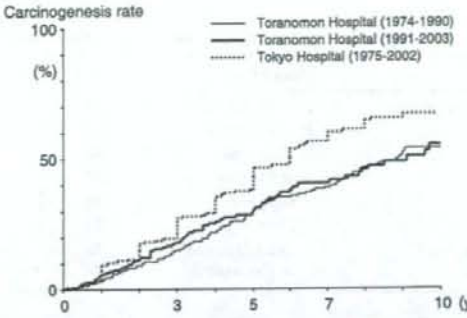

Fig. 1. Cumulative hepatocellular carcinogenesis rates in 183 patients who were diagnosed with HCV-related cirrhosis at Toranomon Hospital between 1974 and 1990. The 5th and 10th year rates were 28.9 and 54.0%, respectively (solid thin line). HCC appearance curves were also drawn in the internal (solid thick line) and external (dotted thick line) validation cohorts. The cancer appearance rate of Tokyo Hospital was significantly higher than those of the other two cohorts from Toranomon Hospital ($P=0.0015$, log-rank test).

Carcinogenesis rate in Tokyo Hospital was significantly higher than that of Toranomon Hospital (log-rank test $P=0.0015$). The risk factors for carcinogenesis were explored using non-time dependent proportional hazard analysis. In the final step of multivariate analysis, AFP ($P<0.001$), age ($P=0.001$), sex ($P=0.030$), and platelet count ($P=0.031$), were identified as independent significant predictors of future HCC appearance (Table 2). The hazard ratio of patients with AFP value of ≥20 ng/ml was 2.30 compared with those with lower AFP value, and the hazard ratio of patients of ≥55 years of age was 2.02 compared with younger patients. Child–Pugh score did not affect the carcinogenesis rate independently.

As for 23 patients with normal aminotransferases initially, 5- and 10-years carcinogenesis rates were 27.3 and 39.4%, respectively.

3.2. Simulation of carcinogenesis rates in patients with each prognostic factor

Simulated carcinogenesis curves were generated in each patient group with the Cox proportional hazard model by substituting the corresponding value for each parameter. Based on the four significant covariates, a total of 16 carcinogenesis curves were drawn, and simulated carcinogenesis rates were also estimated in the subgroups. To facilitate the practical use of the prediction model for carcinogenesis rate, we tabulated the results of estimated HCC appearance rates at the end of the 5th and 10th year (Table 3), in which calculated rates for a patient could be easily found for a given set of patient parameters (AFP, age, platelet and gender).

The model showed that when a patient is a male younger than 55 years, with a platelet count less than 100,000/mm^3 and an AFP value less than 20 ng/ml, the estimated hepatocarcinogenesis rates are 19% at the end of the 5th

Table 2
Factors associated with hepatocarcinogenesis (compensated cirrhosis, $n=183$, 1974–1990 cohort of Toranomon Hospital)

Factors	Category	No. of primary cohort	B	SE	Hazard ratio (95% CI)	P
Alpha-fetoprotein	0: <20 (ng/ml)	97			1	
	1: ≥20 (ng/ml)	69	0.83	0.20	2.30 (1.55–3.42)	<0.001
Age	0: <55 (year)	80			1	
	1: ≥55 (year)	103	0.74	0.22	2.02 (1.32–3.08)	0.001
Sex	0: Female	91			1	
	1: Male	92	0.46	0.21	1.58 (1.05–2.38)	0.030
Platelet count	0: ≥100,000/mm^3	87			1	
	1: <100,000/mm^3	96	0.43	0.20	1.54 (1.04–2.28)	0.031

year and 43% at the 10th year. The highest carcinogenesis rates were computed for males 55 years or older with a low platelet count and a high AFP value (64% at the 5th year, 93% at the 10th year), while the lowest estimated rates were found in females younger than 55 years with a high platelet count and a low AFP value (9% at the 5th year, 21% at the 10th year).

3.3. Validation of the prediction values of carcinogenesis rate

The reliability of the estimated HCC development rates was validated using internal (Toranomon Hospital, 1991–2003) and external (Tokyo National Hospital, 1975–2002) cohorts consisting of patients with HCV-related cirrhosis. Table 4 shows brief characteristics of patients in the two cohorts.

Since, HCC development curves were coarse and unreliable when a subgroup consisted of fewer patient number than 15, six figures of carcinogenesis curves were shown in principal subgroups consisting of ≥20 patients in each validation cohort (Fig. 2). When the parameters for all of the four significant covariates were at their worst (male ≥55 years, AFP≥20 ng/ml, and platelet count <100,000/mm^3), the simulated carcinogenesis rates were 64% at the end of the 5th year and 93% at the 10th year. On the other hand, the actual carcinogenesis rates in the internal and external validation cohorts were 54.9 and 61.5% at the 5th year, and 100 and 100% at the 10th year, respectively. The latter curves corresponded significantly with the simulation-generated carcinogenesis rate (Fig. 2a). Similarly, the other five simulated carcinogenesis curves were compared with both internal and external validation cohorts (Fig. 2b–f). Although the remaining 10 curves were not shown because of lack of sufficient patient number in the subgroup, actual carcinogenesis curves for the internal and external cohorts showed very analogous rates to the simulated ones, indicating that the simulation effectively predicted the future carcinogenesis rates. When we compared actual carcinogenesis rates in the validation groups with their calculated simulation values, 74.0% (375/507) and 70.4% (357/507) of the validation values for their 5th and 10th rates were coincident with those of predicted ones and stayed in an interval between +10% and −10% of simulated values. Although those patients in a large cohort consisting of 15 patients or more (e.g. Fig. 2a–f) usually showed a reliable and consistent values with simulated ones, those in a small cohort often revealed a labile and different values from simulated ones.

When a combined patient group of the three cohorts was analyzed, the same factors proved to affect the HCC appearance rate significantly: AFP (hazard ratio 2.19, $P<0.001$), age (1.96, $P<0.001$), sex (1.80, $P<0.001$), and platelet count (1.51, $P=0.009$). Hazard ratios with 95% confidence interval and P-values were also calculated in the individual validation groups (Table 5).

