development among IFN-treated patients. However, this information is difficult to determine because of the paucity of large-scale, long-term cohort studies.

The 70-kDa glycoprotein α-fetoprotein (AFP), encoded by a gene located on chromosome 4, is the major serum protein during fetal life.[10] Shortly before birth, AFP is replaced by albumin as the major serum protein,[11,12] and thereafter, serum AFP levels remain extremely low throughout life (<10 ng/mL). Because serum AFP levels are frequently elevated in patients with HCC and germ-cell tumors, measurement of AFP is widely used as a serological marker for these tumors.[8,13] However, AFP levels are sometimes elevated in patients with chronic viral hepatitis and cirrhosis who do not have HCC.[3,19] While one possible explanation for this elevation is liver inflammation, in patients with CHC, the relationship between AFP and markers of liver inflammation such as alanine aminotransferase (ALT) is unclear. Moreover, although several reports suggest that pre-IFN treatment ALT and AFP levels in patients or those in patients who did not undergo subsequent treatment are associated with the development of HCC, it is unclear whether post-IFN treatment ALT and AFP levels are associated with hepatocarcinogenesis in patients with CHC. Hence, to clarify these associations we conducted a large-scale, long-term cohort study of patients with CHC to analyze the influence of ALT and AFP levels before and after IFN therapy on hepatocarcinogenesis in addition to other host and virological factors.

Patients and Methods

Patients. Patients chronically infected with HCV who had histologically proven chronic hepatitis or cirrhosis and had undergone IFN treatment between 1992 and 2010 were enrolled in the cohort. HCC was definitively ruled out by ultrasonography, dynamic computed tomography (CT), and/or magnetic resonance imaging (MRI) on enrollment. Patients were excluded if they had a history of HCC at the time of liver biopsy, autoimmune hepatitis, primary biliary cirrhosis, excessive alcohol consumption (≥50 g/day), hepatitis B surface antigen, or antihuman immunodeficiency virus antibody. Based on these criteria, a total of 2,689 patients were initially enrolled. Of these, 223 (8.3%) patients were excluded from the cohort because of loss to follow-up. In the remaining 2,466 patients, 133 and 515 patients were excluded from this analysis because of short follow-up and retreatment with IFN-based therapy during the follow-up period, respectively. Thus, the cohort comprising 1,818 patients was analyzed in the present study. Written informed consent was obtained from all patients and the Ethical Committee of Musashino Red Cross Hospital approved this study, which was conducted in accordance with the Declaration of Helsinki.

Histological Evaluation. To obtain liver specimens, laparoscopic or ultrasound-guided liver biopsies were performed with 13G or 15G needles, respectively. The median length of specimen was 18 mm (range, 11-41 mm), and the mean number of portal tracts was 17 (range, 9-35). The stage of fibrosis and the grade of inflammatory activity were scored by two pathologists according to the classification of Desmet et al.[24] The percentage of steatosis was quantified by determining the average proportion of hepatocytes affected.

IFN Therapy and Definitions of Response to IFN Therapy. All patients had chronic HCV infection at liver biopsy, which was confirmed by the presence of HCV-RNA in serum. All IFN therapies were initiated within 48 weeks after liver biopsy. Among the 1,818 patients, 535 received IFNα or IFNβ monotherapy for 24 weeks, 244 patients received IFNα ribavirin (RBV) combination therapy for 24 weeks, 299 patients received pegylated (PEG-) IFNα monotherapy for 48 weeks, and 760 patients received PEG-IFNα RBV combination therapy for 48-72 weeks.

Patients negative for serum HCV-RNA 24 weeks after IFN therapy completion were defined as SVRs. Patients who remained positive for HCV-RNA 24 weeks after therapy completion were defined as non-SVRs. HCV-RNA was determined by the qualitative Amplicor or TaqMan HCV assay (Roche Molecular Diagnostics, Tokyo, Japan).

Data Collection and Patient Follow-up. At enrollment, patient characteristics, biochemical,

Address reprint requests to: Yasuhiro Asahina, M.D., Ph.D., Professor, Department of Liver Disease Control, Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. E-mail: asahina.gast@tmd.ac.jp; fax: +81-3-5803-0268.

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Additional Supporting Information may be found in the online version of this article.

Table 1. Characteristics of Patients Enrolled in the Present Study

Factors	Value
Patients, n	1818
Sex, n (%)	
Male	833 (45.8)
Female	985 (54.2)
Age (SD), year	57.1 (12.0)
BMI (SD), kg/m^2	23.1 (3.2)
Fibrosis stage, n (%)	
F1/2	1384 (76.1)
F3/4	434 (23.9)
Activity grade, n (%)	
A0/1	964 (53.0)
A2/3	854 (47.0)
%Severe steatosis (≥10%)	23.7
Albumin (SD), g/dL	4.0 (0.38)
ALT (SD), IU/L	78.3 (71.0)
γ-GTP (SD), IU/L	49.8 (50.6)
T. Bilirubin (SD), mg/dL	0.73 (0.34)
Fasting blood sugar (SD), mg/dL	113.4 (37.8)
LDL-Cholesterol (SD), mg/dL	101.6 (28.9)
T. Cholesterol (SD), mg/dL	176.2 (38.4)
AFP (SD), ng/mL	11.3 (28.3)
WBC counts (SD),/μL	4990 (1516)
Hb (SD), g/dL	14.0 (1.7)
Platelet counts (SD), x10^3/μL	164 (54)
HCV load (SD), KIU/mL	1097 (1263)
HCV genotype, n (%)*	
1a	11 (0.56)
1b	1183 (67.4)
2a	361 (20.6)
2b	180 (10.3)
Others	20 (1.1)
%Core 70 a.a. mutation†	34.2
%ISDR wild or 1 mutation‡	63.9
IFN regimen, n (%)	
IFN mono	758 (35.0)
IFN + RBV	275 (12.7)
PEG-IFN mono	307 (14.2)
PEG-IFN + RBV	758 (38.2)

Unless otherwise indicated, data are given as mean (SD).
*HCV genotype was determined in 1755 patients.
†HCV core mutation was determined in 409 patients with genotype 1b.
‡ISDR was determined in 1264 patients with genotype 1b.
Abbreviations: BMI, body mass index; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; LDL, low-density lipoprotein; AFP, α-fetoprotein; WBC, white blood cell; Hb, hemoglobin; HCV, hepatitis C virus; ISDR, interferon sensitivity determining region; IFN, interferon; RBV, ribavirin; PEG, pegylated.

hematological, virological, and histological data were collected. Age was determined at the time of primary liver biopsy. Patients were examined for HCC by abdominal ultrasonography, dynamic CT, and/or MRI every 3-6 months. Serum ALT and AFP levels were measured every 1-6 months. The surveillance protocols were in accordance with the standard of care in Japan. If HCC was suspected on the basis of the screening examination, additional procedures (e.g., dynamic CT, dynamic MRI, CT during hepatic arteriography, CT during arterial portography, contrast-enhanced ultrasonography, and tumor biopsy) were used to confirm the diagnosis. HCC diagnosis was confirmed by needle biopsy, histology of surgically resected specimens, or characteristic radiological findings. To evaluate the effects of changes in serum ALT and AFP levels during IFN therapy on hepatocarcinogenesis, the average integration values of ALT and AFP in each patient were calculated before and after IFN therapy. Data obtained more than 1 year prior to HCC development were used to exclude AFP elevation caused by HCC itself.

Follow-up was between the date of primary liver biopsy and HCC development or the last medical attendance until June 2011. The mean follow-up period was 6.1 years (range, 1.0-20.8 years).

Statistical Analyses. Categorical data were compared by the chi-square test or Fisher's exact test. Distributions of continuous variables were analyzed with Student t test for two groups. All tests of significance were two-tailed and $P < 0.05$ was considered statistically significant. The cumulative incidence curve was determined by the Kaplan-Meier method, and differences among groups were assessed using the log-rank test. Factors associated with HCC risk were determined by the Cox proportional hazard model. As covariates in the multivariate stepwise Cox model, age, sex, stage of liver fibrosis, grade of histological activity, presence of hepatic steatosis, serum albumin levels, γ-glutamyl transpeptidase (γ-GTP) level, fasting blood sugar levels, platelet counts, pre-IFN ALT levels, pre-IFN AFP levels, post-IFN ALT levels, post-IFN AFP levels, and virological response were included. HCC development was the dependent variable. Time zero was defined as the time of primary liver biopsy. The proportional assumption was supported by log[-log(survival)] versus log(time) plots that showed parallel lines. Statistical analyses were performed using the Statistical Package for the Social Sciences software v. 18.0 (SPSS, Chicago, IL).

Results

Patient Characteristics and Factors Associated With Risk of HCC. Table 1 shows patient characteristics at the time of enrollment. During follow-up, HCC developed in 179 patients. The cumulative incidence of HCC for 5 and 10 years was 6.5% and 15.0%, respectively. The final virological response to IFN therapy was determined in all patients. The overall rate of SVRs was 50.2% (913/1818). The cumulative incidence in SVRs was 2.3% and 5.5%, respectively, which was significantly lower than that in non-SVRs (6.9% and 21.9%, respectively; log-rank test, $P < 0.0001$).

Univariate analysis demonstrated factors that increase the risk for HCC development (Table 2). According to

multivariate stepwise Cox analysis, older age, male gender, advanced fibrosis, severe steatosis, lower serum albumin levels, non-SVR, and higher post-IFN treatment ALT and AFP levels, but not pre-IFN treatment ALT and AFP levels, were identified as independent factors that were significantly associated with HCC development (Table 2).

Association of Post-IFN Treatment ALT and AFP Levels With HCC Development in SVRs and Non-SVRs. Because our multivariate analysis identified post-IFN treatment ALT and AFP levels as independent factors associated with HCC risk, we determined the cutoff values of these factors for predicting the development of HCC by receiver operator characteristics (ROC) analysis. The area under the ROC curve for post-IFN treatment ALT and AFP levels were higher than that for pre-IFN treatment ALT and AFP levels, suggesting that quantification of post-IFN treatment ALT and AFP levels rather than pre-IFN treatment levels of these values is useful for predicting HCC (Fig. 1A). From this ROC analysis, ALT <40 IU/L and AFP <6.0 ng/mL were identified as cutoff values. Negative predictive values were extremely high at 0.960 in each value, suggesting patients with ALT and/or AFP levels below these cutoff values are at a lower risk for HCC.

As shown in Fig. 1B, the hazard ratio determined by Cox proportional hazard analysis after adjustment for age, sex, stage of liver fibrosis, degree of liver steatosis, serum albumin levels, and virological response to therapy demonstrated that the hazard ratio for HCC was dependent on post-IFN treatment ALT and AFP levels. These hazard ratios increased predominantly when post-IFN treatment ALT and AFP levels were more than the cutoff values.

