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, $\leq$38 IU/L[a])	69 (17–372)	181
Alanine transaminase (normal, $\leq$50 IU/L[a])	56 (9–282)	181
Platelet (normal, 149–315$\times 1000^3$/mm^3)	95 (33–213)	183
ICG R15[b] (normal, $\leq$10%)	27 (6–81)	173
Prothrombin time (normal, $\geq$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 $\geq$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).

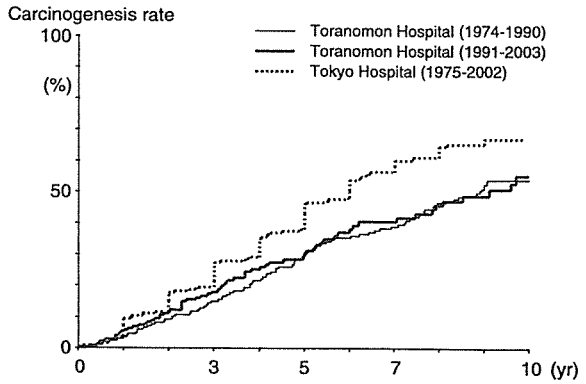

Fig. 1. Cumulative hepatocellular carcinogenesis rates in 183 patients who were diagnosed with HCV-related cirrhosis at Toranomon Hospital between 1974 and 1990. The 5th and 10th year rates were 28.9 and 54.0%, respectively (solid thin line). HCC appearance curves were also drawn in the internal (solid 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 $\geq$20 ng/ml was 2.30 compared with those with lower AFP value, and the hazard ratio of patients of $\geq$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

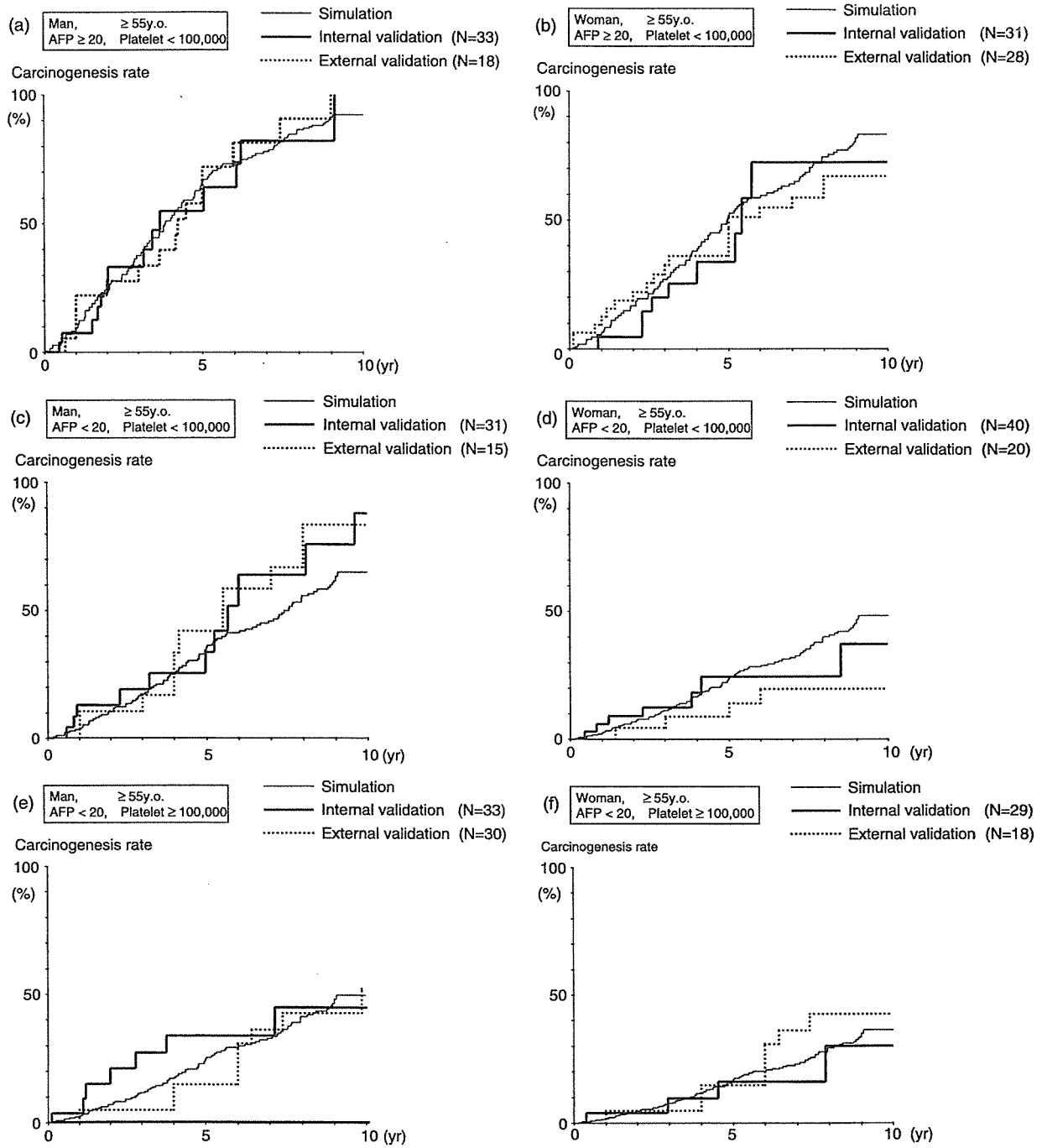

Fig. 2. Simulated carcinogenesis curves with actual carcinogenesis rates of internal and external validation cohorts, according to four significant predictors (gender, age, alpha-fetoprotein [AFP], and platelet count). *Thin solid lines*: simulated carcinogenesis curves, *bald lines*: actual curves of 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