In addition, we evaluated the 'group factor' (study group, internal, and external validation groups) as a covariate in ordinary proportional hazard analysis for a combined patient group. Although, the internal and validation groups showed a slightly low (0.90) and high (1.26) hazard ratios for HCC development compared with that of the study group, the other four factors proved to show higher hazard ratios in the model (Table 6).

Table 3
Simulated carcinogenesis rates in stratified patient groups according to gender, age, platelet count, and alpha-fetoprotein value

Gender	Age (years)	Platelet	Alpha-feto-protein (ng/ml)	Simulated carcinogenesis rate (%)	
				5-year	10-year
Men					
	<55	<100,000/mm^3	<20	19	43
			≥20	42	77
		≥100,000/mm^3	<20	13	31
			≥20	32	65
	≥55	<100,000/mm^3	<20	32	65
			≥20	64	93
		≥100,000/mm^3	<20	23	50
			≥20	50	83
Women					
	<55	<100,000/mm^3	<20	13	30
			≥20	30	61
		≥100,000/mm^3	<20	9	21
			≥20	22	47
	≥55	<100,000/mm^3	<20	22	49
			≥20	49	83
		≥100,000/mm^3	<20	16	37
			≥20	37	69

Table 4
Patient profiles and laboratory data of two cohorts for validation: an internal cohort (Toranomon Hospital from 1991 to 2003, $n=302$) and an external cohort (Tokyo National Hospital, $n=205$)

	Internal cohort (Toranomon Hospital, 1991–2003)		External cohort (Tokyo National Hospital, 1975–2002)	
Demography and backgrounds				
Total number	302		205	
Sex (M/F)	166/136		111/94	
Age (year)[a]	59 (28–80)		62 (13–83)	
Diagnostic method				
Peritoneoscopy and/or biopsy	128		115	
Clinical diagnosis	174		90	
Interferon therapy				
Yes	105 (34.8%)		12 (5.9%)	
No	197		193	
Observation period (year)[a]	5.3 (0.5–13.9)		7.5 (0.5–30.8)	
Laboratory examination	Internal cohort (Toranomon Hospital, 1991–2003)	Valid data	External cohort (Tokyo National Hospital, 1975–2002)	Valid data
Platelet ($\times 1000^3$/mm^3)[a]	91.5 (25–223)	302	100 (19–310)	205
Alpha-fetoprotein (ng/ml)[a]	14 (1–380)	296	15 (2–365)	205

[a] Expressed by median (range).

3.4. Estimation of carcinogenesis rates by number of unfavorable risk factors

The prognostic model showed that the HCC development rate was significantly affected by the following four unfavorable factors: high AFP ($\geq$20 ng/ml), older age ($\geq$55 years), low platelet count ($<100{,}000$/mm^3), and male sex. Although, limitation of predictability could not be avoided because of different values of hazard ratios, we attempted to make more convenient HCC prediction curves. Five carcinogenesis curves were generated according to the number of unfavorable risk factors among the four significant covariates: no factors, one, two, three, and four unfavorable factors. When no unfavorable factor was found in a cohort of HCV-cirrhosis, the hepatocarcinogenesis rates were 9% at the end of the 5th year and 21% at the 10th year. Similarly, when one, two, three and four factors were found in a cohort, the carcinogenesis rates were 16, 28, 46, and 64% at the 5th year, and 35, 55, 78, and 93% at the 10th year, respectively (log-rank test, $P=0.0001$).

To validate the reliability of the concise prediction curves, the actual carcinogenesis curves were generated by the product-limit method for the 1991–2003 internal cohort of our hospital (Fig. 3). All actual carcinogenesis curves fitted well with the simulated curves, except for the subgroup with 'no unfavorable factors': none of 11 patients in this subgroup developed HCC during a median observation period of 10.0 years (25 percentile 8.1 years, 75 percentile 10.8 years).

4. Discussion

Ten-year-rate of HCC development has been reported as 50–80% in some cohorts of HCV-positive cirrhosis [10–13,28], and the cohorts in our hospital showed 54–55%, and Tokyo Hospital 68%. However, the reasons for the significant differences found in the rates among various hospitals have not been fully elucidated until recently. Many risk factors have been identified as important for the development of HCC in patients with hepatitis or cirrhosis [10,13,29,30], but of even greater interest is the precise prediction of HCC. In order to establish a reliable method for predicting carcinogenesis risk in a variety of patients with HCV-positive cirrhosis (compensated and decompensated), we investigated a large cohort of patients with few dropout cases, using a multivariate proportional model.

In the final step of multivariate analysis, AFP, age, platelet and gender were independently associated with HCC development in the primary cohort of our hospital. A total of 16 simulated carcinogenesis curves were drawn according to the four binary factors. Surprisingly, the estimated carcinogenesis curves significantly differed from each other among the stratified subgroups in our hospital, depending on demographic and background characteristics. In the case of a patient with HCV-cirrhosis, the combination of age, gender, AFP and platelet count could give important prognostic information about future carcinogenesis risk. When HCC appearance rates were simulated under 16 conditions according to the four binary variables identified by multivariate analysis, the 5th year rate varied from 9 to 64%, and 10th year rates from 21 to 93%. On the other hand, aminotransferase level and Child–Pugh score were poor predictors of carcinogenesis in patients with HCV-cirrhosis.