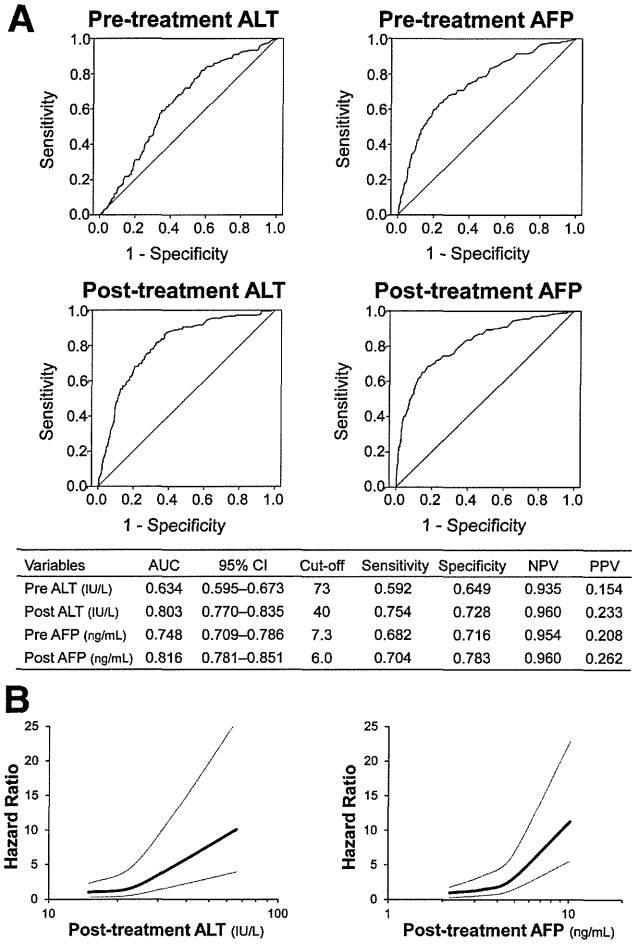
As shown in Fig. 2, the cumulative incidence of HCC was closely related to post-IFN treatment ALT and AST levels and was significantly lower in patients whose post-IFN treatment ALT and AFP levels was suppressed to <40 IU/L and 6.0 ng/mL, respectively. This suppressive effect was also notable in non-SVRs (Fig. 2C,D). Moreover, the cumulative incidence of HCC was significantly higher even in SVRs whose post-IFN treatment ALT and AFP levels were not <40 IU/L and 10 ng/mL, respectively (Fig. 2E,F).

Changes in ALT and AFP Levels With IFN Therapy and HCC Development. In the entire cohort, the mean ALT and AFP levels significantly decreased after IFN therapy (ALT = 78.4 to 36.6 IU/L, 95% confidence interval [CI] = 38.6-45.0, $P < 0.0001$; AFP = 11.3 to 6.9 ng/mL, 95% CI = 3.25-5.69, $P < 0.0001$; paired Student t test), and this significant decrease was found not only in SVRs, but also non-SVRs (Fig. 3A). Because post-IFN treatment ALT and AFP levels rather than pre-IFN treatment levels were

Table 2. Factors Associated With Hepatocellular Carcinoma

Risk Factor	Hazard Ratio (95% CI)	*P* Value
Univariate analysis		
Age (by every 10 year)	1.82 (1.52-2.20)	<0.0001
Sex		
Female	1	
Male	1.61 (1.20-2.17)	<0.0001
Fibrosis stage		
F1/F2	1	
F3/F4	4.90 (3.64-6.61)	<0.0001
Activity grade		
A0/A1	1	
A2/A3	3.38 (2.41-4.74)	<0.0001
Degree of steatosis		
<10%	1	
≥10%	3.84 (2.62-5.63)	<0.0001
Albumin (by every 1 g/dL)	0.18 (0.22-0.25)	<0.0001
Pre-ALT (by every 40 IU/L)	1.04 (0.96-1.08)	0.525
Post-ALT (by every 40 IU/L)	1.68 (1.55-1.81)	<0.0001
γ-GTP (by every 40 IU/L)	1.17 (1.08-1.27)	<0.0001
Fasting blood sugar (by every 100 mg/dL)	1.82 (1.35-2.45)	<0.0001
Pre-AFP (by every 10 ng/mL)	1.07 (1.05-1.09)	<0.0001
Post-AFP (by every 10 ng/mL)	1.08 (1.06-1.12)	<0.0001
Platelet counts (by every $10^4/\mu L$)	0.88 (0.85-0.90)	<0.0001
Genotype		
Non-1	1	
1	2.27 (1.51-3.45)	<0.0001
Core 70 mutation		
Wild	1	
Mutant	2.79 (1.19-6.53)	0.018
ISDR		
More than 1 mutation	1	
Wild or 1 mutation	1.27 (0.87-1.85)	0.216
Virological response		
SVR	1	
Non-SVR	3.66 (2.51-5.35)	<0.0001
Multivariate analysis		
Age (by every 10 year)	2.18 (1.71-2.81)	<0.0001
Sex		
Female	1	
Male	2.66 (1.86-3.80)	<0.0001
Fibrosis stage		
F1/F2	1	
F3/F4	2.27 (1.58-3.27)	<0.0001
Degree of steatosis		
<10%	1	
≥10%	2.29 (1.49-3.50)	<0.0001
Albumin (by every 1 g/dL)	0.35 (0.23-0.55)	<0.0001
Post-ALT (by every 40 IU/L)	1.81 (1.55-2.12)	<0.0001
Post-AFP (by every 10 ng/mL)	1.06 (1.02-1.10)	0.007
Virological response		
SVR	1	
Non-SVR	1.58 (1.01-2.48)	0.044

Hazard ratios for development of hepatocellular carcinoma were calculated by the Cox proportional hazards analysis. ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transpeptidase; AFP, alpha-fetoprotein; ISDR, interferon sensitivity determining region; SVR, sustained virological responder. As covariates in the multivariate stepwise Cox model, age, sex, stage of liver fibrosis, grade of histological activity, presence of hepatic steatosis, serum albumin levels, γ-GTP level, fasting blood sugar levels, platelet counts, pre-IFN ALT levels, pre-IFN AFP levels, post-IFN ALT levels, post-IFN AFP levels, and virological response were included.



Variables	AUC	95% CI	Cut-off	Sensitivity	Specificity	NPV	PPV
Pre ALT (IU/L)	0.634	0.595–0.673	73	0.592	0.649	0.935	0.154
Post ALT (IU/L)	0.803	0.770–0.835	40	0.754	0.728	0.960	0.233
Pre AFP (ng/mL)	0.748	0.709–0.786	7.3	0.682	0.716	0.954	0.208
Post AFP (ng/mL)	0.816	0.781–0.851	6.0	0.704	0.783	0.960	0.262

Fig. 1. Predictive values for ALT and AFP, and hazard ratios (HRs) according to post-IFN treatment ALT and AFP levels. (A) ROC curve for prediction of HCC. Area under the ROC curve, 95% CI, cutoff value, sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) are shown in the bottom of the figure. (B) Spline curves of HR (solid line) and 95% CI (dotted line) for HCC development according to post-IFN treatment ALT and AFP levels. Curves were fitted using polynomial regression.

significantly associated with the development of HCC in non-SVRs, we determined the effects of changes in ALT and AFP levels by IFN therapy on hepatocarcinogenesis. Even in non-SVRs with equal or higher pre-IFN treatment ALT or AFP levels than the cutoff values, the cumulative incidence of HCC was significantly suppressed in patients whose post-IFN treatment ALT or AFP level was reduced to less than the cutoff values (Fig. 3B). In contrast, persistence of post-IFN treatment ALT or AFP levels to more than the cutoff values after IFN therapy was associated with a significantly higher incidence of HCC (Fig. 3B).

Relation Between AFP and ALT or Histological Findings. Univariate analysis using logistic regression determined factors that were associated with post-IFN treatment ALT or AFP levels (Supporting Table). Although many clinical factors were associated with post-IFN ALT and/or AFP levels, post-IFN ALT and AFP levels were not correlated with each other ($r^2 = 0.050$). Therefore, the cumulative incidence of HCC was significantly higher in patients with higher post-IFN treatment AFP levels, even when patients were stratified by post-IFN treatment ALT levels (Fig. 4A,B).

As shown in Fig. 4C-F, the cumulative incidence of HCC development was significantly lower in patients whose post-IFN treatment AFP level was <6.0 ng/mL in all subgroups stratified by stage of fibrosis and grade of activity. Therefore, reduction in post-IFN treatment AFP levels reduces HCC risk even in patients with advanced fibrosis. Although pre-IFN treatment AFP levels correlated with the advance of histological fibrosis and grade of activity, such correlations became less significant with post-IFN treatment AFP levels (data not shown).

Platelet Counts and Aspartate Aminotransferase-to-Platelet Ratio Index (APRI) in Patients Without Advanced Fibrosis. Because a substantial amount of HCC cases developed in the patients without histologically advanced fibrosis (Fig. 4C), we characterized these individuals using platelet counts and APRI,[25] which are the readily available surrogate markers for liver fibrosis. We first determined the cutoff values of platelet counts and APRI for predicting HCC development by ROC analyses. Accordingly, platelet counts $<150 \times 10^3/\mu L$ and APRI >0.96 were identified as cutoff values, and the areas under the ROC curve for platelet counts and APRI were 0.715 (95% CI: 0.675-0.755) and 0.740 (95% CI: 0.701-0.779), respectively (Supporting Figure). Even in individuals without advanced fibrosis (F1 and F2 patients), the proportion of patients with platelet counts $<150 \times 10^3/\mu L$ or APRI >0.96 was significantly higher in patients with HCC than in those without HCC (platelet counts, 53.0% [35/66] versus 31.3% [387/1238], $P = 0.0002$; APRI, 53.0% [35/66] versus 26.4% [325/1229], $P < 0.0001$). Moreover, the cumulative incidence of HCC development was significantly higher in patients with platelet counts $<150 \times 10^3/\mu L$ or APRI >0.96 in the subgroups without advanced fibrosis (Supporting Figure). Therefore, patients with low platelet counts or high APRI still have a substantial risk for HCC development even though they were diagnosed with mild fibrosis by liver biopsy.