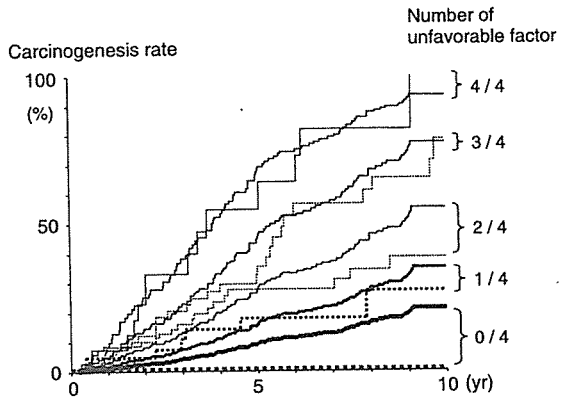

Fig. 3. Simulated HCC appearance curves with actual appearance rates of internal and external validation cohorts, according to the number of unfavorable risk factors. Five solid curves show simulated carcinogenesis rates drawn according to the number of unfavorable risk factors; none (the thickest line), 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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Classification of hepatitis B virus genotypes by the PCR-Invader method with genotype-specific probes

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Abstract

Hepatitis B virus is a worldwide public health problem. A simple and effective test to identify viral genotypes would greatly aid efforts to understand and control the spread of this disease. A serial invasive signal amplification reaction assay (PCR-Invader assay) was developed for distinguishing the known eight genotypes (A–H) and four subgenotypes (Aa, Ae, Ba, Bj) of hepatitis B virus (HBV). The preS/S and core regions were amplified by multiplex PCR and delivered to 12 wells containing genotype-specific Invader probes. By observing the fluorescence patterns in the wells, HBV sub/genotypes can be assigned. A total of 505 serum samples containing HBV/HBsAg in Japan was examined by PCR-Invader and compared the results with those from ELISA assays with monoclonal antibodies against epitopes on gene products of the preS2 region and with a genotype-specific probe assay (GSPA) based on the preS1 region. Genotypes determined by the PCR-Invader agreed with those of the ELISA method in 98.2% of cases and with the GSPA method in 97.1% of cases. Co-infection with two distinct genotypes was correctly identified by the PCR-Invader in four serum samples, as determined by GSPA. Thus, the PCR-Invader assay is a useful tool for detecting the 10 known HBV sub/genotypes.

Keywords: Hepatitis B virus; PCR-Invader; Subgenotype; Ba; Bj

1. Introduction

Nearly 400 million people worldwide are chronically infected with hepatitis B virus (HBV). HBV is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. The large number of infections and serious disease associations make HBV a significant public health problem. A simple and effective test of viral genotypes would greatly enhance efforts to curb the epidemic.

HBV is classified into eight genotypes (A–H), based on complete nucleotide sequences and assuming an intergenotype divergence of >8% (Okamoto et al., 1988; Arauz-Ruiz et al., 2002). HBV genotypes seem to have characteristic geographical distributions. In Europe, Africa and the USA, the predominant genotypes are A and D. In East Asia, genotype B and C dominate. In Japan, except for Tohoku and Okinawa, genotype C is found in more than 90% of cases (Orito et al., 2001; Kidd-Ljunggren et al., 2002).

In addition, several subgenotypes have been reported. Two subgenotypes have been identified in genotype B. Subgenotype Ba is found in Asia (except Japan) and has a sequence in the core region resulting from a recombination with genotype C. Subgenotype Bj is found almost exclusively in Japan (Sugauchi et al., 2002). In Taiwan, genotype Ba had the strong association with the earlier development of HCC in patients younger than 50 years (Kao et al., 2000). Individuals with subgenotype Bj test positive for viral antibodies (HBeAb), whereas those with Ba show a high rate of positive for the viral antigen (HBeAg) (Kobayashi et al., 2005). Moreover, Ba is very susceptible to the YMDD mutation and breakthroughs tend to occur early. Similarly, two subgenotypes have been identified in genotype A. Subgenotype Ae is prevalent in Europe and Aa is common in Africa and Asia (Kramvis et al., 2002). A third subgenotype Ac is also found in Africa (Kurbanov et al., 2005).

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The clinical aspects of each genotype are an active area of study in Japan. For example, genotype C poses a clearly greater risk for hepatocellular carcinoma than genotype B. Furthermore, the age of onset appears to be younger in genotype C than genotype B (Kobayashi et al., 2006). In Japan, the incidence of hepatitis has begun to increase, a result of infections by genotype A from Europe, USA, Africa and Philippines. This genotype seems to easily move from acute to chronic hepatitis (Kobayashi et al., 2002; Suzuki et al., 2005).

These epidemiological and clinical investigations emphasize the importance of the genotypical distinction of HBV. An effective diagnostic tool would be of great value. Sequence analysis and PCR-RFLP have traditionally been used to genotype HBV (Mizokami et al., 1999; Stuyver et al., 2000). However, these methods are too expensive or too difficult to be routinely used. A cheaper and simpler method is needed for large-scale surveys.

The specificity and sensitivity of the Invader assay to identify viral genotypes were examined by examining single nucleotide polymorphisms (SNPs) from PCR product.

2. Materials and methods

2.1. Source of patients

A total of 505 serum samples containing HBsAg were obtained from Toranomon Hospital (Kanagawa, Japan). Procurement of all serum samples was approved by and was in accordance with the human research ethics committee of Toranomon Hospital. All serum samples were stored frozen at −80 °C until use.