We recognized that the HCC development rate should be evaluated more specifically for each subgroup than for the entire cohort of HCV-positive cirrhosis patients. Integration of the four predictive factors could provide useful information about HCV-related carcinogenesis in actual clinical practice. The reported diversity of carcinogenesis

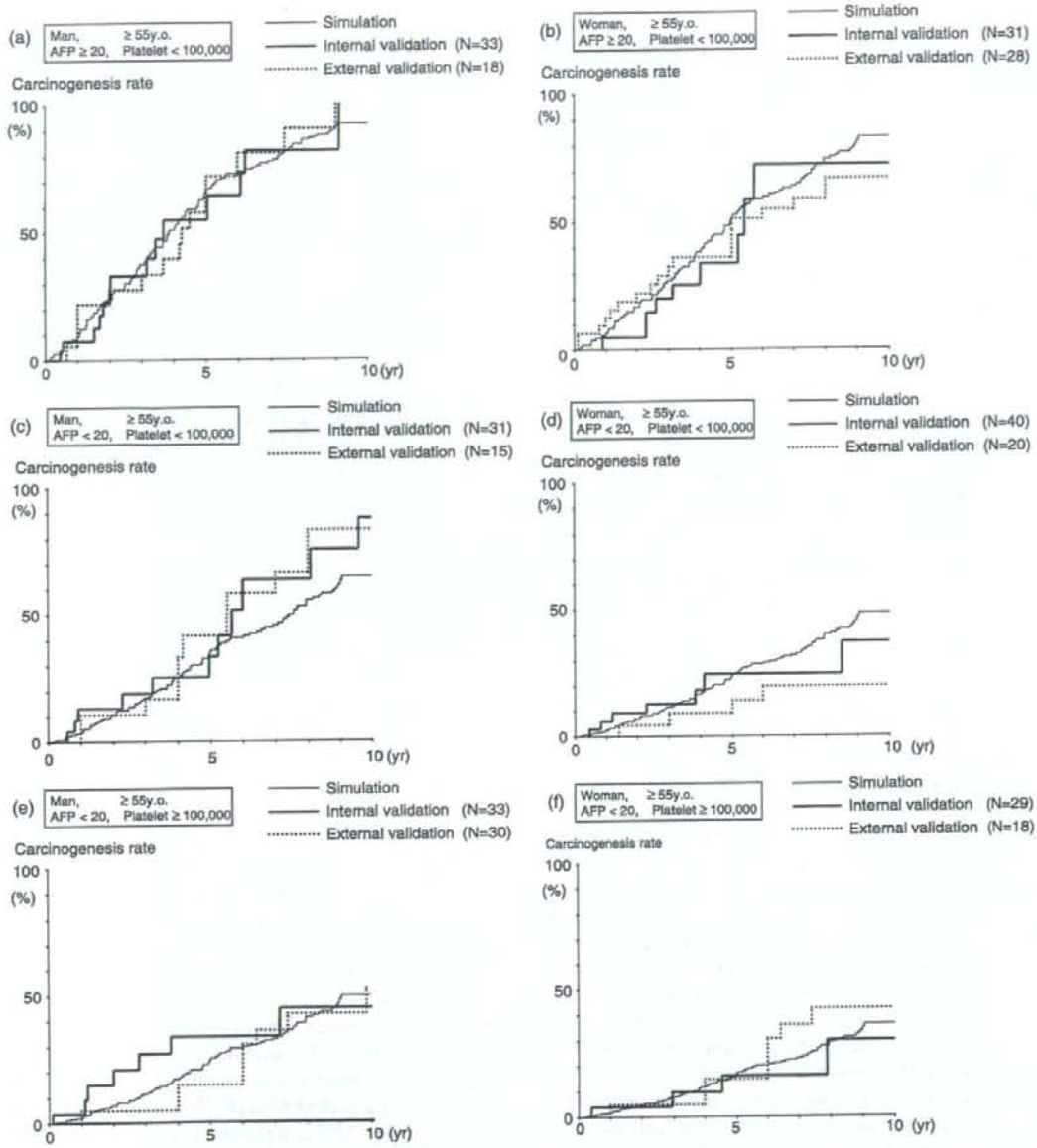

Fig. 2. Simulated carcinogenesis curves with actual carcinogenesis rates of internal and external validation cohorts, according to four significant predictors (gender, age, alpha-fetoprotein [AFP], and platelet count). ***Thin solid lines*****: simulated carcinogenesis curves,** ***bald lines*****: actual curves of internal cohort (Toranomon Hospital, 1991–2003),** ***bald dotted lines*****: actual curves of external cohort (Tokyo National Hospital, 1975–2002). (a) Carcinogenesis curves for subgroup of man, age ≥ 55 years, AFP ≥ 20 ng/ml, and platelet count < 100,000/mm^3. (b) Subgroup of woman, age ≥ 55 years, AFP ≥ 20 ng/ml, and platelet count < 100,000/mm^3. (c) Subgroup of man, age ≥ 55 years, AFP < 20 ng/ml, and platelet count < 100,000/mm^3. (d) Subgroup of woman, age ≥ 55 years, AFP < 20 ng/ml, and platelet count < 100,000/mm^3. (e) Subgroup of man, age ≥ 55 years, AFP < 20 ng/ml, and platelet count ≥ 100,000/mm^3. (f) Subgroup of woman, age ≥ 55 years, AFP < 20 ng/ml, and platelet count ≥ 100,000/mm^3.**

rates also explains the inconsistency of estimated carcinogenesis rates from untreated cirrhosis caused by HCV. One of the reasons why carcinogenesis rates differed between the two hospitals seemed to originate from the difference of age of the patient populations. Current study did aim at precise prediction of carcinogenesis rate of each cirrhotic patient in different hospital and different period of time.

Validation of such a model is essential before these tools can gain widespread clinical use [31]. The best way to validate these models is to assess their performance in sets

Table 5
Significance of four factors associated with hepatocarcinogenesis in the internal validation group ($n=302$) and external validation group ($n=205$, 1975–2002 cohort of Tokyo National Hospital)

Factors	Internal validation cohort (1991–2003 Toranomon Hospital)		External validation cohort (1975–2002 Tokyo National Hospital)	
	Hazard ratio (95% CI)	P	Hazard ratio (95% CI)	P
Alpha-fetoprotein	1		1	
	2.13 (1.21–3.78)	0.009	2.23 (1.55–3.23)	<0.001
Age	1		1	
	3.36 (1.56–7.23)	0.002	1.55 (0.96–2.48)	0.071
Sex	1		1	
	1.78 (0.99–3.19)	0.040	2.01 (1.38–2.92)	<0.001
Platelet	1		1	
	1.49 (0.83–2.67)	0.18	1.40 (0.97–2.02)	0.070

of patients who are independent in place and time [32]. This external validity is particularly important when models are used to predict outcomes in daily practice, because it is well known that prognostic models do not perform as well in patients outside the clinical context in which they are developed [33]. This study shows that our prognostic model accurately predicts carcinogenesis rates for patients with HCV-cirrhosis from a chronologically different group and a geographically different referral center, and therefore supports the generalization and reliability of the model. The two validation cohorts (302 and 205 patients) were classified into 16 groups according to their risk factors, and the values for the actual and model-predicted survival of each risk group were compared graphically using actual Kaplan–Meier curves. The model provided a very good fit with the carcinogenesis data of each risk group in the validation cohorts (Fig. 2a–f).