Hepatic Steatosis and Post-IFN ALT and AFP Levels in SVRs. To characterize SVRs without ALT and AFP normalization after IFN therapy, we evaluated the percentage of severe hepatic steatosis in these patients. Accordingly, the percentages of severe hepatic steatosis were significantly higher in SVRs without ALT and AFP normalization than in those with normal

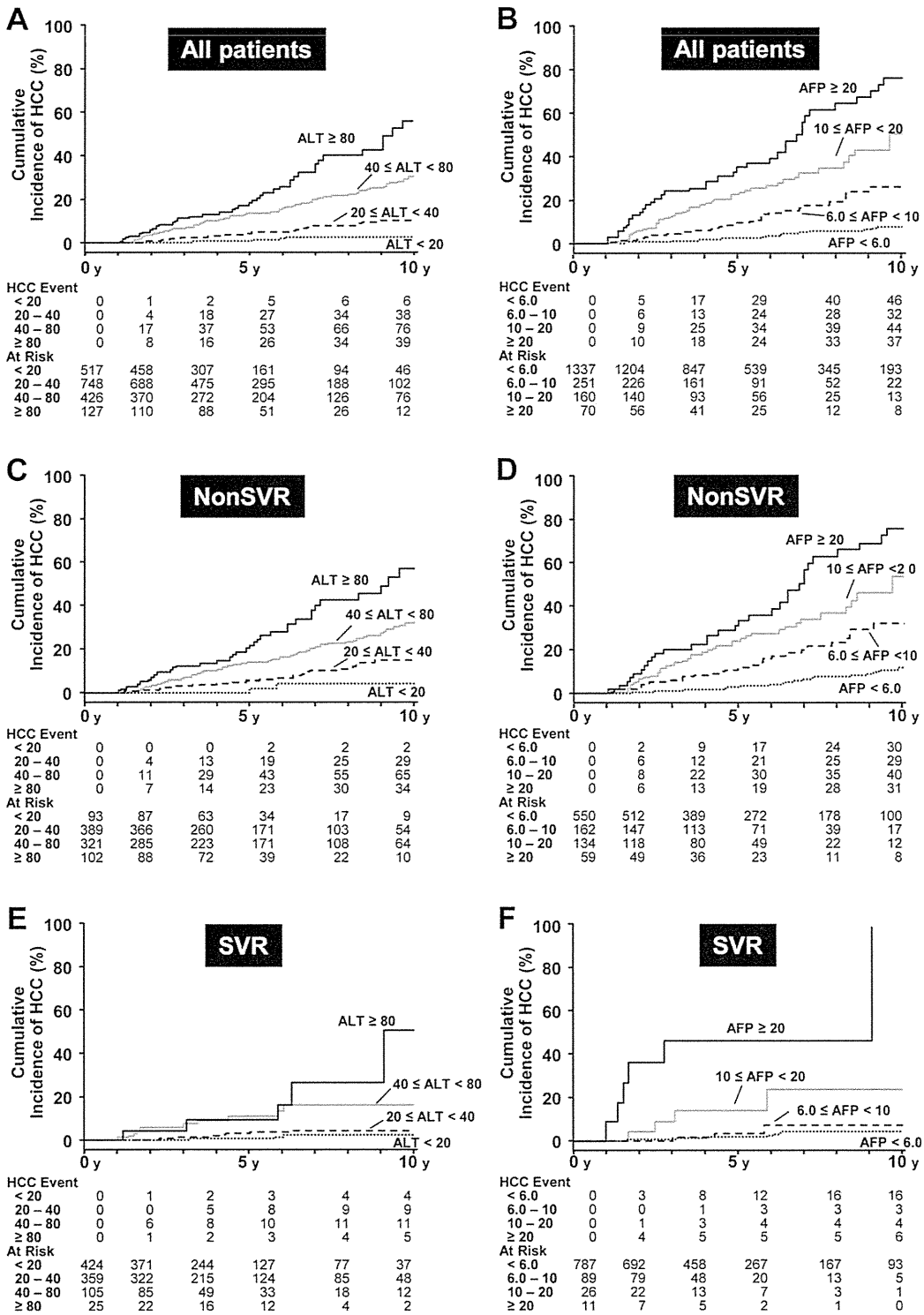
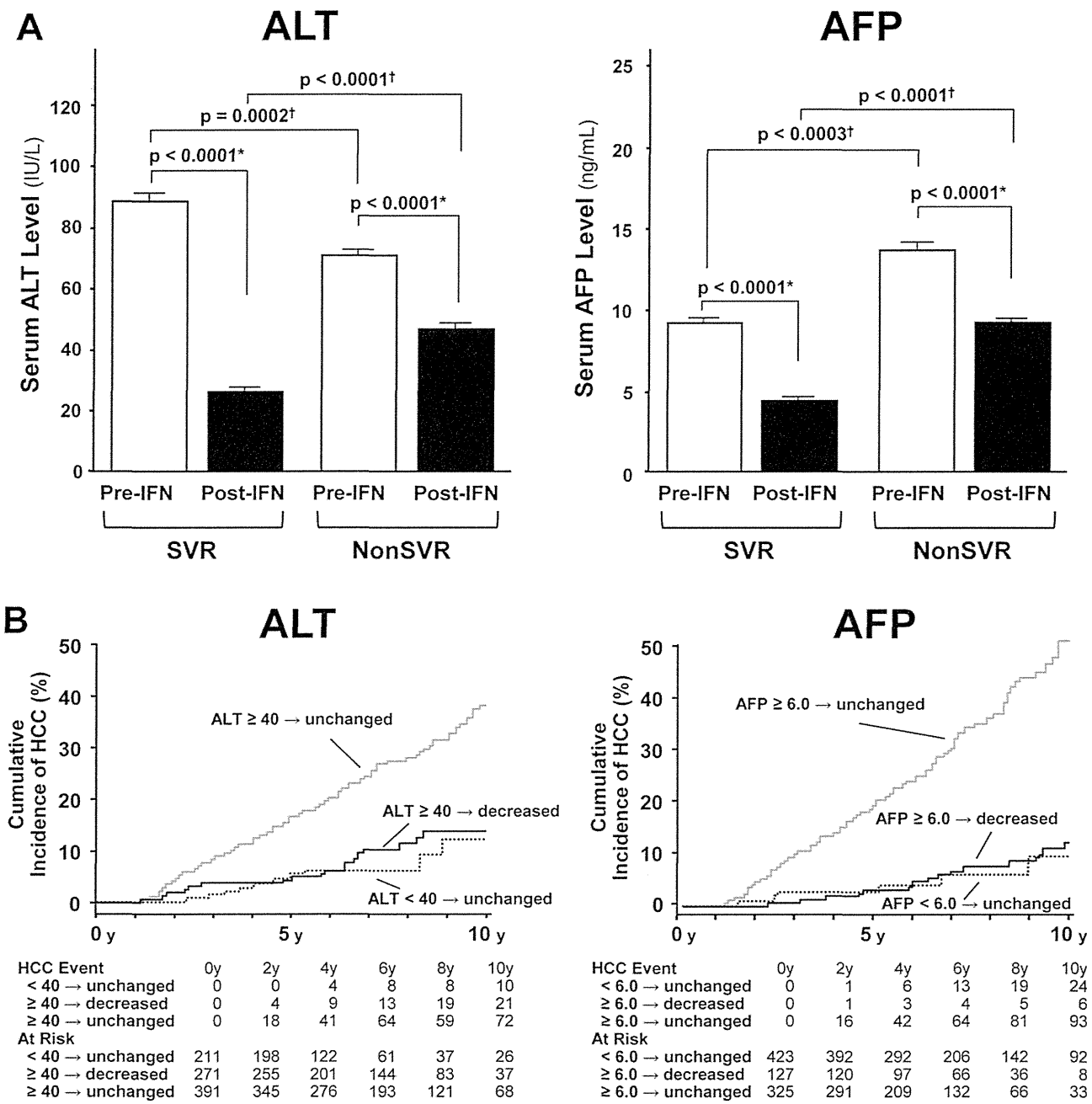


Fig. 2. Cumulative incidence of HCC according to post-IFN treatment ALT and AFP levels. (A) Entire cohort stratified by post-IFN treatment ALT levels (log-rank test: $P < 0.0001$). (B) Entire cohort stratified by post-IFN treatment AFP levels (log-rank test: $P < 0.0001$). (C) Non-SVRs stratified by post-IFN treatment ALT levels (log-rank test: $P < 0.0001$). (D) Non-SVRs stratified by post-IFN treatment AFP levels (log-rank test: $P < 0.0001$). (E) SVRs stratified by post-IFN treatment ALT levels (log-rank test: $P < 0.0001$). (F) SVRs stratified by post-IFN treatment AFP levels (log-rank test: $P < 0.0001$). The number of HCC events and patients at risk at each timepoint are shown below the graphs.

ALT and AFP (ALT, 37.9% [36/95] versus 13.8% [77/557], $P < 0.0001$; AFP, 31.6% [31/98] versus 14.8% [82/554], $P < 0.0001$). Therefore, it is likely that presence of hepatic steatosis is associated with ALT and/or AFP elevation, and it is one of the risks for HCC development even after achieving SVR.



HCC Event	0y	2y	4y	6y	8y	10y
< 40 → unchanged	0	0	4	8	8	10
≥ 40 → decreased	0	4	9	13	19	21
≥ 40 → unchanged	0	18	41	64	59	72
At Risk						
< 40 → unchanged	211	198	122	61	37	26
≥ 40 → decreased	271	255	201	144	83	37
≥ 40 → unchanged	391	345	276	193	121	68

HCC Event	0y	2y	4y	6y	8y	10y
< 6.0 → unchanged	0	1	6	13	19	24
≥ 6.0 → decreased	0	1	3	4	5	6
≥ 6.0 → unchanged	0	16	42	64	81	93
At Risk						
< 6.0 → unchanged	423	392	292	206	142	92
≥ 6.0 → decreased	127	120	97	66	36	8
≥ 6.0 → unchanged	325	291	209	132	66	33

Fig. 3. Changes in pre- and post-IFN treatment ALT and AFP levels, and their effects on HCC development. (A) Mean serum pre- (open columns) and post-IFN treatment (solid columns) ALT and AFP levels in SVRs and non-SVRs. Error bars indicate the standard error. *Paired Student *t* test. †Unpaired Student *t* test. (B) Cumulative incidence of HCC stratified by changes in pre- and post-IFN treatment ALT and AFP levels (log-rank test: $P < 0.0001$). ALT <40 → unchanged, patients with ALT <40 IU/L before IFN therapy unchanged after IFN therapy; ALT ≥40 → decreased, patients with ALT ≥40 IU/L before IFN therapy decreased to ALT <40 IU/L after IFN therapy; ALT ≥40 → unchanged, patients with ALT ≥40 IU/L before IFN therapy unchanged at ALT not <40 IU/L after IFN therapy. AFP <6.0 → unchanged, patients with AFP <6.0 ng/mL before IFN therapy unchanged at AFP <6.0 ng/mL after IFN therapy; AFP ≥6.0 → decreased, patients with AFP ≥6.0 ng/mL before IFN therapy decreased to AFP <6.0 ng/mL after IFN therapy; AFP ≥6.0 → unchanged, patients with AFP ≥6.0 ng/mL before IFN therapy unchanged at AFP ≥6.0 ng/mL after IFN therapy.