2.2. Determination of HBV genotypes by ELISA and GSPA

The genotypes of HBV were determined by ELISA (HBV genotype EIA, Institute of Immunology, Tokyo) and GSPA (Genome Science Laboratories, Tokyo) with commercial kits. The ELISA method used five monoclonal antibodies directed to epitopes on the preS2 region (Usuda et al., 1999, 2000). The GSPA (genotype-specific probes assay) method used seven DNA probes of corresponding sequence on preS1 region (Kato et al., 2003). The viral samples were classi-

Table 1
Invader genotype-specific oligos

HB1978-p2	ACGGACGCGGAGẎRTTCGAGAYCTCCTYGAC
HB1978-p1	CGCGCCGAGGĠGTGCRDGAYCTCCTAGA
HB1978-io	CTGTGGAGTTACTCTCTTTTTTGCCTTCTGACTTCTTTCCKTCC
HB1984-p1	CGCGCCGAGGĠGATCTACTHGAHACMSG
HB1984-p2	ACGGACGCGGAGȦGATCTYSTRGAYACMG
HB1984-io	TCGTTTTTGCCTTCTGACTTCTTTCCTTCMGHVNGT
HBV2020-p1	CGCGCCGAGGḦGCHTTRRAATCTCCTG
HBV2020-p2	ACGGACGCGGAGĠGCCTTAGAGTCTCCS
HBV2020-io	GACACCGCCTCHGCYYTGTATMGGGAT
HB2056-p1	CGCGCCGAGGĠTGRTGAKGTGAGCAAT
HB2056-p2	ACGGACGCGGAGȦTGRTGAGGWGWRCAATG
HB2056-io	CAACACACAATAGCTTGCCTGAKTGCYGTT
HB2059-p1	CGCGCCGAGGĊGTATRGTGAGSYGWVMH
HB2059-io	MCCCCAACAYWSAAYVVCTTGCCTGAKWGCA
HB2160-p2	ACGGACGCGGAGĠYTATGTCAAYRTTAATATGGG
HB2160-p1	CGCGCCGAGGȦTTATGTTAACACTMMCAYGGG
HB2160-io	AGTAATTTRSAAGAYCCAGCATCCAGGGAWYTAGTAGTCAC
HBV2345-p1	CGCGCCGAGGḰDYRTCTAACWAYASWAGTY
HBV2345-p2	ACGGACGCGGAGĠGYCYCKDYRTCTAA
HBV2345-io	AGGGAGTTCTTCTTCTAGGGRWCCYGCVTCT
HB2887-p2	ACGGACGCGGAGĊAGAATCTTTYCAYCAGC
HB2887-p1	CGCGCCGAGGȦCRAAYCTKBCKGTYCCC
HB2887-io	KTCTTSSRAACMWMRRCWASRSMWKGGGKT
HB2901-p1	CGCGCCGAGGḰCCCAAYCCKCTGGG
HB2901-p2	ACGGACGCGGAGĊCYCAATCCNCTGGG
HB2901-io	CTCGRMAAGGCATGGGGACRAATCTTTCYGTA
HB2950-p2	ACGGACGCGGAGȦAAGCCAACTCMGAMAATC
HB2950-p1	CGCGCCGAGGĠGAGCCAAYTCAAACAATC
HB2950-io	TCCCCGATCATCAGTTGGACCCTGCRTTCT
HB2980-p2	ACGGACGCGGAGĊTCAAYCCVMACAAGGAC
HB2980-p1	CGCGCCGAGGṪTCAACCCCAACAAGGAT
HB2980-io	TGCATTCAAAGCCAACTCAGAAAATCCAGATTGGGACA
HB3008-p2	ACGGACGCGGAGĠCTGGCCAGKKGT
HB3008-p1	CGCGCCGAGGṪSKGGCCAGYSGTC
HB3008-io	GAATGCTCCYACTCCTACCTGRTKGGCTC

p1, primary probe (FAM); p2, primary probe (RED); underlined sequence represents the 5′ flap of probe; amino-blocked 3′ end of all primary probes.
·: cleavage site of primary probes.