We could not draw meaningful and reliable carcinogenesis curves in the remaining 10 risk groups, because of small patient numbers. The significance of current study might be the prediction of hepatocarcinogenesis in these small patient groups.

We also tried to predict carcinogenesis risk using a simplified process in the same patient group, using few unfavorable risk factors instead of individual items of the risk factors. The clinical characteristics of the 302 patients in the internal validation cohort, for whom complete information was available, are summarized in Table 4, together with the characteristics of the 183 patients used to develop the model. Since, both groups of patients were very similar in terms of their risk variables, the estimated carcinogenesis curves showed good agreement: all actual carcinogenesis curves fitted well with the simulated curves, except for a subgroup with 'no unfavorable factors'. The reason for the inconsistency was that none of the 11 patients in the subgroup developed HCC, and because the 'best' subgroup might include a significant number of patients with far better liver function tests for cirrhosis. Since, the external validation cohort included older patients with low platelet counts, the differences in the proportion of unfavorable risk factors would produce contradictory results in this kind of analysis when only using few risk factors.

For pragmatic purposes, a good prognostic model, in addition to being generalizable, needs to be based on readily accessible variables and can be calculated easily at the bedside [34]. Our model employs four variables that are readily available for every patient with cirrhosis, and includes the responses to four yes/no questions. With the help of a pocket table (Table 3), a calculator is even not needed to determine the carcinogenesis risk of a given patient and their estimated median carcinogenesis rate. Since, there is considerable diversity in carcinogenesis risk among individual patients with HCV-cirrhosis, these results will be useful for stratification of patients in future cancer prevention trials. Even though predictability of carcinogenesis risk in individual patients is limited in this kind of statistics [35], this study will be helpful to realize the diversity of carcinogenesis rate in the same 'HCV-related cirrhosis'.

In conclusion, our four-variable model is a simple and useful tool for predicting carcinogenesis rates in patients with cirrhosis caused by HCV. Prediction models for HCC

Table 6
Multivariate analysis for a combined patient group of study cohort, internal validation cohort, and external validation cohort

Factors	Category	Hazard ratio (95% confidence interval)	P
Alpha-fetoprotein	0: <20 (ng/ml)	1	
	1: ≥20 (ng/ml)	2.22 (1.77–2.79)	<0.001
Age	0: <55 (year)	1	
	1: ≥55 (year)	1.90 (1.44–2.51)	<0.001
Sex	0: Female	1	
	1: Male	1.90 (1.50–2.40)	<0.001
Platelet count	0: ≥100,000/mm^3	1	
	1: <100,000/mm^3	1.46 (1.16–1.84)	0.001
Patient groups	0: Study cohort	1	
	1: Internal validation cohort	0.90 (0.66–1.23)	0.52
	2: External validation cohort	1.26 (1.04–1.57)	0.023

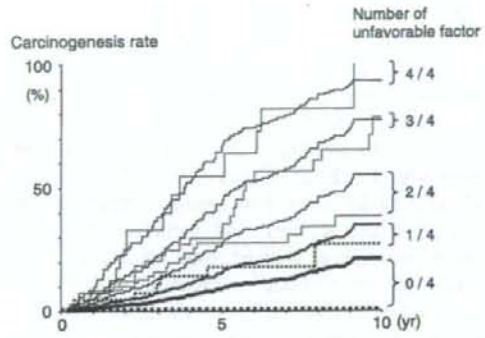

Fig. 3. Simulated HCC appearance curves with actual appearance rates of internal and external validation cohorts, according to the number of unfavorable risk factors. Five solid curves show simulated carcinogenesis rates drawn according to the number of unfavorable risk factors; none (the thickest line), one, two, three, and four (the thinnest line). Five dotted curves indicate actual HCC appearance curves of the validation cohort (Toranomon Hospital, 1991–2003).

development that combine several variables of patient data to indicate the probability of clinical outcome are powerful tools for assisting physicians in the decision-making process. Our model can be used for prediction of HCC in daily clinical practice by hepatologists, for education and information for individual patients, for selection of a candidate for a cancer prevention program, and for a proper stratification of cirrhotic patients in clinical trials for the purpose of cancer prevention. The consistency and reproducibility of the present model should also be confirmed by other institutions outside Japan.

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＜速　報＞

LDL cholesterol と HCV core region は C 型慢性肝炎に対する Peginterferon/Ribavirin 併用療法の重要な治療前効果予測因子である

芥田　憲夫[1]　鈴木　文孝[1]　川村　祐介[1]　八辻　寛美[1]
瀬崎ひとみ[1]　鈴木　義之[1]　保坂　哲也[1]　小林　正宏[1]
小林万利子[2]　荒瀬　康司[1]　池田　健次[1]　熊田　博光[1]

目的：Peginterferon（PEG-IFN）/Ribavirin（RBV）併用療法中に HCV RNA が陰性化しない治療抵抗例では Core region の aa70 と aa91（Core aa70/91）の置換が関与していることを著者らは報告してきた[1][2]．また最近では，脂質代謝改善薬が抗 HCV 療法の治療成績を改善する可能性が示唆されていることから[3][4]，脂質要因が併用療法の治療成績に如何なる影響を及ぼしているか検討した．

方法：PEG-IFN/RBV 併用療法 48 週間（PEG-IFNα2b は 1.5μg/kg/週，RBV は 10.9mg/kg/日の投与量中央値）を施行した genotype 1b・高ウイルス量（≥100KIU/ml）の日本人 130 例を対象とした．