Discussion

This large-scale, long-term cohort study establishes important findings, which demonstrate a strict association between hepatocarcinogenesis and post-IFN treatment ALT and AFP levels in patients with CHC. This association was notable in both SVR and non-SVR subgroups, and suppression of these values by IFN therapy reduced the hepatocarcinogenesis risk despite failure of HCV eradication. These data, which

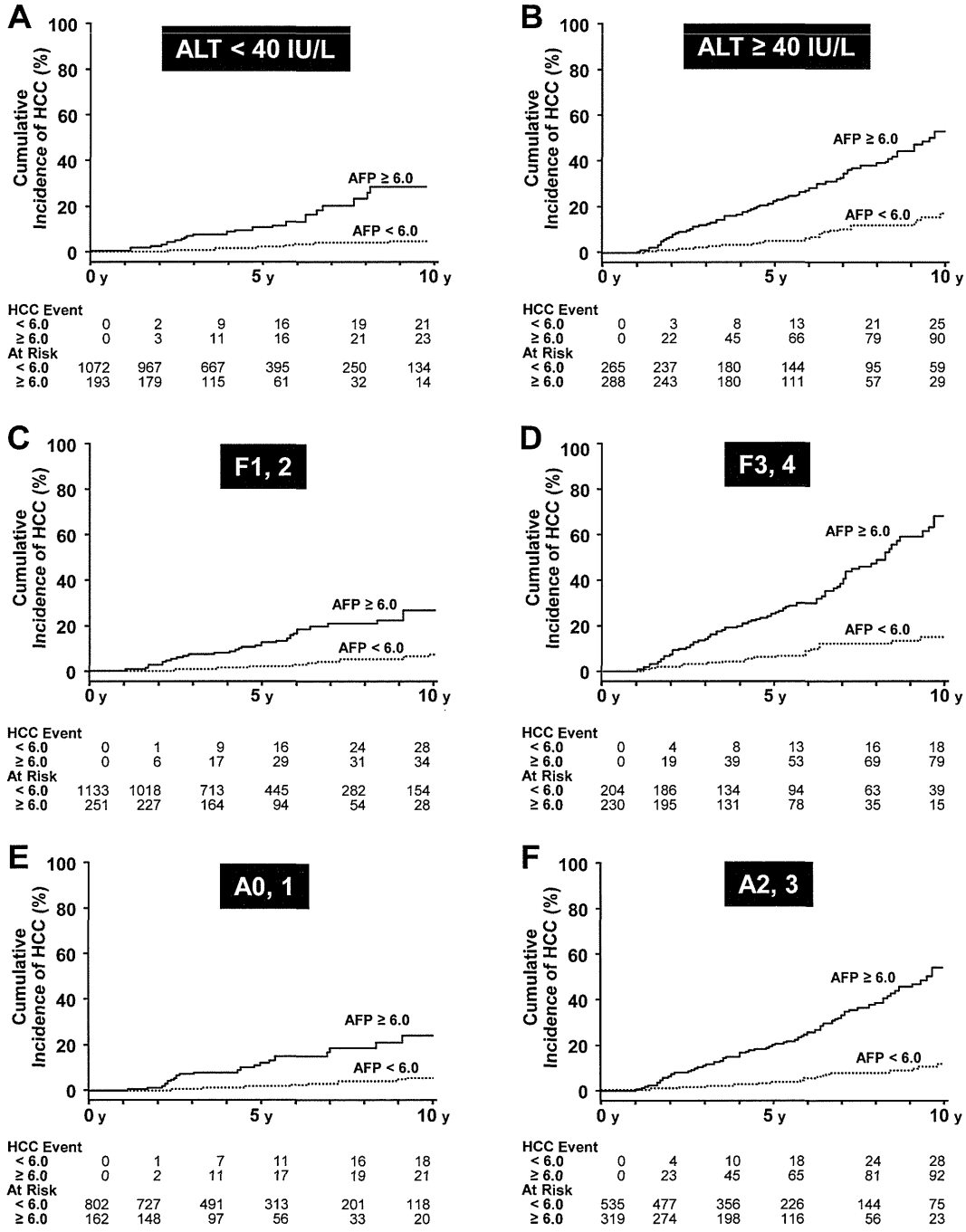


Fig. 4. Relationship between AFP and ALT or histological findings. (A) The cumulative incidence of HCC stratified by post-IFN treatment AFP levels in subgroups according to the post-IFN treatment ALT (log-rank test: $P < 0.0001$ in both subgroups). (B) Cumulative incidence of HCC stratified by post-IFN treatment AFP levels in subgroups according to the histological stage of fibrosis (log-rank test: $P < 0.0001$ in both subgroups). (C) Cumulative incidence of HCC stratified by post-IFN treatment levels of AFP in subgroups according to the histological grade of activity (log-rank test: $P < 0.0001$ in both subgroups).

demonstrate the efficacy of IFN against HCC development associated with suppression of AFP, have clinically important implications for physicians.

Although there have been reports on the association between baseline pretreatment AFP levels and HCC risk,[26-35] little is known regarding the effects of IFN therapy on change in post-IFN treatment AFP and its relation to HCC risk.[36] Although a previous report demonstrated that a decrease in AFP levels in patients receiving IFN therapy reduced the incidence of HCC,[37] this study was performed in a small number of patients (n = 382), and cutoff values, relation to ALT, or histological findings were not determined. Our study, performed in a large well-characterized

cohort, had a greater advantage in that it allowed determination of cutoff values for post-IFN treatment ALT and AFP levels useful for predicting HCC development. Although a higher cutoff value of 20 ng/mL was used to determine the incidence of HCC in the previous study,[36] we propose a lower value for negatively predicting HCC. From our results, those with AFP levels ≥6.0 ng/mL have a substantial HCC risk, even if it is <20 ng/mL. Therefore, post-IFN treatment AFP levels should be <6.0 ng/mL to suppress HCC risk in patients with CHC.

It should be noted that AFP produced by HCC itself was carefully excluded in our study. Serum AFP elevation is frequently observed in patients with advanced CHC in the absence of HCC.[19-23] Although the precise mechanisms accounting for this observation are unknown, Hu et al.[38] found a correlation between AFP and measures of liver disease activity, suggesting that AFP production is enhanced in the presence of necroinflammatory injury of the liver. However, in our study post-IFN treatment ALT and AFP levels were not correlated, and the cumulative incidence of HCC was significantly higher in patients with higher post-IFN treatment AFP levels, even when patients were stratified by post-IFN treatment ALT levels. Moreover, multivariate analysis confirmed that AFP and ALT are independently associated with HCC risk. Therefore, observed elevation in AFP levels in patients with subsequent HCC development is not necessarily caused by necroinflammation of the liver. Alternatively, increased AFP levels have been reported during liver regeneration following hepatic resection and during recovery from massive hepatic necrosis,[39-41] suggesting that elevated AFP levels are a surrogate for proliferative activity of liver cells, which may cause hepatocarcinogenesis in patients with CHC.

Other possible reasons accounting for HCC risk related to AFP are the close association between AFP levels and the stage of liver fibrosis, which is consistent with a previous report.[35] However, we further clarified the fact that correlation between post-IFN treatment AFP levels and liver fibrosis was less notable in patients without subsequent development of HCC (data not shown). Cumulative incidence of HCC was significantly higher in patients with higher post-IFN treatment AFP levels at each stage when patients were stratified by the histological stage of fibrosis (Fig. 4). Therefore, post-IFN treatment AFP is not just a surrogate marker for liver fibrosis, and elevation of post-IFN treatment AFP as a potential risk for hepatocarcinogenesis is not only the result of advanced liver fibrosis. Conversely, suppression of post-IFN treatment AFP levels may reduce HCC risk even in patients with advanced fibrosis.

This study has a few limitations, the first being the heterogeneity of our cohort, which included various treatment regimens with different treatment responses. However, we obtained similar results in a more uniform subgroup of HCV genotype 1b patients treated with PEG-IFNα/RBV (data not shown). The second limitation is the ethnic homogeneity of the Japanese population. Because the baseline incidence of HCC development differs among population groups, longer-term longitudinal studies in larger cohorts with various population subgroups are required to verify the generality of our results.

With the development of potent direct-acting antiviral agents combinations, IFN-free therapy is likely to be approved in the near future. This raises the question of whether posttreatment ALT and/or AFP levels will remain a significant predictor of HCC risk. Moreover, it is uncertain whether the suppressive effect of viral eradication by IFN-free regimens on hepatocarcinogenesis will be identical to that obtained by IFN-based regimens. Therefore, it is extremely interesting to prove these issues in future studies.

In conclusion, post-IFN treatment ALT and AFP levels are strictly associated with hepatocarcinogenesis risk in patients with CHC. Measurement of these values is useful for predicting future HCC risk in IFN-treated patients. Suppression of these values after IFN therapy reduces HCC risk even in patients without HCV eradication, while SVRs with increased ALT and/or AFP levels are at risk for HCC development. The present results have potentially important clinical implications for physicians and may influence their decisions regarding treatment strategy and HCC surveillance for individual patients.

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ORIGINAL ARTICLE—Liver, Pancreas, and Biliary Tract

Genetic variation near interleukin 28B and the risk of hepatocellular carcinoma in patients with chronic hepatitis C

Yasuhiro Asahina · Kaoru Tsuchiya · Takashi Nishimura · Masaru Muraoka · Yuichiro Suzuki · Nobuharu Tamaki · Yutaka Yasui · Takanori Hosokawa · Ken Ueda · Hiroyuki Nakanishi · Jun Itakura · Yuka Takahashi · Masayuki Kurosaki · Nobuyuki Enomoto · Mina Nakagawa · Sei Kakinuma · Mamoru Watanabe · Namiki Izumi

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Abstract
Background We aimed to clarify the association between single nucleotide polymorphism (SNP) located near *interleukin 28B* and hepatocellular carcinoma (HCC).
Methods A cohort comprising 792 patients treated with interferon for chronic hepatitis C was investigated. SNPs at rs8099917 and rs12979860 were determined. Cumulative incidence and HCC risk were analyzed by Kaplan–Meier and Cox proportional hazard analyses for a mean follow-up period of 4.9 years. Fibrosis progression rate (FPR) was determined in these patients with a known time of infection ($n = 294$).
Results Cumulative HCC incidence was significantly higher in rs8099917 nonTT (minor homozygote or heterozygote) patients than in rs8099917 TT (major homozygote) patients (20.8 vs. 10.5 % over 10 years, logrank test, $p = 0.002$). This difference was notable in patients infected with genotype 1 and those treated with pegylated interferon and ribavirin. Among nonSVRs, interferon had a limited effect in suppressing alanine aminotransferase (ALT) and/or α-fetoprotein (AFP) levels in nonTT patients. The suppression of these values after interferon therapy was associated with a lower incidence of HCC. FPR were similar in TT and nonTT patients.
Conclusions rs8099917 nonTT is related to higher HCC development in patients with HCV genotype 1 and those treated with pegylated interferon and ribavirin. Higher HCC incidence observed in nonTT patients partly results from the limited suppression of ALT and/or AFP by interferon in these patients.

Keywords Hepatocarcinogenesis · Fibrosis · Interferon · Alanine aminotransferase · α-Fetoprotein

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Y. Asahina · K. Tsuchiya · T. Nishimura · M. Muraoka · Y. Suzuki · N. Tamaki · Y. Yasui · T. Hosokawa · K. Ueda · H. Nakanishi · J. Itakura · Y. Takahashi · M. Kurosaki · N. Izumi
Department of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610, Japan

Y. Asahina (✉) · M. Nakagawa · S. Kakinuma · M. Watanabe
Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan
e-mail: asahina.gast@tmd.ac.jp

Y. Asahina · S. Kakinuma
Department of Liver Disease Control, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

T. Nishimura
Department of Gastroenterology, Faculty of Medicine, Shiga University of Medical Science, Setatsukinowa-cho, Otsu-shi, Shiga 520-2192, Japan

M. Muraoka · Y. Suzuki · N. Enomoto
First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, 1110 Shimogato, Chuo-shi, Yamanashi 409-3898, Japan

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Introduction

Hepatitis C virus (HCV) infection is one of the major causes of chronic hepatitis, which progresses to hepatocellular carcinoma (HCC) in many patients [1]. In the last two decades, interferon (IFN) therapy has been used to treat chronic hepatitis C (CH-C) with the goal of altering its natural progression. Although HCV eradication with IFN therapy in CH-C patients reportedly prevents HCC development [2–4], factors responsible for HCC development in IFN-treated patients are difficult to determine because of the prolonged clinical course of CH-C.