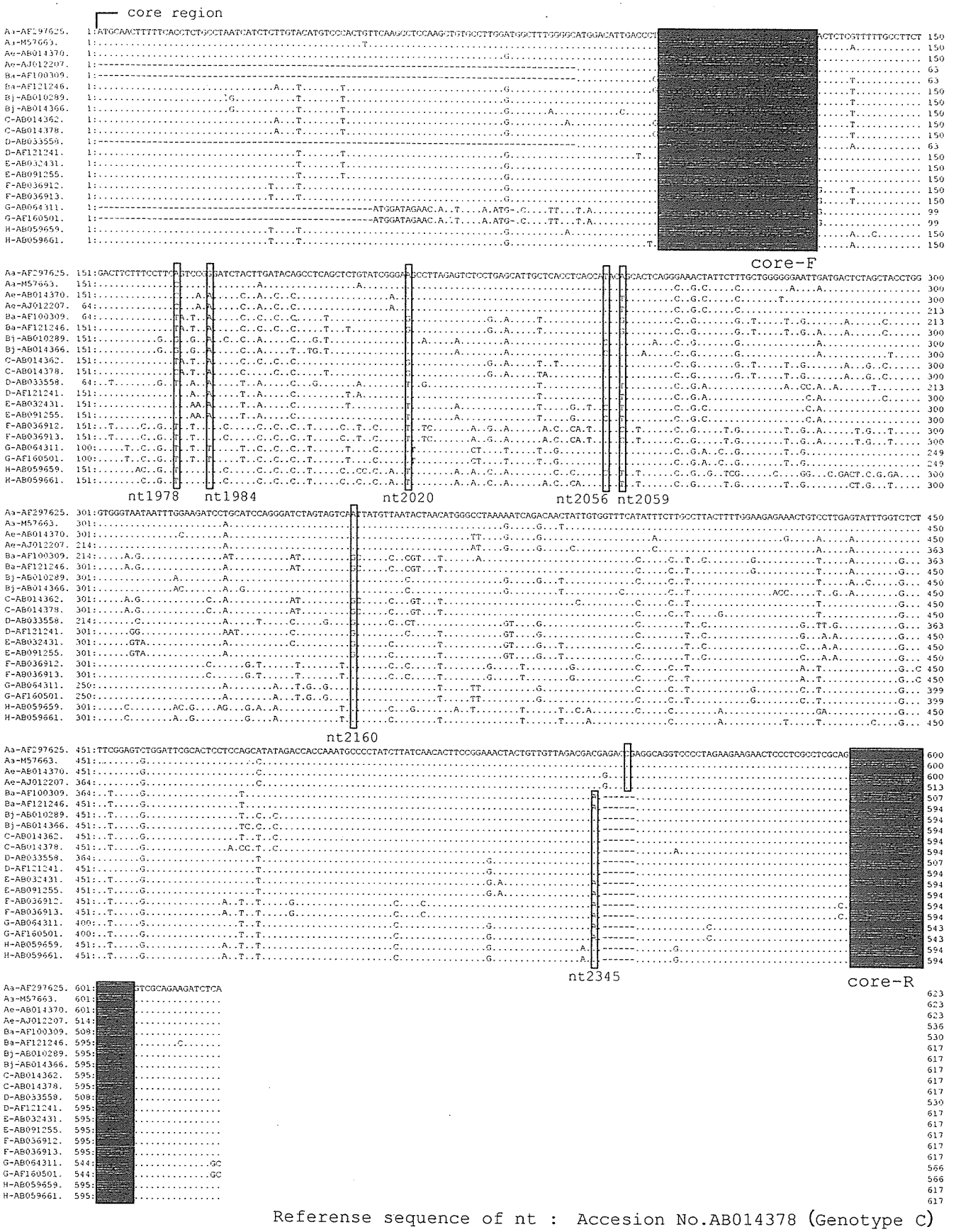

Fig. 1. Cleavage points for genotyping in core domain. sequence of each genotypes obtained from genebank. Shaded boxes represent primer (core-F, core-R) binding region. White boxes represent cleavage point (nt1978, 1884, 2020, 2056, 2059, 2160, 2345). Start codon is shown above.

fied as genotypes A–F by ELISA and as genotypes A-G by GSPA.

2.3. Design of genotype-specific Invader probes

Consensus nucleotides and neighbor regions within sub/genotypes were found by analyzing 491 individual sequences of the HBV complete genome from the National Center for Biotechnology Information (NCBI) database. Twelve nucleotide, in the core region (L1–L7:nt1978, nt1984, nt2020, nt2056, nt2059, nt2160, nt2345) (Fig. 1) and *S* region (L8–L12:nt2887, nt2901, nt2950, nt2980, nt3008) were selected as cleavage points for Invader reaction to distinguish HBV sub/genotypes (Table 2). Primary probe/invasive oligonucleotides were designed by the Invader technology creator (TWT, Madison, WI, USA) (Table 1). Invader probes had 1–10 sequence variations in equal amounts in one reaction. The hybridized region of L7 probes was maintained in all sub/genotypes and this well was used as a positive control (Table 2). (Preference sequence: accession no. AB014378).

2.4. Extraction of HBV-DNA and amplification of core/S region

Nucleic acids were extracted from 100 μl of serum with a commercial kit (Smitest EX-R&D, (Genome Science Laboratories, Fukushima)). HBV *S* and core genes were amplified in the same test tube by a multiplex PCR method with primer pairs (for *S* gene, sense: 5′-GGTCACYATATTCTTGGGAACAAGAK-CTA-3′ [nucleotide (nt) 2815–2843] and anti-sense: 5′-CTGA-CTGCCGATTGGTRGAGGCAG-3′ [nt 3151–3128]; for the core gene, sense: 5′-TATAAAGAATTTGGAGCTWCTGTGG-AGTT-3′ [nt 1916–1944] and anti-sense: 5′-GCGGCGA-TTGAGAYCTKCKT-3′ [nt 2414–2395]). Extracted DNA was amplified in a 25-μl reaction mixture containing 1 μM each of the above primers with 0.8 mM each of dUTPs in 2× Multiplex PCR Master Mix containing dNTPs (QIAGEN, Hilden, Germany). The reaction mixture was preheated at 50 °C for 2 min and then at 95 °C for 15 min. Amplification was carried out for 50 cycles (94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s [7 min in the last cycle]) in a GeneAMP PCR system 9700 thermocycler (Roche, Basel, Switzerland) utilizing a 96-well plate (Applied Biosystems, Foster City, CA, USA) without a mineral oil overlay.

2.5. Invader reaction

The reactions were performed in 384-well plates with Cleavage XI Invader core reagent kit (Amplified DNA) (TWT) and containing 2 μl of purified viral DNA amplicon by a AMPure reagent (Agencourt Bioscience, Beverly, MA, USA) (Nevilie et al., 2002). The plates were incubated at 65 °C for 15 min in the DNA thermocycler (PTC-200; MJ Research, Watertown, MA, USA) and fluorescent intensities were measured at zero time and then 15 min later. Second fluorescent intensities were measured continuously on a Cytoflour 4000 fluorescence plate reader (Applied Biosystems) for FAM (carboxyfluorescein) (wavelength/bandwidth: excitation, 485/20 nm; emission, 530/25 nm) and RED (REDmond RED) (excitation, 560/20 nm; emission, 620/40 nm) dyes. The genotype was identified by calculation of fold-over-zero (FOZ) values. Twenty-one sera without HBV-DNA were used to determine the cut-off value of FOZ. The mean plus 5 S.D. of the FOZ value of these sera in each well was calculated to 1.21–1.51 (L1–12) of FAM-FOZ and 1.20–1.64 (L1–12) of RED-FOZ. Therefore, the cut-off level was set at 2.00.