Core aa70/91 の置換は変異特異的 primer を用いた PCR 法で aa70 と aa91 を各々測定し，Double wild type（aa70：arginine（wild）かつ aa91：leucine（wild））とそれ以外の Non double wild type に分類．治療効果判定は 12 週目で RNA 量が $2\log_{10}$ 以上低下もしくは RNA 陰性化した症例を Early virologic response（EVR），治療終了後 24 週目で RNA 陰性化が持続している症例を Sustained virological response（SVR）とし，脂質要因を含む治療前 28 因子（年齢，性別，PEG-IFN 量/体重，RBV 量/体重，組織学的 staging，AST，ALT，γGTP，白血球数，ヘモグロビン値（Hb），血小板数，血清鉄，血清フェリチン，ICG R15，アルブミン，クレアチニンクリアランス，輸血歴，肝疾患家族歴，BMI，肝細胞脂肪化，空腹時血糖，尿酸，総コレステロール（TC），中性脂肪，HDL コレステロール（HDL-C），LDL コレステロール（LDL-C），HCV RNA 量，Core aa70/91 置換）を用いて多変量解析（logistic regression analysis）を行い治療効果に寄与する独立要因を求めた．

成績：EVR 率は全体で 75%，SVR 判定可能な連続 104 例における SVR 率は ITT 解析で 45%．

EVR に関する単変量解析では Core aa70/91 置換（Double wild type），TC（≥170mg/dl），LDL-C（≥86mg/dl），白血球数（≥4,500/mm³），γGTP（＜109IU/l）の 5 要因で EVR と EVR 以外の症例との間に統計学的に傾向差もしくは有意差が認められた（P＜0.1，chi-squared test）．多変量解析で EVR に寄与する独立因子は LDL-C，Core aa70/91 置換，白血球数であった（P＜0.05，logistic regression analysis）．

更に SVR に関する単変量解析では Core aa70/91 置換（Double wild type），年齢（＜55 歳），性別（男性），PEG-IFN 量/体重（≥1.25μg/kg），RBV 量/体重（≥11.0mg/kg），staging（F1），AST（＜60IU/l），白血球数（≥4,500/mm³），Hb（≥14.0g/dl），ICG R15（＜10%），アルブミン（≥3.9g/dl），γGTP（＜109IU/l），LDL-C（≥86mg/dl）の 13 要因で SVR と SVR 以外の症例との間に統計学的に傾向差もしくは有意差が認められた（P＜0.1，chi-squared test）．多変量解析で SVR に寄与する独立因子は LDL-C，Core aa70/91 置換，性別，ICG R15，AST であった（P＜0.05，logistic regression analysis）．

この様に LDL-C と Core aa70/91 置換は EVR と SVR に共通した治療前効果予測因子であることが確認された（Table）．

考案：LDL-C と Core aa70/91 は PEG-IFN/RBV 併用療法における重要な治療前効果予測因子であることが示唆された．血清中の HCV 粒子は HCV-LDL 複合体を形成し，LDL receptor を介して endocytosis により細胞内に進入する[5]．この様な感染メカニズムに重要な LDL-C が日本の genotype 1b に対する PEG-IFN/RBV 治療反応性に影響するという成績は非常に重要であり，この機序に関しては更なる検討を要する．

索引用語：LDL cholesterol，HCV core region，Peginterferon/Ribavirin

文献：1）Akuta N, Suzuki F, Sezaki H, et al. Intervirology 2005; 48: 372—380　2）Akuta N, Suzuki F, Sezaki H, et al. J Med Virol 2006; 78: 83—90　3）Ikeda M, Abe K, Yamada M, et al. Hepatology 2006; 44: 117—125　4）Fujita N, Kaito M, Kai M, et al. J Viral Hepat 2006; 13: 441—448　5）Agnello V, Abel G, Elfahal M, et al. Proc Natl Acad Sci USA 1999; 96: 12766—12771

1）虎の門病院肝臓病センター
2）虎の門病院肝臓研究室

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Table Factors associated with treatment efficacy to combination therapy with peginterferon plus ribavirin for 48 weeks in patients infected with HCV genotype1b, identified by multivariate analysis

Factor	[Category]	Odds ratio (95% confidence interval)	P
(Factor for EVR)			
Amino acid substitution in core region	1 : double wild type *	1	
	2 : non double wild type	0.041 (0.007-0.255)	0.001
LDL cholesterol (mg/dl)	1 : < 86	1	
	2 : ≥ 86	9.920 (2.642-37.25)	0.001
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Amino acid substitution in core region	1 : double wild type *	1	
	2 : non double wild type	0.072 (0.012-0.422)	0.003
LDL cholesterol (mg/dl)	1 : < 86	1	
	2 : ≥ 86	7.543 (1.067-53.30)	0.043

* The pattern of wild at aa 70 and wild at aa 91 was evaluated as double wild type, and the other patterns were as non double wild type.

Only common variables for prediction of EVR and SVR that achieved statistical significance ($P < 0.05$) on multivariate logistic regression are shown.

Normal reference ranges : 86-135 mg/dl for LDL cholesterol.

英文要旨

Low density lipoprotein cholesterol levels and amino acid substitutions in HCV core region are important pretreatment predictors of response to treatment with peginterferon plus ribavirin in Japanese patients with chronic hepatitis C

Norio Akuta[1], Fumitaka Suzuki[1], Yusuke Kawamura[1], Hiromi Yatsuji[1], Hitomi Sezaki[1], Yoshiyuki Suzuki[1], Tetsuya Hosaka[1], Masahiro Kobayashi[1], Mariko Kobayashi[2], Yasuji Arase[1], Kenji Ikeda[1], Hiromitsu Kumada[1]

We evaluated 130 consecutive Japanese adults of HCV genotype 1b who received treatment with peginterferon (PEG-IFN) plus ribavirin (RBV) for 48 weeks, to investigate the pretreatment predictive factors of early virologic response (EVR) and sustained virological response (SVR). 75% of patients could achieve EVR, and 45% were SVR. Multivariate analysis identified low density lipoprotein cholesterol (LDL-C) (≥86mg/dl) and amino acid (aa) substitutions in HCV core region (Double wild type: arginine at aa 70 and leucine at aa 91) as independent and significant determinants of EVR. Furthermore, multivariate analysis identified LDL-C (≥86mg/dl), aa substitutions in core region (Double wild type), gender (male), ICG R15 (<10%), AST (<60IU/l) as determinants of SVR. In conclusion, LDL-C and aa substitutions in core region are important pretreatment predictors of response to treatment with PEG-IFN plus RBV in Japanese patients infected with genotype 1b.