Recent studies demonstrated that single nucleotide polymorphisms (SNPs) near *interleukin (IL) 28B* were strongly associated with the virological response to pegylated IFNα (PEG-IFNα) and ribavirin (RBV) combination therapy [5–7]. However, it remains unclear if the SNPs near *IL28B* are associated with further consequences of CH-C, such as HCC and liver fibrosis, in IFN-treated patients because of the paucity of adequate cohort studies. To address the important question of whether SNPs near *IL28B* are associated with the development of HCC, we analyzed the influence of this polymorphism on HCC risk in a large-scale, long-term cohort of IFN-treated patients.

Methods

Patients

Patients chronically infected with HCV who had histologically proven chronic hepatitis or cirrhosis and had undergone IFN treatment between 1992 and 2010 were enrolled in the original cohort [8]. In this cohort comprising 1,818 patients, a subgroup of 792 patients who were available for genotyping of the SNPs near *IL28B* (rs8099917 and rs12979860) was assessed in the present study. Patients were excluded from the original cohort if they had a history of HCC at the time of liver biopsy, autoimmune hepatitis, primary biliary cirrhosis, excessive alcohol consumption (≥50 g/day), hepatitis B surface antigen, or anti-human immunodeficiency virus antibody. HCC was definitively ruled out by ultrasonography, dynamic computed tomography (CT), and/or magnetic resonance imaging (MRI) on enrollment. Written informed consent was obtained from all patients, and the Ethical Committee of Musashino Red Cross Hospital approved this study, which was conducted in accordance with the Declaration of Helsinki.

Genotyping for SNPs near *IL28B* (rs8099917 and rs12979860)

Genetic polymorphisms in tagged SNPs located near *IL28B* (rs8099917 and rs12979860) were determined by direct sequencing of PCR-amplified DNA, as reported previously [9].

Histological evaluation

Laparoscopic or ultrasound-guided liver biopsy was undertaken using 13-gauge or 15-gauge needles, respectively. The median length of specimens was 18 mm (range 11–40 mm), and the median number of portal tracts was 18 (range 9–34). Fibrosis stage and grade of inflammatory activity were scored by two pathologists according to Desmet et al.'s classification [10]. In case of interobserver disagreement in histological staging or grading, the diagnosis was confirmed by consensus.

IFN therapy and definitions of response to IFN therapy

All patients had chronic HCV infection at liver biopsy, which was confirmed by the presence of HCV-RNA in serum. All IFN therapies were initiated within 48 weeks after liver biopsy. Of 792 patients, 71 patients received IFNα or IFNβ monotherapy for 24 weeks, 54 received IFNα/RBV combination therapy for 24 weeks, 118 received PEG-IFNα monotherapy for 48 weeks, and 549 received PEG-IFNα/RBV combination therapy for 48–72 weeks.

Patients negative for serum HCV-RNA 24 weeks after IFN therapy completion were defined as sustained virological responders (SVRs). Patients who remained positive for HCV-RNA 24 weeks after therapy completion were defined as nonSVRs. HCV-RNA was determined by the qualitative Amplicor or TaqMan HCV assay (Roche Molecular Diagnostics, Tokyo, Japan).

Data collection and patient follow up

At primary liver biopsy, patient characteristics and biochemical, hematological, virological, and histological data were evaluated. Age at primary liver biopsy was determined. Patients were examined for HCC by abdominal ultrasonography, dynamic computed tomography, and/or magnetic resonance imaging every 3–6 months. Serum alanine aminotransferase (ALT) and α-fetoprotein (AFP) levels were measured every 1–6 months. Surveillance protocols were in accordance with the standard of care in Japan. If HCC was suspected on the basis of the screening examination, additional procedures (e.g., dynamic CT, dynamic MRI, CT during hepatic arteriography, CT during arterial portography, contrast-enhanced ultrasonography, and tumor biopsy) were used to confirm the diagnosis. HCC diagnosis was confirmed by needle biopsy, histology of surgically resected specimens, or characteristic radiological findings. To evaluate the effect of changes in serum ALT and AFP levels during IFN therapy on

hepatocarcinogenesis, mean integration values of ALT and AFP in each patient were calculated before and after IFN therapy. In patients who developed HCC, data obtained more than 1 year prior to HCC development were used to exclude AFP elevation caused by HCC itself.

Follow-up was between the date of primary liver biopsy and HCC development or the last medical attendance until June 2011. The mean follow-up period was 4.9 years (range 1.0–18.6 years).

Determination of changes in fibrosis stage over time

Changes in fibrosis stage over time were determined in patients who showed evidence of a single blood transfusion as a known time of HCV infection. Two hundred ninety-four patients had a single blood transfusion before 1992, indicating the known time of HCV infection (rs8099917 TT, $n = 217$; rs8099917 nonTT, $n = 77$). In this subgroup, 221 (75.2 %) patients were infected with HCV genotype 1. Annual fibrosis progression rate (FPR) was calculated as the fibrosis stage at liver biopsy divided by HCV infection duration, which was determined by the period between blood transfusion and liver biopsy (mean duration, 35.1 years; range 12.0–60.0 years).

Statistical analyses

Categorical data were compared by Chi-square or Fisher's exact tests. Continuous variable distributions were analyzed with Student's *t*- or Mann–Whitney *U* test. All tests of significance were two-tailed. $p < 0.05$ was considered significant. The cumulative incidence curve was determined by the Kaplan–Meier method, and differences between groups were assessed using the logrank test. Factors associated with HCC risk were determined by the Cox proportional hazard model. As covariates in the multivariate stepwise Cox model, age, sex, stage of liver fibrosis, pre- and post-IFN treatment ALT and AFP levels, virological response, and *IL28B* genotype were included. HCC development was the dependent variable. Time zero was defined as the time of primary liver biopsy. The proportional assumption was supported by log[−log(survival)] vs. log(time) plots, which showed parallel lines. Statistical analyses were performed using the Statistical Package for the Social Sciences software (version 18.0) (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics and the SNPs near *IL28B*

Patient characteristics are demonstrated in Table 1. Frequency of the rs8099917 genotype was as follows: major homozygote (TT), 74.2 % (588/792); heterozygote (TG), 24.2 % (192/792); and minor homozygote (GG), 1.5 % (12/792). Genotypic distribution of this SNP was consistent with that in a recent report on Japanese patients [5]. The frequency of the rs12979860 genotype was as follows: major homozygote (TT), 73.4 % (581/792); heterozygote (TG), 25.1 % (199/792); and minor homozygote (GG), 1.5 % (12/792). The genotypic discrepancy between rs8099917 and rs12979860 was found only in seven patients. Therefore, the genotypes of the two SNPs (rs8099917 and rs12979860) were 99.1 % identical. All seven patients had a major homozygote (TT) in rs8099917 but a heterozygote (CT) in rs12979860, and HCC developed in one of seven patients at 2.2 years after initiation of the follow-up.

Response to IFN therapy

The final responses to IFN therapy (SVR or nonSVR) were determined in all patients. SVR rate was significantly higher in TT patients than in nonTT patients (58.3 vs. 27.9 %, $p < 0.001$) (Table 1). SVR rates for each therapeutic regimen in TT and nonTT patients, respectively, were as follows: IFN monotherapy, 35.7 % (20/56) vs. 26.7 % (4/15), $p = 0.759$; IFNα/RBV combination therapy, 46.3 % (19/41) vs. 15.4 % (2/13), $p = 0.057$; PEG-IFNα monotherapy, 63.2 % (55/87) vs. 35.5 % (11/31), $p = 0.008$; PEG-IFNα/RBV combination therapy, 61.6 % (249/404) vs. 27.6 % (40/145), $p < 0.001$.

Factors associated with the SNPs near *IL28B*

NonTT patients were significantly associated with higher γ-glutamyl transpeptidase levels, lower low-density lipoprotein cholesterol levels, higher hepatic steatosis frequency, glutamine or histidine mutations at amino acid position 70 (70QH) in the HCV core region, and one or no mutation in the IFN sensitivity-determining region in the HCV nonstructural 5A gene (Table 1).

Cumulative incidence of HCC according to the SNPs near *IL28B*

During follow-up, 53 patients developed HCC (Table 1). At 3, 5, and 10 years, the overall cumulative incidence of HCC was 3.4, 7.4, and 13.1 %, respectively. The cumulative incidence of HCC at 5 and 10 years was significantly higher in nonTT patients than in TT patients (13.0 and 20.8 % vs. 5.4 and 10.5 %, respectively; logrank test, $p = 0.002$) (Fig. 1a). Among SVRs, no significant difference was found in the cumulative HCC incidence between TT and nonTT patients (Fig. 1b). However, the cumulative incidence of HCC among nonSVRs was significantly

Table 1 Characteristics of patients and comparison between the SNPs near *IL28B*

Characteristics	Total	rs8099917 TT	rs8099917 nonTT	*p* value*
Patients, *n*	792	588	204	
Sex, *n* (%)				0.329†
Male	310 (39.1)	236 (40.1)	74 (36.3)	
Female	482 (60.9)	352 (59.9)	130 (63.7)	
Age (SD), year	58.6 (10.7)	58.5 (10.6)	58.8 (11.0)	0.684‡
BMI (SD), kg/m^2	22.8 (3.2)	22.9 (3.2)	22.7 (3.3)	0.382‡
Fibrosis stage, *n* (%)				0.751†
F1/2	612 (77.3)	456 (77.6)	156 (76.5)	
F3/4	180 (22.7)	132 (22.4)	48 (23.5)	
%Severe steatosis (≥10%)	25.3	21.4	35.4	<0.001†
ALT level (SD), IU/L	63.4 (52.5)	64.9 (50.1)	59.0 (42.9)	0.170‡
γ-GTP level (SD), IU/L	45.9 (45.3)	41.5 (43.5)	58.3 (47.9)	<0.001‡
LDL-C level (SD), mg/dL	99.8 (26.8)	102.0 (26.6)	93.6 (26.8)	0.034‡
AFP level (SD), ng/mL	10.3 (26.7)	8.24 (12.2)	16.4 (47.9)	<0.001‡
Platelet counts (SD), ×10^3/μL	164 (52)	163 (51)	167 (56)	0.422‡
HCV load (SD), KIU/mL	1550 (1465)	1612 (1465)	1392 (1457)	0.107‡
HCV genotype, *n* (%)[a]				0.065†
1a	8 (1.0)	5 (0.9)	3 (1.5)	
1b	588 (74.8)	422 (72.4)	166 (81.7)	
2a	118 (15.0)	96 (16.5)	22 (10.8)	
2b	63 (8.0)	52 (8.9)	11 (5.4)	
Others	9 (1.1)	8 (1.4)	1 (0.5)	
%Core 70 a.a. mutation[b]	34.5	26.2	57.1	<0.001†
%ISDR wild or 1 mutation[c]	67.4	64.0	76.1	0.005†
Duration (SD), year	4.9 (3.0)	5.0 (3.1)	4.8 (2.8)	0.480‡
IFN regimen, *n* (%)				0.798†
IFN mono	71 (9.0)	56 (9.5)	15 (7.4)	
IFN + RBV	54 (6.8)	41 (7.0)	13 (6.4)	
PEG-IFN mono	118 (14.9)	87 (14.8)	31 (15.2)	
PEG-IFN + RBV	549 (69.3)	404 (68.7)	145 (71.1)	
SVR, *n* (%)	400 (50.5)	343 (58.3)	57 (27.9)	<0.001†
HCC, *n* (%)	53 (6.7)	30 (5.1)	23 (11.3)	0.002†

* Comparison between *IL28B* major and minor genotypes

† Chi-square test

‡ Student's *t*-test

[a] HCV genotype was determined in 786 patients (*n*: *IL28B* major = 583, minor = 203)

[b] HCV core mutation was determined in 313 patients with genotype 1b

[c] ISDR was determined in 585 patients with genotype 1b

higher in nonTT patients than in TT patients (15.5, and 24.8 vs. 7.2 %, and 15.4 % at 5, and 10 years, respectively; logrank test, $p = 0.016$) (Fig. 1c). Similar results were obtained when the rs12979860 genotype was used as a reference. That is, the cumulative incidences of HCC at 5 and 10 years in overall patients were 13.1 and 20.5 % in nonCC patients and 5.2 and 10.4 % in CC patients (logrank test, $p = 0.001$); those in SVRs were 3.8 and 4.9 % in CC patients and 4.9 and 4.9 % in nonCC patients; and those in nonSVRs were 15.9 and 25.1 % in nonCC patients and 6.8 and 15.0 % in CC patients (logrank test, $p = 0.008$).