2.6. Cloning and sequencing HBV-DNA

HBV-DNA of four genotypes and two subgenotypes (i.e., Ae, Ba, Bj, C, D) were amplified by sense (seq-F): 5′-CCCGTATAAAGAATTTGGAGCTTC-3′ [nt 1912–1925] and anti-sense (seq-R): 5′-CCCGTATAAAGAATTTGGAGCTTC-3′ [nt 1736–1715] for examination of detection sensitivity. The amplified products were cloned into pCRII-TOPO-vector (Invitrogen, Carlsbad, CA, USA) and sequenced by the dideoxy method using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) in a model 3100 fluorescent DNA sequencer (Applied Biosystems). The cloning sequence used to identify co-infection was described (Kato et al., 2001).

2.7. Detection sensitivity

Detection sensitivity of PCR-Invader was examined with single or mixed genotype plasmids containing PCR products

Table 2
Nucleotides for determining sub/genotypes

Core gene	Nt position	1978		1984		2020		2056			
	Nucleotide	G	Y	G	A	A	G	C	T		
	Genotype	Bj	Ba, C	Aa	Ae, E	Bj, E	Ba, C	Bj	Ba, C, E, G		
	Nt position	2059		2160		2345					
	Nucleotide	G		A	G	[a]	–				
	Genotype	Ba		Bj	Ba, C, E	A	Not A				
S gene	Nt position	2887		2901		2950		2980		3008	
	Nucleotide	A	C	T	C	G	A	T	C	A	C
	Genotype	B, C	D	C, H	B	C	B	C	B	Aa, E	Ae

Nt 2059 is used to detect only genotype Ba.
[a] A six-nt insertion (ARGACC) at nt 2345 indicates genotype A.

of sub/genotype Ae, Ba, Bj, C and D. The detection limit of infection with a single genotype was determined with a 1:1000 dilution of each plasmid. Detection limits of co-infection with HBV strains of two distinct genotypes were determined with mixed plasmids of different genotypes.

3. Results

3.1. Detection sensitivity

The detection threshold for PCR-Invader was 10 copies of HBV DNA per reaction and the variation on the detection limits of each genotype were within a 10-fold difference (Table 3). In samples with two genotypes, PCR-Invader was able to detect both genotypes, even if the ratio of the genotypes was 1000:10 (Table 4).

3.2. Determination of 10 HBV genotypes

Ten sera classified in different genotype by PCR-Invader were picked up from all samples which were measured. The fluorescent detection of L1–12 was in accord with the predicted pattern (Fig. 2). Clear difference was seen on L1, 3, 4, 5 and 6 with the core region probe and also for subgenotypes Ba and Bj. Subgenotypes Aa and Ae were distinguished by L2 and L12 (Fig. 3). These same sera are confirmed by sequencing (Table 5).

3.3. Comparison of HBV genotypes determined by PCR-Invader and by ELISA

In 168 serum samples containing HBV, genotypes were determined by PCR-Invader and ELISA. Genotypes determined by PCR-Invader agreed with those found by ELISA in 165 samples (98.2%). For three samples (1.8%), the results did not agree. When the genotypes for these three samples were confirmed by sequencing and GSPA (Table 6A), they supported the HBV genotypes determined by PCR-Invader. In addition, three samples were found to be coinfected with B (one Ba and two Bj) and C.

Table 5
Highest homology strain of 10 samples classified in different HBV genotypes

Genotype	Accession no.	Homology (%)
Ae	AY128092	98
Aa	AB116094	99
Ba	AB073832	98
Bj	AB073846	97
C	AF461363	100
D	X97849	99
E	AB205192	99
F	AY179735	99
G	AF405706	100
H	AB179747	99

Accession numbers are those that showed the highest homogeny to the complete genome.

3.4. Comparison of HBV genotypes determined by PCR-Invader and GSPA

The genotyping results from PCR-Invader and GSPA showed excellent correlation: 333 of 343 (97.1%) samples were in agreement. Ten samples (2.9%) could not be genotyped by GSPA: three were determined to be Ae, one to be Ba, four to be Bj and two to be C by PCR-Invader (Table 6B). The genotypes from PCR-Invader were confirmed by sequencing analysis. PCR-

Table 3
Genotype detection sensitivity

Plasmid copy	Genotypes				
	HBV/Ae FAM/RED	HBV/Ba FAM/RED	HBV/Bj FAM/RED	HBV/C FAM/RED	HBV/D FAM/RED
10^3	+ (1.1/30.9)	+ (1.1/32.1)	+ (13.0/1.0)	+ (1.0/32.7)	+ (1.1/25.4)
10^2	+ (1.0/28.3)	+ (1.0/31.3)	+ (13.0/1.0)	+ (1.1/36.4)	+ (1.1/24.0)
10^1	+ (1.0/27.5)	+ (1.1/30.3)	+ (9.4/0.9)	+ (1.1/32.8)	+ (1.1/23.4)
10^0	+ (1.0/25.2)	+ (1.0/4.9)	− (1.0/0.9)	+ (1.0/31.3)	− (1.1/1.1)

Plasmids of each density were examined as templates. Samples in which a genotype-specific signal was detected are noted as positive. FOZ values of FAM/RED at L6(nt2160).