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1) Department of Hepatology, Toranomon Hospital, Tokyo, JAPAN
2) Liver Research Laboratory, Toranomon Hospital, Tokyo, JAPAN

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＜速　報＞

LDL cholesterol と HCV core region はC型慢性肝炎に対する Peginterferon/Ribavirin 併用療法の重要な治療前効果予測因子である

芥田　憲夫[1]　鈴木　文孝[1]　川村　祐介[1]　八辻　寛美[1]
瀬崎ひとみ[1]　鈴木　義之[1]　保坂　哲也[1]　小林　正宏[1]
小林万利子[2]　荒瀬　康司[1]　池田　健次[1]　熊田　博光[1]

目的：Peginterferon（PEG-IFN）/Ribavirin（RBV）併用療法中にHCV RNAが陰性化しない治療抵抗例ではCore regionのaa70とaa91（Core aa70/91）の置換が関与していることを著者らは報告してきた[1][2]．また最近では，脂質代謝改善薬がC型HCV療法の治療成績を改善する可能性が示唆されていることから[3][4]，脂質要因が併用療法の治療成績に如何なる影響を及ぼしているか検討した．

方法：PEG-IFN/RBV併用療法48週間（PEG-IFNα2bは1.5μg/kg/週，RBVは10.9mg/kg/日の投与量中央値）を施行したgenotype 1b・高ウイルス量（≥100KIU/ml）の日本人130例を対象とした．

Core aa70/91の置換は変異特異的primerを用いたPCR法でaa70とaa91を各々測定し，Double wild type（aa70：arginine（wild）かつaa91：leucine（wild））とそれ以外のNon double wild typeに分類．治療効果判定は12週目でRNA量が$2\log_{10}$以上低下もしくはRNA陰性化した症例をEarly virologic response（EVR），治療終了後24週目でRNA陰性化が持続している症例をSustained virological response（SVR）とし，脂質要因を含む治療前28因子（年齢，性別，PEG-IFN量/体重，RBV量/体重，組織学的staging，AST，ALT，γGTP，白血球数，ヘモグロビン値（Hb），血小板数，血清鉄，血清フェリチン，ICG R15，アルブミン，クレアチニンクリアランス，輸血歴，肝疾患家族歴，BMI，肝細胞脂肪化，空腹時血糖，尿酸，総コレステロール（TC），中性脂肪，HDLコレステロール（HDL-C），LDLコレステロール（LDL-C），HCV RNA量，Core aa70/91置換）を用いて多変量解析（logistic regression analysis）を行い治療効果に寄与する独立要因を求めた．

成績：EVR率は全体で75%，SVR判定可能な連続104例におけるSVR率はITT解析で45%．

EVRに関する単変量解析ではCore aa70/91置換（Double wild type），TC（≥170mg/dl），LDL-C（≥86mg/dl），白血球数（≥4,500/mm³），γGTP（<109IU/l）の5要因でEVRとEVR以外の症例との間に統計学的に傾向差もしくは有意差が認められた（P<0.1，chi-squared test）．多変量解析でEVRに寄与する独立因子はLDL-C，Core aa70/91置換，白血球数であった（P<0.05，logistic regression analysis）．

更にSVRに関する単変量解析ではCore aa70/91置換（Double wild type），年齢（<55歳），性別（男性），PEG-IFN量/体重（≥1.25μg/kg），RBV量/体重（≥11.0mg/kg），staging（F1），AST（<60IU/l），白血球数（≥4,500/mm³），IIb（≥14.0g/dl），ICG R15（<10%），アルブミン（≥3.9g/dl），γGTP（<109IU/l），LDL-C（≥86mg/dl）の13要因でSVRとSVR以外の症例との間に統計学的に傾向差もしくは有意差が認められた（P<0.1，chi-squared test）．多変量解析でSVRに寄与する独立因子はLDL-C，Core aa70/91置換，性別，ICG R15，ASTであった（P<0.05，logistic regression analysis）．

この様にLDL-CとCore aa70/91置換はEVRとSVRに共通した治療前効果予測因子であることが確認された（Table）．

考案：LDL-CとCore aa70/91はPEG-IFN/RBV併用療法における重要な治療前効果予測因子であることが示唆された．血清中のHCV粒子はHCV-LDL複合体を形成し，LDL receptorを介してendocytosisにより細胞内に進入する[5]．この様な感染メカニズムに重要なLDL-Cが日本のgenotype 1bに対するPEG-IFN/RBV治療反応性に影響するという成績は非常に重要であり，この機序に関しては更なる検討を要する．

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英文要旨

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1) Department of Hepatology, Toranomon Hospital, Tokyo, JAPAN
2) Liver Research Laboratory, Toranomon Hospital, Tokyo, JAPAN

Amino Acid Substitutions in the Hepatitis C Virus Core Region are the Important Predictor of Hepatocarcinogenesis

Norio Akuta,[1] Fumitaka Suzuki,[1] Yusuke Kawamura,[1] Hiromi Yatsuji,[1] Hitomi Sezaki,[1] Yoshiyuki Suzuki,[1] Tetsuya Hosaka,[1] Masahiro Kobayashi,[1] Mariko Kobayashi,[2] Yasuji Arase,[1] Kenji Ikeda,[1] and Hiromitsu Kumada[1]