Ten subjects [rs8099917 TT, $n = 9$; nonTT, $n = 1$: SVR, $n = 8$; nonSVR, $n = 2$: mean follow-up period = 4.3 years (range 1.1–8.3 years)] were lost to follow-up during the last 2 years. These patients were censored from the cumulative incidence analyses at the time of the last visit.

In this study cohort, only three (one TT, $n = 1$; nonTT, $n = 2$) patients died during follow-up, and no patient underwent liver transplantation. These deaths were HCC-related. Therefore, it is unlikely that competing risks would have affected our results regarding differences in HCC incidence between TT and nonTT patients.

Because the SNPs near *IL28B* affects treatment responses particularly in patients infected with HCV genotype 1 and/or those treated with PEG-IFNα/RBV combination therapy, the cumulative incidences of HCC were analyzed in a subgroup of the patients. In patients infected with HCV genotype 1 ($n = 596$), the cumulative incidence of HCC was significantly higher in nonTT patients than in TT patients (15.2 and 24.9 % vs. 6.4 and 10.5 % at 5, and 10 years, respectively; logrank test, $p = 0.001$) (Fig. 1d). In patients treated with PEG-IFNα/RBV combination therapy ($n = 549$), the cumulative incidence of HCC was also

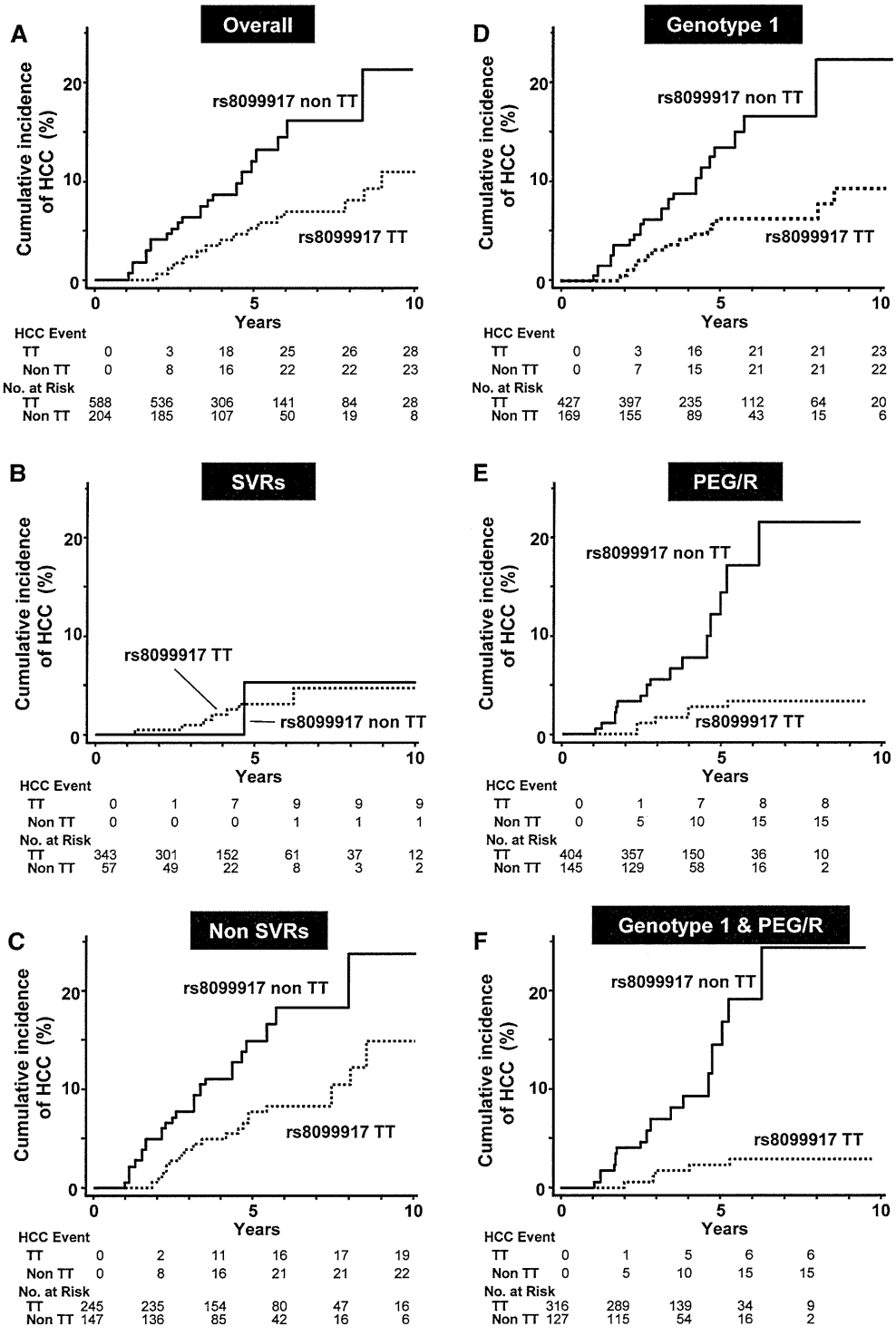

Fig. 1 Cumulative incidence of HCC according to genetic variation near *IL28B*. **a** Data for the entire patient group. Logrank test: $p = 0.002$. **b** Data for SVRs. Logrank test: $p = 0.775$. **c** Data for nonSVRs. Logrank test: $p = 0.016$. **d** Data for patients with HCV genotype 1. Logrank test: $p = 0.001$. **e** Data for patients who were treated with PEG-IFNα/RBV combination therapy. Logrank test: $p < 0.001$. **f** Data for patients with HCV genotype 1 who were treated with PEG-IFNα/RBV combination therapy. Logrank test: $p < 0.001$

significantly higher in nonTT patients than in TT patients (17.9, and 22.7 vs. 2.6, and 3.6 % at 5, and 9 years, respectively; logrank test, $p < 0.001$) (Fig. 1e). Particularly, in patients infected with HCV genotype 1 who were treated with PEG-IFNα/RBV ($n = 443$), the cumulative incidence of HCC was significantly higher in nonTT patients than in TT patients (19.5, and 24.5 vs. 2.2, and 3.2 % at 5, and 9 years, respectively; logrank test, $p < 0.001$) (Fig. 1f). Among patients infected with HCV genotype non-1 or those treated with other than PEG-IFNα/RBV therapy, no significant difference was found in the cumulative HCC incidence between TT and nonTT patients.

Influence of the SNPs near *IL28B* on progression of fibrosis over time

Among the 294 patients with evidence of a single blood transfusion, the annual FPR was similar between TT and nonTT patients ($p = 0.758$, Fig. 2). No difference was found in age at blood transfusion (26.0 [SD, 9.7] years old vs. 26.5 [SD, 9.6] years old, $p = 0.658$) and duration of HCV infection (34.7 [10.0] years vs. 36.1 [9.9] years, $p = 0.291$) between TT and nonTT patients.

Mean ALT and AFP levels after IFN therapy according to the SNPs near *IL28B*

Because we recently reported that post-IFN treatment ALT and AFP levels are significantly associated with hepatocarcinogenesis [8], the influence of ALT and AFP levels after IFN treatment was determined in TT and nonTT patients to address possible reasons associated with higher HCC development observed in nonSVRs with rs8099917 nonTT. Overall, mean serum ALT and AFP levels were reduced after IFN therapy. However, the reduction observed in mean ALT and AFP levels after IFN therapy was less in nonTT patients than in TT patients among nonSVRs (Fig. 3). The cutoff values of ALT and AFP after IFN treatment for predicting patients without HCC developments were determined as ALT <40 IU/L and AFP <6.0 ng/mL by the receiver–operator characteristics curves analysis in the original cohort [8]. The cumulative incidence of HCC development in nonSVRs was less in patients whose post-IFN ALT or AFP levels were below these cutoff values (Fig. 4a, b). Even in patients whose ALT ≥40 IU/L or AFP ≥6.0 ng/mL before IFN therapy, patients with a reduction of ALT <40 IU/L or AFP <6.0 ng/mL after IFN therapy showed significantly lower cumulative development of HCC than those without reduction in both TT and nonTT subgroups (Fig. 4c–f). However, the proportion of patients with reduction of ALT <40 IU/L or AFP <6.0 ng/mL after IFN therapy in nonSVRs was significantly smaller in nonTT patients than TT patients (Fig. 5).

As reported in the recent study [8], the persistence of post-IFN treatment ALT or AFP levels to more than the cutoff values after IFN therapy was associated with a significantly higher incidence of HCC in both SVRs and nonSVRs (Supplementary Figure). In contrast, even in nonSVRs with an equal or higher pre-IFN treatment ALT or AFP level than the cutoff values, the cumulative incidence of HCC was significantly suppressed in patients whose post-IFN treatment ALT or AFP level was reduced to less than the cutoff values (Supplementary Figure).