Table 4
Detection of mixed plasmids

	HBV/C				
	10^5 FAM/RED	10^4 FAM/RED	10^3 FAM/RED	10^2 FAM/RED	10^1 FAM/RED
HBV/Ae	C (1.2/26.9)[a]	C (1.7/26.3)[a]	Ae + C (6.8/24.2)[a]	Ae + C (17.7/23.0)[a]	Ae + C (21.4/21.3)[a]
HBV/Ba	C (14.6/1.4)[b]	Ba + C (14.1/3.0)[b]	Ba + C (14.2/9.3)[b]	Ba + C (13.7/12.6)[b]	Ba + C (14.3/12.9)[b]
HBV/Bj	C (17.3/1.3)[b]	Bj + C (15.4/2.7)[b]	Bj + C (13.6/7.1)[b]	Bj + C (14.1/10.5)[b]	Bj + C (13.1/13.4)[b]

Add plasmid (10 copies).
[a] FOZ values of FAM/RED at L6(nt2160). HBV/Ae was detected positive when FAM-FOZ became more than 2.0.
[b] FOZ values of FAM/RED at L11(nt2980). HBV/B was detected positive when RED-FOZ became more than 2.0.

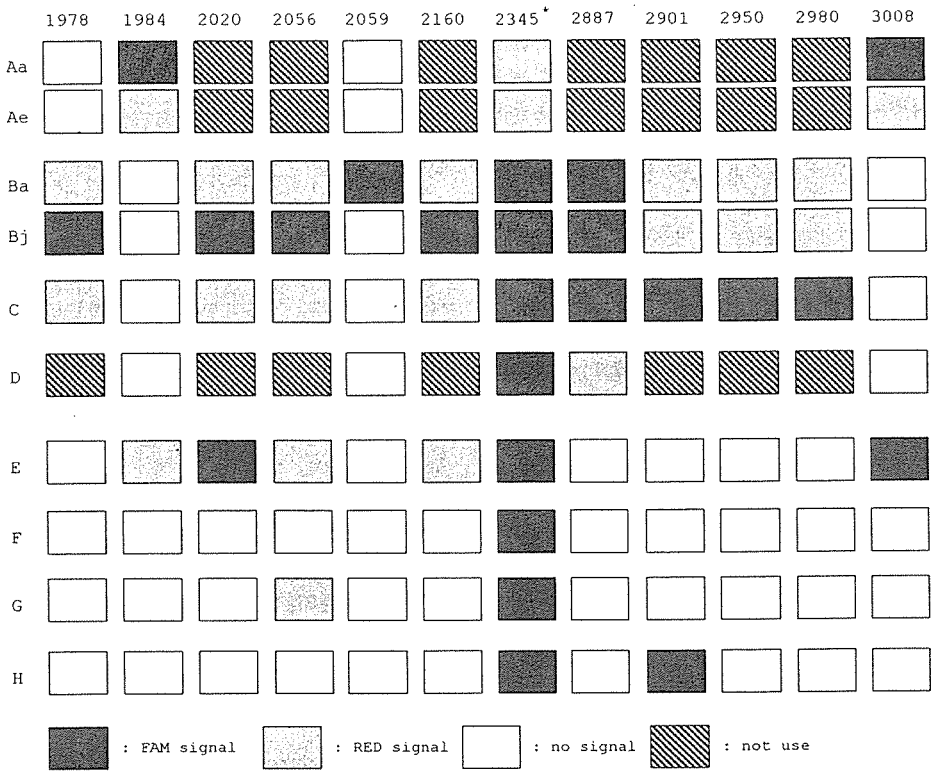

Fig. 2. Genotype-specific fluorescence patterns. Dark gray boxes represent FAM signals, light gray boxes represent RED signals and signal is not shown for the white boxes. Shaded box signals were not conserved in each genotype and, therefore, not used for detection. A few genotype A strains show FAM signals at nt 2345.

Table 6
Comparison of HBV genotypes determined by PCR-Invader with those ELISA/GSPA

(A)

Invader	Genotype	ELISA genotype				
		A	B	C	D/E	F
A	Ae	16				
	Aa	4				
B	Ba		16	1[a]		
	Bj		37			
Ba+C				1		
Bj+C			1	1		
C		1[a]	1[a]	87		
D					1	
F						1

(B)

Invader	Genotype	GSPA genotype				
		A	B	C	D	Except A–G[c]
A	Ae	31				3[b]
	Aa	4				
B	Ba		1			1[b]
	Bj		16			4[b]
C				275		2[b]
D					2	

[a] Discordant results between ELISA and PCR-Invader.
[b] Discordant results between GSPA and PCR-Invader.
[c] The case that a signal was detected only well for HBV-positive.

Table 7
HBV genotypes of clones from four serum determined mixed genotypes by GSPA and PCR-Invader

Sample number	GSPA	PCR-Invader	Cloning sequence	
			Clone	Accession
72	B, C	Bj, C	20	Bj (AB073848)
80	B, C	Bj, C	15	C (AF458664)
84	B, C	Bj, C	3	Bj (D23678)
			3	C (D50518)
112	B, C	Bj, C	21	Bj (D23678)
			3	C (AB050018)

BLAST searches were conducted on the sequences found for the clones. Accession numbers are those that showed the highest homogeny to the complete genome.

Invader also detected multiple HBV genotypes in some individual samples. For example, four serum samples were found to contain Bj and C by both methods. Cloning confirmed that the samples contained two distinct genotypes each (Table 7).

