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

2.4. Statistical analysis and predictive model for carcinogenesis

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

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

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

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

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

3. Results

3.1. Rate of hepatocellular carcinogenesis and risk factors

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

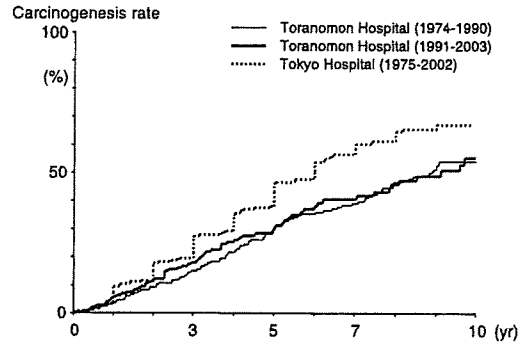

Fig. 1. Cumulative hepatocellular carcinogenesis rates in 183 patients who were diagnosed with HCV-related cirrhosis at Toranomon Hospital between 1974 and 1990. The 5th and 10th year rates were 28.9 and 54.0%, respectively (solid thin line). HCC appearance curves were also drawn in the internal (solid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

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

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

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

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

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

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

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

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

3.3. Validation of the prediction values of carcinogenesis rate

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

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

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

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

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

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

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

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

[a] Expressed by median (range).

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

The prognostic model showed that the HCC development rate was significantly affected by the following four unfavorable factors: high AFP (≥ 20 ng/ml), older age (≥ 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

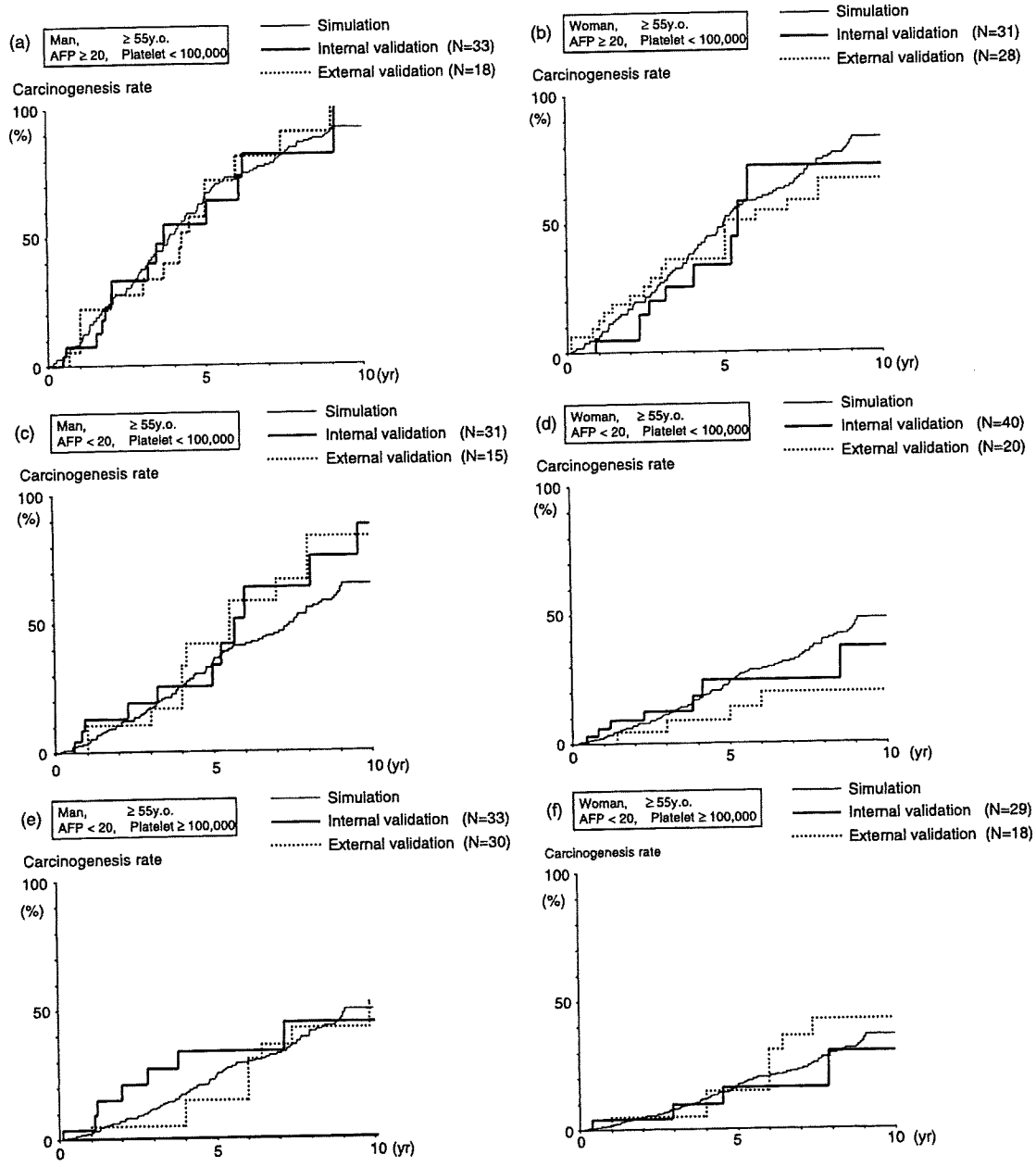

Fig. 2. Simulated carcinogenesis curves with actual carcinogenesis rates of internal and external validation cohorts, according to four significant predictors (gender, age, alpha-fetoprotein [AFP], and platelet count). *Thin solid lines*: simulated carcinogenesis curves, *bald lines*: actual curves of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