We showed previously that amino acid (aa) substitutions in hepatitis C virus core region (HCV-CR) are negative predictors of virologic response to pegylated interferon (IFN) plus ribavirin therapy. HCV-CR induces hepatocellular carcinoma in transgenic mice, but the clinical impact is still unclear. To evaluate the impact of aa substitutions in HCV-CR on hepatocarcinogenesis, we performed a follow-up study on 313 noncirrhotic consecutive naïve patients infected with HCV genotype 1b who received IFN monotherapy. The median follow-up was 14.7 years. A sustained virologic response (SVR) after the first IFN was achieved by 65 patients (20.8%) (group A). Of 248 patients (79.2%) of non-SVR after first IFN, 112 (35.8%) did not receive additional IFN (group B), and the remaining 136 (43.5%) received multicourse IFN monotherapy (group C). As a whole, cumulative hepatocarcinogenesis rates in double wild-type (arginine at aa 70/ leucine at aa 91) of HCV-CR were significantly lower than those in nondouble wild-type. Multivariate analyses identified 3 parameters (fibrosis stage 3, nondouble wild-type of HCV-CR, and group B) that tended to or significantly influenced hepatocarcinogenesis independently. With regard to hepatocarcinogenesis rates in group C according to HCV-CR and the mean alanine aminotransferase (ALT) during IFN-free period, significantly higher rates were noted in patients of nondouble wild-type with ALT levels of more than 1.5 times the upper limit of normal (25.7%) compared with the others (2.4%). *Conclusion:* Amino acid substitutions in the HCV-CR are the important predictor of hepatocarcinogenesis. In multicourse IFN therapy to nondouble wild-type, we emphasize the importance of reducing the risk of hepatocarcinogenesis by mean ALT during an IFN-free period below 1.5 times the upper limit of normal. (HEPATOLOGY 2007;46: 1357-1364.)

Hepatitis C virus usually causes chronic infection, which can result in chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC).[1-5] In patients with chronic HCV, treatment with IFN can induce viral clearance and marked biochemical and histological improvement.[6,7]

For chronic HCV infection, peginterferon (PEG-IFN) plus ribavirin (RBV) combination therapy is an expensive treatment modality that is accompanied by severe side effects and high sustained virological response (SVR). Patients who do not achieve SVR need to be identified before the start of combination therapy to avoid unnecessary side effects and high costs. Thus, safer IFN monotherapy should be considered to reduce the risk of hepatocarcinogenesis in patients unsuitable for PEG-IFN plus RBV therapy. We studied previous determinants of response to PEG-IFN plus RBV in patients with high titers of HCV genotype 1b (≥100 KIU/mL), which is dominant in Japan. Our results identified substitution of amino acids (aa) 70 and/or 91 in the HCV core region (HCV-CR) as an independent and significant negative predictor associated with virological response.[8-10] Furthermore, we reported that multicourse IFN monotherapy reduces the risk of hepatocarcinogenesis and increases survival even if patients fail to achieve SVR after a single-course IFN, and

Abbreviations: aa, amino acid(s); HCV-CR, hepatitis C virus core region; MU, million units; PEG-IFN, peginterferon; RBV, ribavirin; SVR, sustained virologic response.

From the [1]Department of Hepatology and [2]Liver Research Laboratory, Toranomon Hospital, Tokyo, Japan.

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Address reprints requests to: Norio Akuta, M.D., Department of Hepatology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo, 105-0001, Japan. E-mail: akuta-gi@umin.ac.jp; fax: (81) 44-860-1623.

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that low ALT levels during an IFN-free period is associated with lower rates of hepatocarcinogenesis.[11] Hence, multicourse IFN monotherapy might be expected to reduce the risk of hepatocarcinogenesis in patients who have negative predictors for PEG-IFN plus RBV.

Despite numerous lines of epidemiological evidence connecting HCV infection and the development of HCC, it remains controversial whether HCV itself plays a direct or indirect role in the pathogenesis of HCC.[12] It has become evident that HCV-CR has oncogenic potential through the use of transgenic mice, but the clinical impact of HCV-CR on hepatocarcinogenesis is still unclear.[13] Whether substitution of aa 70 and/or 91 in HCV-CR as a predictor of virological response for PEG-IFN plus RBV therapy also affects hepatocarcinogenesis awaits further investigation.

The present study included 313 consecutive naïve cases infected with HCV genotype 1b in whom 15 years had elapsed since induction of IFN monotherapy. The aims of the study were: (1) to evaluate the clinical impact of aa substitutions in the HCV-CR on hepatocarcinogenesis; (2) to analyze the predictive factors associated with hepatocarcinogenesis in patients who received IFN monotherapy; and (3) to evaluate the long-term efficacy of multicourse IFN monotherapy on hepatocarcinogenesis as examined through analysis of the outcomes of single and multicourses of IFN.

Patients and Methods

Patients. Among 573 consecutive HCV-infected patients in whom IFN monotherapy was induced between February 1987 and August 1992 at Toranomon Hospital, 313 were selected in the present study based on the following criteria: (1) patients naïve to IFN monotherapy; (2) patients infected with HCV genotype 1b alone; (3) patients with chronic hepatitis, without cirrhosis or HCC, as confirmed via biopsy examination within 6 months of enrollment; (4) patients not treated with IFN plus RBV combination therapy during follow-up time; (5) patients negative for hepatitis B surface antigen (radioimmunoassay, Dainabot, Tokyo, Japan), positive for anti-HCV (third-generation enzyme immunoassay, Chiron Corp., Emeryville, CA), and positive for HCV RNA qualitative analysis with PCR (nested polymerase chain reaction or Amplicor, Roche Diagnostic Systems, CA); (6) patients free of coinfection with human immunodeficiency virus; (7) patients not treated with antiviral or immunosuppressive agents within 6 months of enrollment; (8) lifetime cumulative alcohol intake <500 kg (mild to moderate alcohol intake); (9) patients free of other types of hepatitis, including hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, and autoimmune liver disease; (10) patients without or with well-controlled diabetes; and (11) patients who consented to the study.

With regard to the clinical features of 313 patients at the start of the first course of IFN monotherapy, there were 223 men and 90 women aged 15-66 years with a median age of 47 years. The numbers of patients with fibrosis stages 1, 2, and 3 were 179, 107, and 27, respectively. The median ALT level was 138 IU/L (range, 24-636 IU/L), and the median platelet count was $17.4 \times 10^4/\mu L$ (range, 8.9×10^4-$39.2 \times 10^4/\mu L$). The median viremia level was 4.0 Meq/mL (range, <0.5-67.0 Meq/mL). The median follow-up time was 14.7 years (range, 0.1-20.1 years).