4. Discussion

Accurate genotyping of HBV samples is critical to understanding and ultimately controlling the spread of HBV. Although various methods have been developed to genotype HBV-DNA, none provides a rapid, accurate and reproducible system. A simple assay based on the PCR-Invader method was developed for distinguishing the eight known HBV genotypes and

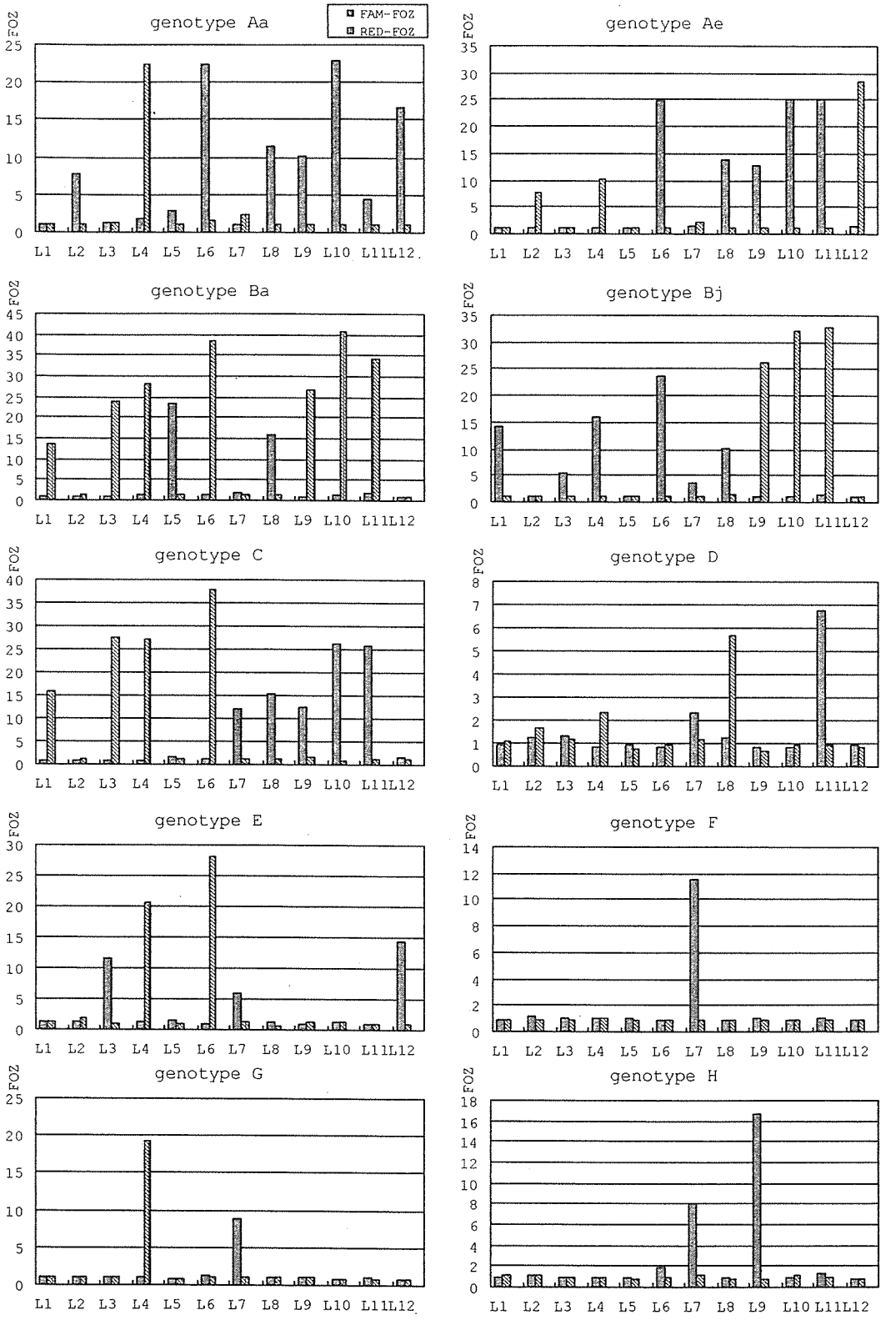

Fig. 3. Fluorescence detection pattern. Grey bars represent FAM-FOZ at L1~12 and shaded bars represent RED-FOZ at L1~12. The cut-off level was set at a FOZ (FAM, RED) of 2.0.

four subgenotypes. The probes were based on 12 conserved nucleotides in the *S* and core regions in each sub/genotype (Fig. 4). PCR-Invader for HBV genotyping are devised several points: (i) genotype-specific consensus nucleotides and neighboring regions in HBV-genome were found from 491 individual sequences; (ii) corresponding to variations in sub/genotypes, Invader probe were designed multiplex; (iii) accuracy of classification was ensured by using both core and *S* domain.