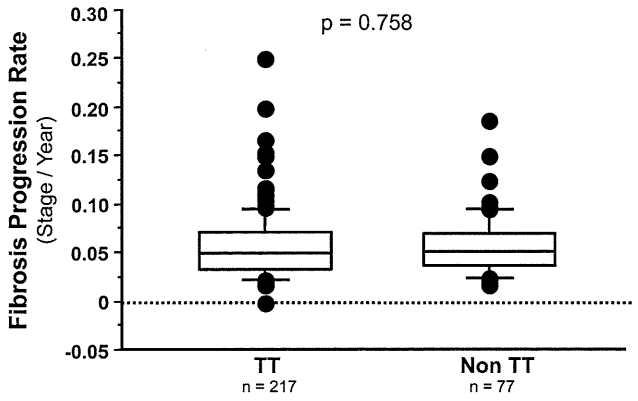

Fig. 2 Changes in fibrosis staging over time. Analysis in patients who showed evidence of a single blood transfusion as a known time of HCV infection ($n = 292$)

Influence of the SNPs near *IL28B* on HCC risk

Univariate analysis demonstrated that nonTT was one of the factors that increased the risk ratio for HCC development (Table 2). In the multivariate Cox model, age, sex, stage of fibrosis, pre-IFN treatment AFP level, post-IFN treatment ALT and AFP levels were independently associated with HCC risk among covariates including age, sex, stage of fibrosis, pre- and post-IFN treatment ALT and AFP levels, virological response and the SNPs near *IL28B* (Table 3). In patients infected with HCV genotype 1 who were treated with PEG-IFNα/RBV combination therapy, the SNPs near *IL28B* as well as age, sex, post-IFN treatment ALT level and pre-IFN treatment AFP level were identified as independent factors associated with the development of HCC among covariates including age, sex, stage of liver fibrosis, pre- and post-IFN treatment ALT and AFP levels, and virological response (Table 4). Although pre-IFN treatment AFP levels were significantly higher in patients with nonTT (Table 1; Fig. 3), our results for the multivariate analysis in this subgroup suggests that higher HCC incidence in nonTT patients is not fully explained by higher pre-IFN treatment AFP levels.

Discussion

By analyzing a large-scale, long-term cohort, we demonstrated that rs8099917 nonTT is significantly associated with HCC development particularly in patients infected with HCV genotype 1 who were treated with PEG-IFNα/RBV combination therapy. The possible relationship between the SNPs near *IL28B* and the risk of HCC development is controversial [11–13] mainly because of the lack of a longitudinal cohort study such as ours. Another possible reason for this controversy is the influence of antiviral therapy because the SNPs near *IL28B* are

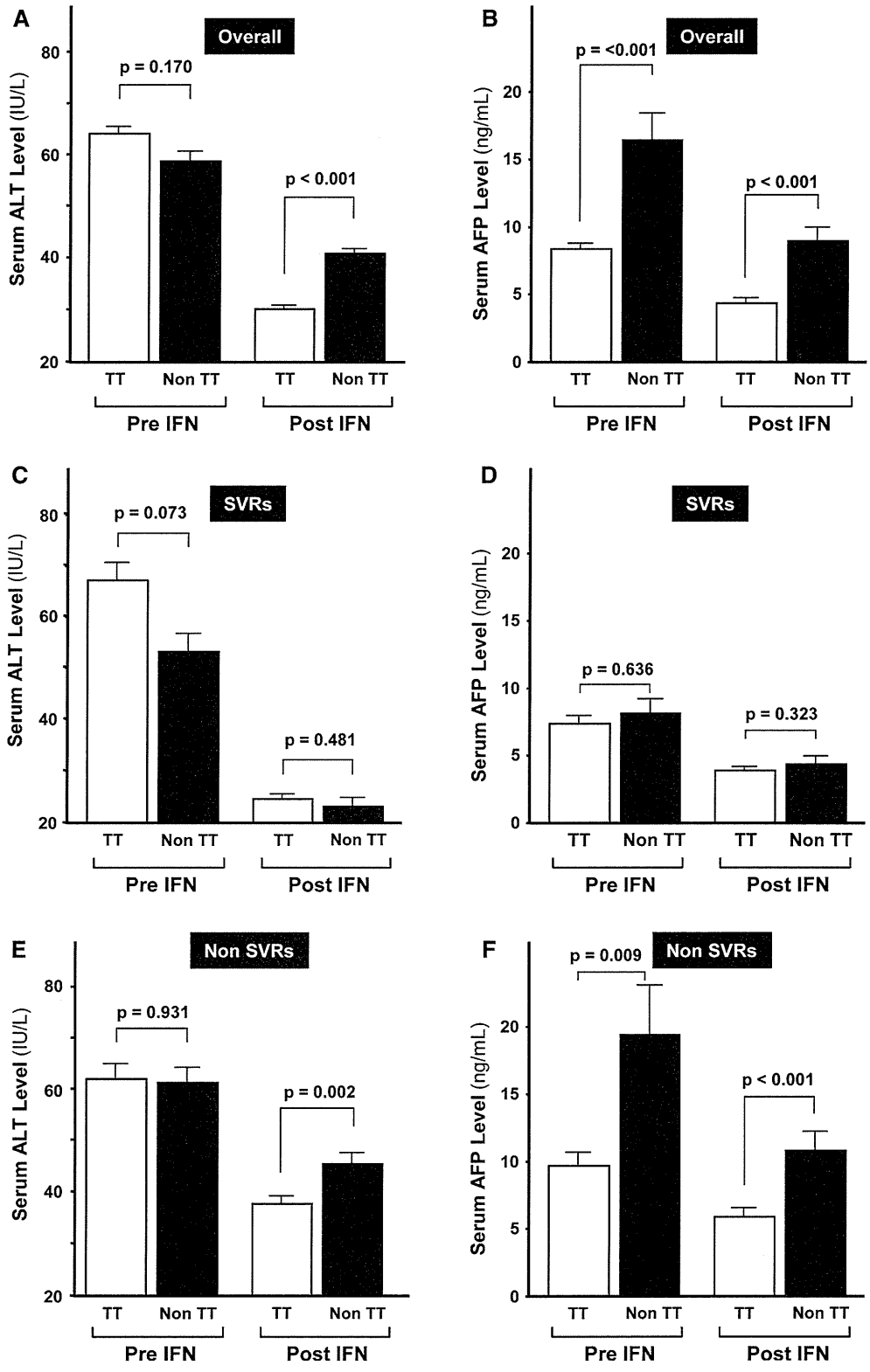

Fig. 3 Mean integration ALT and AFP values before and after interferon therapy in rs8099917 TT and nonTT patients. *Error bars* indicate the standard error. *p* values were determined by unpaired Student's *t* test

strongly associated with the antiviral response. Recent cross-sectional studies in patients without IFN treatment could not ascertain the relationship between the SNPs near *IL28B* and HCV-related HCC [12, 13]. From this viewpoint, our cohort is unique in that it includes only IFN-treated patients. In the Kaplan–Meier analyses, significantly higher incidence of HCC in nonTT was observed in patients infected with HCV genotype 1 and/or those treated with PEG-IFNα/RBV combination therapy, whereas it was not in patients infected with HCV genotype non-1 and

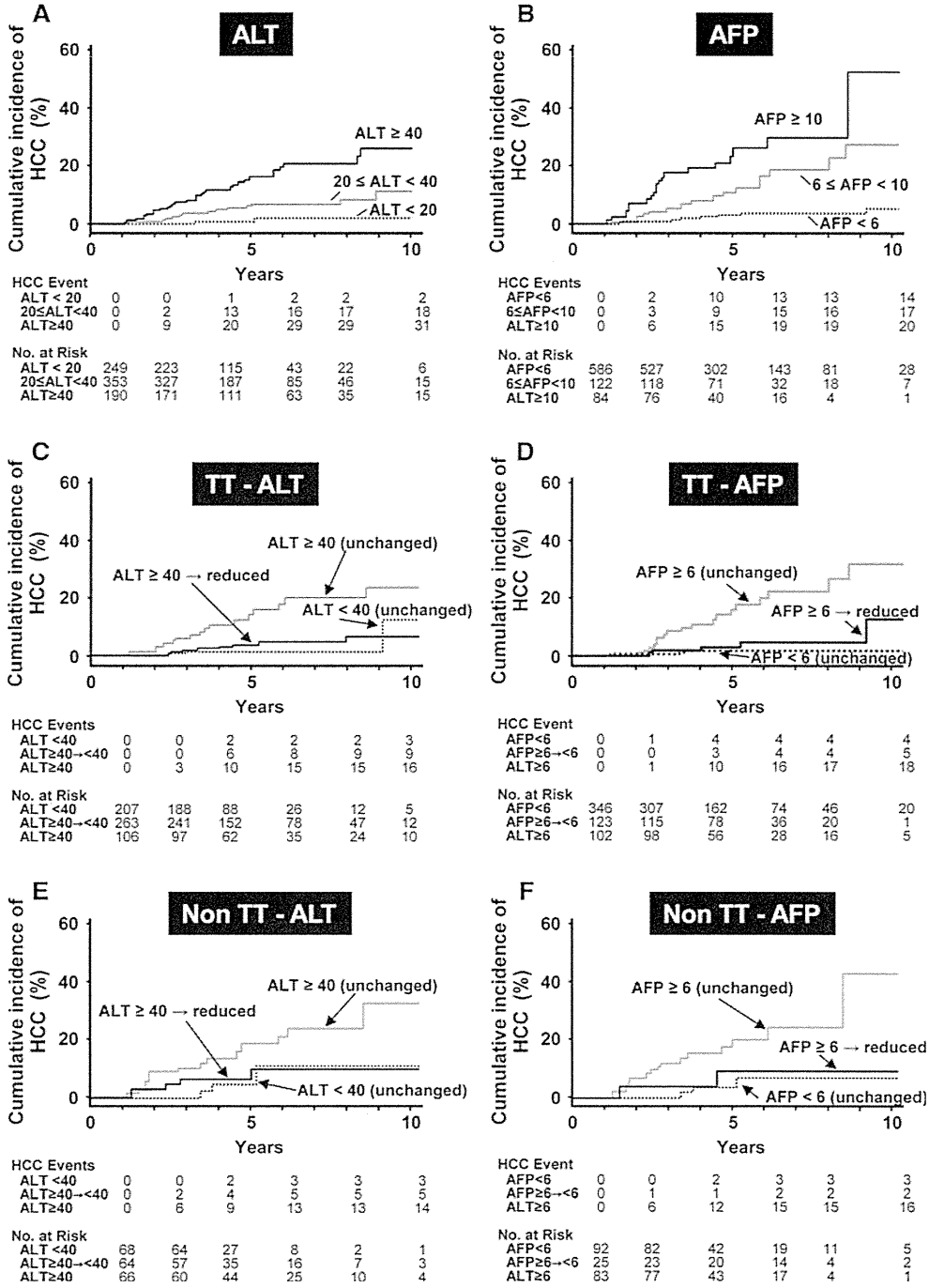

Fig. 4 Cumulative incidence of HCC stratified by mean integration values of post-IFN ALT and AFP levels. **a** Stratified by post-IFN treatment levels of ALT in all patients. Logrank test: $p < 0.001$. **b** Stratified by post-IFN treatment levels of AFP in all patients. Logrank test: $p < 0.001$. **c** According to changes in mean ALT levels before and after interferon therapy in patients with rs8099917 TT. Logrank test: $p < 0.001$. **d** According to changes in mean AFP levels before and after interferon therapy in patients with rs8099917 TT. Logrank test: $p < 0.001$. **e** According to changes in mean ALT levels before and after interferon therapy in patients with rs8099917 nonTT. Logrank test: $p = 0.040$. **f** According to changes in mean AFP levels before and after interferon therapy in patients with rs8099917 nonTT. Logrank test: $p < 0.001$

those treated other than PEG-IFNα/RBV. Moreover, our multivariate analyses demonstrated that an independent association between rs8099917 nonTT and HCC development was only found in patients infected with HCV genotype 1 who were treated with PEG-IFNα/RBV combination therapy. Because the SNPs near *IL28B* affects antiviral response particularly in patients infected with HCV genotype 1 and/or those treated with PEG-IFNα/RBV therapy, impact of the SNPs near *IL28B* on HCC risk may be indirect and is largely influenced by treatment effect.