Furthermore, at the first course of IFN monotherapy, 222 patients (70.9%) received IFN-α alone; 83 patients (26.5%) received IFN-β alone; and the remaining 8 patients (2.6%) received a combination of IFN-α and IFN-β. A median IFN dose per day of 6 million units (MU) (range, 1-10 MU) was administered. As a whole, a median total dose of IFN of 525 MU (range, 22-3,696 MU) was administered during a median period of 23.9 weeks (range, 0.6 to 205.4 weeks). Patients mainly received IFN monotherapy, including initial aggressive induction therapy (every day within 8 weeks, followed by 3 times per week).

The study protocol was approved by the Human Ethics Review Committee of Toranomon Hospital.

Methods. The primary measure of efficacy of treatment was sustained virological response (SVR), defined as negative HCV RNA via qualitative analysis with PCR at 24 weeks after cessation of IFN therapy. Patients who achieved SVR after the first course of IFN monotherapy were classified as group A. Patients who did not achieve SVR after the first course of IFN monotherapy were classified into 2 groups based on whether they received other courses of IFN monotherapy. Patients who did not receive further courses of IFN monotherapy based on concerns about adverse effects, lack of time for treatment, physician recommendation based on the appearance of depression, and cardiopulmonary disease during and after the first course of IFN, or the lower levels of ALT, were classified as group B. Patients who received 2 or more courses of IFN monotherapy were classified as group C.

Laboratory Investigations. Blood samples were frozen at −80°C within 4 hours of collection and were not thawed until used for testing. HCV genotype was determined via PCR using a mixed primer set derived from nucleotide sequences of the NS5 region.[14] In all cases, HCV-RNA viremia level was measured by branched DNA assay version 2.0 (Chiron Corp.) at commence-

ment of therapy using frozen samples, and the results were expressed as 10^6 genomic equivalents per milliliter (Meq/mL). The lower limit of the assay was 0.5 Meq/mL. Samples with undetectable levels using this quantitative assay (<0.5 Meq/mL) were also evaluated via HCV-RNA qualitative analysis with PCR (nested PCR or Amplicor) during and after therapy especially, and the results were expressed as positive or negative. The lower limit of the assay was 100 copies/mL.

Detection of Amino Acid Substitutions in Core Region. We developed a simple and low-cost PCR method for detecting substitutions of aa 70 or aa 91 in HCV-CR of genotype 1b using mutation-specific primer as an alternative to the direct sequencing method. The major protein type was determined based on the relative intensity of the bands for wild (aa 70, arginine; aa 91, leucine) and mutant (aa 70, glutamine/histidine; aa 91, methionine) in agarose gel electrophoresis. If the intensities of the bands were similar, the case was regarded as competitive. The detection rate was 94.4%, the sensitivity was 10 KIU/mL using quantitative assay with PCR (Cobas Amplicor HCV monitor version 2.0 using the 10-fold dilution method), the reproducibility was high, and consistency with direct sequencing was 97.1% in positive cases.[15] In this study, the pattern of arginine (wild) at aa 70 and leucine (wild) at aa 91 was evaluated as double wild-type, while the other patterns were nondouble wild-type. The mutation in this study refers to substitution from consensus sequence. In previous studies, HCV-J was considered as a prototype, and the aa substitution was evaluated by comparison with the consensus sequence prepared from 50 clinical trial samples.[8,16] In this study, the PCR genotyping could be performed in 232 patients; the remaining 81 patients could not be analyzed due to the lack of adequate serum samples obtained before treatment.

Liver Histopathological Examination. Liver biopsy specimens were obtained percutaneously or at peritoneoscopy using a modified Vim Silverman needle with an internal diameter of 2 mm (Tohoku University style, Kakinuma Factory, Tokyo), fixed in 10% formalin, and stained with hematoxylin-eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff after diastase digestion. All specimens for examination contained 6 or more portal areas. Histopathological diagnosis was made by an experienced liver pathologist (H. K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on histopathological assessment according to the scoring system of Desmet et al.[17]

Follow-Up. Clinical and laboratory assessments were performed at least once every month before, during, and after treatment. Adverse effects were monitored clinically by careful interviews and medical examination at least once every month. Patient compliance with treatment was evaluated with a questionnaire. Blood samples were also obtained at least once every month before, during, and after treatment, and were also analyzed for ALT levels and HCV-RNA levels at various time points.

Follow-up time represented the time from the start of the first course of IFN treatment until death or until the last visit.

Diagnosis of HCC. Patients were examined for HCC via abdominal ultrasonography every 3-6 months. If HCC was suspected based on ultrasonographic results, additional procedures such as CT, magnetic resonance imaging, abdominal angiography, and ultrasonography-guided tumor biopsy (if necessary), were used to confirm the diagnosis.

Statistical Analysis. The χ^2 test, Fisher exact probability test, and Mann-Whitney U test were used to compare background characteristics between groups. Multiple comparisons were examined by the Bonferroni test. Cumulative hepatocarcinogeneses were calculated using the Kaplan-Meier technique; differences between survival curves were tested using the log-rank test. Statistical analyses of hepatocarcinogenesis according to groups were calculated using the period from start of the first course of IFN monotherapy. Stepwise Cox regression analysis was used to determine independent predictive factors that were associated with hepatocarcinogenesis. We also calculated the OR and 95% CI. Potential predictive factors associated with hepatocarcinogenesis included the following 11 variables: age, sex, histological stage, viremia level, serum AST, serum ALT, platelet count, aa substitutions in HCV-CR, total IFN dose, total IFN duration, and group of treatment. Each variable was transformed into categorical data consisting of 2 simple ordinal numbers for univariate and multivariate analyses. Variables that achieved statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were tested using the multivariate Cox proportional hazard model to identify significant independent factors. Statistical comparisons were performed using the SPSS software (SPSS Inc., Chicago, IL). All P values of less than 0.05 by the 2-tailed test were considered significant.

Results

Efficacy of IFN Monotherapy. 65 patients (20.8%) achieved SVR after the first course of IFN monotherapy (group A). Of 248 (79.2%) non-SVR patients after the first course of IFN, 112 (35.8%) did not receive a second course of IFN monotherapy (group B), while the remain-