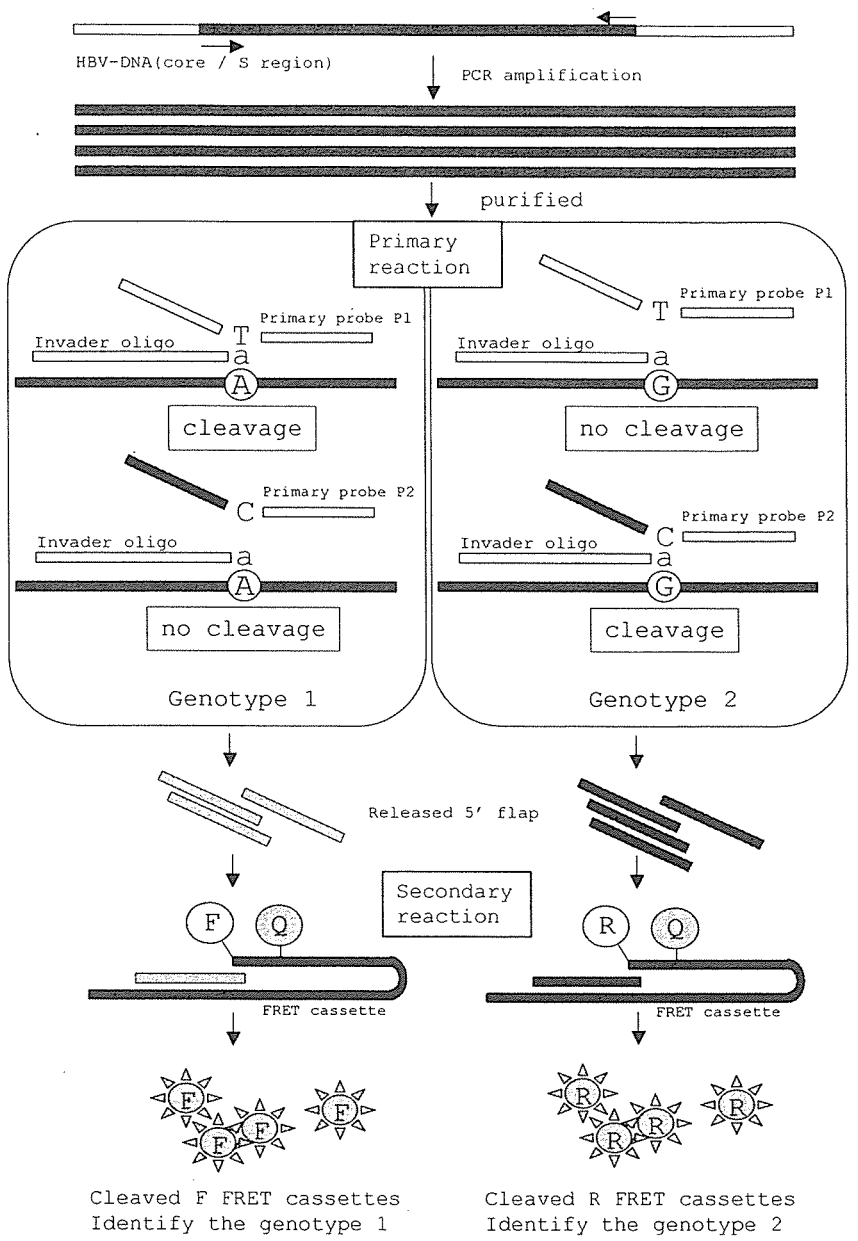

Fig. 4. Schematic illustration of the PCR-Invader reaction, showing discrimination of HBV genotypes. HBV-DNA is amplified by multiplex PCR. Purified PCR product formed invasive complex with genotype-specific primary probe and Invader oligo. Released 5′-flap, product from the primary reaction, promotes cleavage of the generic FRET cassette in secondary reaction, creating a detectable, amplified signal for classified HBV genotypes.

The assay has excellent sensitivity. Five sub/genotypes with genotypes A–D were detected in as few as 10 copies of the HBV DNA per reaction. The PCR-Invader assay was as sensitive as the GSPA method in identifying HBV strains in co-infections of two distinct genotypes. PCR-Invader could detect both genotypes even if the two plasmids were mixed in a ratio of 1000:10. In fact, PCR-Invader distinguished samples that could not be accurately resolved by other methods. The reason may be that, in ELISA method, the monoclonal antibody cannot bind when the mutation occurs in preS2 epitope region. Similarly, with GSPA method, the hybridization probe may not bind when a mutation occurs in a probe-binding site of the preS1 region.

By comparison, with the PCR-Invader, genotypic specificity is not based only on a single cleavage site but on the domain to which the primary probe hybridizes. Thus, even with mutations at several sites in the core and *S* domains, PCR-Invader is still not be likely to misjudge the genotype because it reads detection patterns in 12 different wells.

The PCR-Invader assay also detected multiple genotypes in a single sample, even when one species was represented in only small amounts. Results of clinical samples which had been shown to be coinfected by genotypes B and C by GSPA and PCR-Invader were confirmed by sequence analysis. In these tests, PCR-Invader detected minor populations that accounted for only 5% of the total virus.

PCR-Invader was originally used for typing SNPs in the human genome. However, this technology can be applied to diagnose infectious diseases, identify bacterial species and identify drug-resistance mutations. The sequences of many virus strains around the world are now available in public databases. Although the probes were somewhat difficult to design for the variety of HBV-DNAs, the PCR-Invader assay could detect genotypes without sequence analysis by finding highly conserved domains and cleavage points. Other methods for classification of HBV genotypes have been described, e.g., sequence analysis, real-time PCR, hybridization technology (Yeh et al., 2004) and serological assays. PCR-Invader recommends many synthesized oligo probes for classified genotype, however, there are several advantage points that have low running cost and can classify in detail as subgenotype Ae, Aa, Bj and Ba easily. It takes about approximately only 7 hours to classified HBV genotypes in sera by PCR-Invader. In addition, high-throughput analysis can do at same time by using automatic machinery. In previous report, PCR-Invader method had failure rate of a few percents (Mein et al., 2000); however, there was scarcely the failure in this examination. It is thought that this reason depends on a difference of a DNA amplification enzyme or PCR cycles. PCR-Invader for HBV genotyping was devised to classified clearly about some points: (i) consensus nuclotides and neighbor region about every genotype were found from 491 complete genome; (ii) corresponding to variations in a genotype, Invader probes were designed multiplex; (iii) result was raised accuracy by measuring at 12 points in core and *S* region.

The study of subgenotype classifications is becoming more and more common. Knowing the viral genotypes can provide valuable clinical information on the likely course of an illness, treatment options and predicted convalescence times. The PCR-Invader assay will be useful for large-scale determinations of HBV sub/genotypes in clinical and epidemiological settings of this dangerous disease.

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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

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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/mm^3），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/mm^3），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

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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
(Factor for SVR)			
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

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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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