Because a significantly higher incidence of HCC in nonTT patients was observed even in nonSVRs, higher HCC risk related to nonTT was not fully explained by the poor virological response rates observed in nonTT patients. Although we have reported that higher post-IFN treatment ALT and AFP levels were significantly associated with the risk of HCC [8], the relationship between *IL28B* SNPs and post-IFN treatment ALT and AFP levels has not yet been elucidated. Hence, to further address the higher HCC risk in nonTT patients, we directed our study at post-IFN

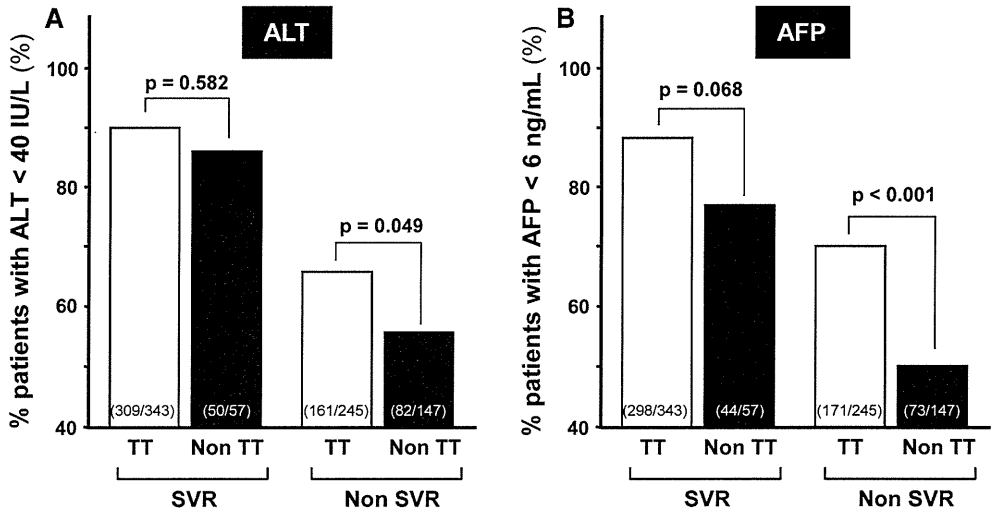

Fig. 5 Proportion of patients with reduction of ALT <40 IU/L or AFP <6.0 ng/mL after IFN therapy. **a** Percentage of patients with ALT <40 IU/L after IFN. **b** Percentage of patients with AFP <6 ng/mL after IFN

Table 2 Univariate analysis for the factors associated with hepatocellular carcinoma

Risk factor	Hazard ratio (95 % CI)	*p* value
IL28B genotype		
rs8099917 TT	1	
rs8099917 nonTT	2.36 (1.37–4.06)	0.002
Age (by every 10 year)	2.22 (1.51–3.28)	<0.001
Sex		
Female	1	
Male	2.17 (1.25–3.75)	0.006
Fibrosis stage		
F1/F2	1	
F3/F4	4.86 (2.82–8.37)	<0.001
γ-GTP (by every 40 IU/L)	1.27 (1.13–1.43)	<0.001
Core 70 mutation		
Wild	1	
Mutant	2.52 (0.94–6.78)	0.066
ISDR		
More than 1 mutation	1	
Wild or 1 mutation	1.08 (0.56–2.06)	0.826
IFN regimen		
IFN mono	1	
IFN + RBV	0.78 (0.31–1.98)	0.602
PEG-IFN mono	0.66 (0.27–1.61)	0.359
PEG-IFN + RBV	0.53 (0.25–1.12)	0.098
Pre-treatment ALT (by every 40 IU/L)	1.13 (1.00–1.22)	0.049
Post-treatment ALT (by every 40 IU/L)	3.02 (2.21–3.96)	<0.001
Pre-treatment AFP (by every 10 ng/mL)	1.09 (1.05–1.13)	<0.001
Post-treatment AFP (by every 10 ng/mL)	1.17 (1.09–1.26)	<0.001
Virological response		
SVR	1	
Non-SVR	3.07 (1.58–5.99)	0.001

Hazard ratios for the development of hepatocellular carcinoma were calculated by the Cox proportional hazards regression analysis

Table 3 Multivariate analysis for the factors associated with hepatocellular carcinoma in all patients

Risk factor	Hazard ratio (95 % CI)	*p* value
IL28B genotype		
rs8099917 TT	1	
rs8099917 nonTT	1.29 (0.72–2.33)	0.395
Age (by every 10 year)	2.59 (1.72–3.87)	<0.001
Sex		
Female	1	
Male	3.30 (1.80–6.06)	<0.001
Fibrosis stage		
F1/F2	1	
F3/F4	2.40 (1.36–4.24)	0.003
Pre-treatment ALT (by every 40 IU/L)	1.04 (0.89–1.17)	0.783
Post-treatment ALT (by every 40 IU/L)	2.58 (1.74–3.81)	<0.001
Pre-treatment AFP (by every 10 ng/mL)	1.38 (1.13–1.68)	0.002
Post-treatment AFP (by every 10 ng/mL)	1.61 (1.04–2.39)	0.028
Virological response		
SVR	1	
Non-SVR	1.64 (0.80–3.39)	0.177

Hazard ratios for the development of hepatocellular carcinoma were calculated by the Cox proportional hazards analysis

treatment ALT and AFP levels, which are considered to be possible biomarkers for the future development of HCC [8, 14]. These further analyses showed notable findings, which demonstrated that a decrease in ALT and AFP levels after IFN therapy is less in nonTT patients among nonSVRs, and

Table 4 Multivariate analysis for the factors associated with hepatocellular carcinoma in patients infected with HCV genotype 1 who were treated with PEG-IFNα/RBV combination therapy

Risk factor	Hazard ratio (95 % CI)	*p* value
IL28B genotype		
rs8099917 TT	1	
rs8099917 nonTT	4.50 (1.61–12.6)	0.004
Age (by every 10 year)	3.19 (1.72–5.99)	<0.001
Sex		
Female	1	
Male	6.17 (2.07–18.5)	0.001
Fibrosis stage		
F1/F2	1	
F3/F4	2.44 (0.86–6.97)	0.093
Pre-treatment ALT (by every 40 IU/L)	0.92 (0.59–1.49)	0.769
Post-treatment ALT (by every 40 IU/L)	2.38 (1.08–5.18)	0.034
Pre-treatment AFP (by every 10 ng/mL)	1.07 (1.01–1.13)	0.025
Post-treatment AFP (by every 10 ng/mL)	1.09 (0.94–1.27)	0.225
Virological response		
SVR	1	
Non-SVR	1.86 (0.46–7.41)	0.382

Hazard ratios for the development of hepatocellular carcinoma were calculated by the Cox proportional hazards analysis

that the proportions of patients with reductions of ALT <40 IU/L or AFP <6.0 ng/mL after IFN therapy in nonSVRs are significantly smaller in nonTT patients (Fig. 5). Although the essential mechanisms responsible for the relationship between elevated levels of ALT or AFP and HCC development are not known, these results suggest that a higher incidence of HCC observed in nonTT patients partly results from the limited suppressive effect of IFN on ALT and AFP levels, and might be reduced even in nonTT patients, whose ALT and/or AFP levels decrease after IFN-based antiviral treatment.

NonTT patients in our study exhibited a significant association with higher γ-glutamyl transpeptidase levels, increased frequency of hepatic steatosis, and increased frequency of the HCV core 70QH mutation; all these factors are associated with HCC development [2]. Therefore, HCC risk found in nonTT patients may also result from those factors coexisting with the *IL28B* minor allele.

Our results demonstrated that the SNPs near *IL28B* appeared to be independent of liver fibrosis. Recently, an association between the *IL28B* major allele and higher cirrhosis prevalence was reported in human immunodeficiency virus–HCV coinfected patients [15]. However, the limitations of this study were that it was a cross-sectional study involving only human immunodeficiency virus coinfected patients; moreover, hepatic elastography was used for determining liver fibrosis. Conversely, Marabita et al. [16] estimated the fibrosis progression rate in 247 patients with a known date of infection, and demonstrated that the *IL28B* genotype has no effect on the risk of developing advanced fibrosis. A recent study on the Swiss and the French cohorts showed a significant relationship between nonTT and a slow FPR; however, this relationship was found only in genotype non1-infected patients, and not in genotype 1-infected patients [17]. Our analysis of the FPR in HCV genotype 1b-dominant patient group demonstrated that the liver FPR did not differ between TT and nonTT patients. Taken together, the SNPs near *IL28B* do not appear to be closely associated with liver fibrogenesis in HCV genotype 1 monoinfected patients.

This study had a few limitations. The first was the heterogeneity of our cohort, which included various treatment regimens with different treatment responses. However, we obtained results in a more uniform subgroup of HCV genotype 1 patients treated with PEG-IFNα/RBV. The second limitation was the ethnic homogeneity of the Japanese population, who had a low minor allele frequency. A recent cross-sectional study in the Swiss cohort demonstrated a poor association between polymorphisms near *IL28B* and HCC occurrence [17]. Although many patients were included in that Swiss study, the number of patients with HCC development was few (3 %), which was inadequate to detect a significant effect of the polymorphism. Because the overall HCC risk varies among population groups (i.e. Japanese > European), longer-term longitudinal studies in larger cohorts with various population subgroups are required to verify the generality of our results. The third limitation involved the subanalyses of the original cohort. However, as shown in the Supplementary Table 1, SVR rates were equivalent between the original and the subcohort, although slight differences were found in proportion of gender, age and ALT levels. Moreover, characteristics of the patients with HCV genotype 1 who were treated with PEG-IFNα/RBV were identical between the original and the subcohort (Supplementary Table 2). Therefore, selection bias was unlikely to have affected our results, particularly in patients with HCV genotype 1 who were treated with PEG-IFNα/RBV, in whom SNPs near *IL28B* were identified as an independent factor associated with HCC development. The fourth limitation was that the effect of liver-supporting therapy such as ursodeoxycholic acid and glycyrrhizin was unclear in the present study, which may reduce ALT level and HCC risk in nonSVRs. However, it is likely that liver-supporting therapy was evenly indicated for both rs8099917 TT and nonTT patients, because we usually excluded the SNPs near *IL28B* from consideration when making decisions on therapeutic

indications of liver-supporting therapy. Moreover, suppressive effect on HCC development by liver-supporting therapy is presumably weak. Therefore, the effect of liver-supporting therapy was unlikely to have affected our results.

In conclusion, rs8099917 nonTT is a risk factor for HCC, in particular in patients infected with HCV genotype 1 who were treated with PEG-IFNα/RBV combination therapy. The effect of the SNPs near *IL28B* on HCC risk may be indirect, and higher HCC development observed in nonTT is presumably because of two reasons: (1) poor IFN efficacy in reducing ALT and/or AFP levels in patients with nonTT, (2) coexisting unfavorable risk factors for HCC. Not only HCV eradication but also suppression of ALT and/or AFP levels after IFN therapy may reduce the risk of hepatocarcinogenesis in nonTT patients.

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