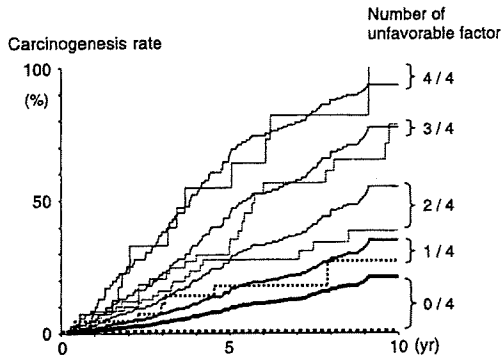

Fig. 3. Simulated HCC appearance curves with actual appearance rates of internal and external validation cohorts, according to the number of unfavorable risk factors. Five solid curves show simulated carcinogenesis rates drawn according to the number of unfavorable risk factors; none (the thickest line), 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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HEPATOLOGY

Origin of neovascular structure in an early stage of hepatocellular carcinoma: Study of alpha-smooth muscle actin immunohistochemistry in serial thin sections of surgically resected cancer

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Abstract

Background: To elucidate the origin of the neovascular structure found in well-differentiated hepatocellular carcinoma (HCC), an immunohistochemical study was performed on sequential thin section specimens.

Method: Eleven surgically resected specimens of well-differentiated HCC were analyzed for neovascular structure using monoclonal alpha-smooth muscle actin (α-SMA) antibody. Each paraffin specimen was serially sliced to a thickness of 3 μm for immunohistochemistry. When a ring-shaped structure was found unrelated to portal triads on α-SMA staining, it was regarded as abnormal neovascularity (non-triadal vessel or unaccompanied vessel).

Results: All of the 11 liver cancers had thin-walled, round- or oval-shaped non-triadal vessels in their well-differentiated parts. Immunohistochemistry of serial thin sections of HCC showed that these non-triadal vessels were connected to portal veins in portal triads in well-differentiated cancer in a total of nine patients (81.8%). This type of neovascular structure found in a well-differentiated cancer seemed to be a surviving portal vein among diminishing and disappearing arteries and bile ducts. All 11 tumors showed isovascular staining on ordinary digital subtraction angiography, and four of the tumors showed negative enhancement on intra-arterial carbon dioxide-enhanced ultrasonography or computerized tomographic (CT) hepatic arteriography, suggesting a relative arterial blood scarcity in the tumor nodules.

Conclusion: At an early stage of HCC, non-triadal vessels originate from ordinary portal veins in intratumoral portal triads. This fact sufficiently explains the reason why a well-differentiated liver cancer can sometimes show arterial blood paucity on CT arteriography or enhanced ultrasonography.

Key words: alpha-smooth muscle actin, hepatocellular carcinoma, neovascularity, non-triadal vessel, portal vein.

INTRODUCTION

Hepatocellular carcinoma (HCC) has been regarded as hypervascular on arterial angiography,[1–3] but early stage HCC often shows isovascular on angiography or dynamic computerized tomography (CT).[4–7] While both an increase in arterial blood flow and a decrease in portal blood flow are directly associated with the growth process of a small HCC,[8] little is known about the structures of tumor corresponding to arterial hypervascularity. Based on findings of imagings, a few authors[9–13] described that isovascular or even relatively

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hypovascular tumors are hemodynamic characteristics of early stage HCC. Substantial and pathological evidence of isovascularity also remained unclear in a well-differentiated HCC.

Recently, we elucidated the relationship between arterial hypervascular characteristics and histological neovascular structure, shown by immunohistological staining using monoclonal alpha-smooth muscle actin (α-SMA) antibody.[14,15] Non-triadal vessels or unaccompanied vessels have proved to be characteristic and pathognomonic for HCC.[16] We described that there were two types of neovascular structure in the literature: type I vessel, principally found in well-differentiated HCC; and type II, exclusively found in moderate to poorly differentiated varieties.[14]

When type I vessels are detected in a nodular lesion, which is difficult to distinguish from regenerative nodules in ordinary histology, a small and well-differentiated HCC can be discriminated from a benign regenerative nodule. Although the appearance of a neovascular structure seems to be an essential event of early stage HCC, the origin of the vascular structure has been scarcely elucidated. The purpose of this study was to explain the nature and origin of a newly developed vascular structure found in well-differentiated HCC in relation to the implication from radiological images. To avoid sampling error in establishing the diagnosis of well-differentiated HCC in a fine-needle biopsy specimen, we only examined surgically resected specimens for ordinary histological characteristics and details of neovascular structure.

METHOD

Patients

Among 14 consecutive patients who underwent hepatic resection for well-differentiated HCC from 1991 to 1998, 11 patients were analyzed for hepatic imagings and immunohistochemical study of α-SMA. These patients had sufficient imaging diagnosis for hemodynamics of the tumor before surgical resection, including ultrasonography (US), dynamic CT, digital subtraction angiography (DSA), computerized tomographic arterial portography (CT-AP), computerized tomographic hepatic arteriography (CTHA), and intra-arterial carbon dioxide-enhanced ultrasonography (CO_2-US). The remaining three patients received local ablation therapy, chemotherapy or transcatheter arterial embolization prior to hepatic resection.

All the patients were males aged 53–70 years with a median age of 57 years. Two patients had hepatitis B surface antigen (HBsAg), six patients had second-generation antibody to hepatitis C virus (anti-HCV, enzyme-linked immunosorbent assay, Dainabot, Tokyo, Japan) and positive HCV-RNA. One patient had both HBsAg and anti-HCV, and the remaining two patients had neither. All the patients had liver cirrhosis, but no patient had decompensated cirrhosis with ascites or a history of encephalopathy. Median value for indocyanine green retention rate at 15 min (ICG R15) was 29%, and median platelet count was 109×10^3 counts/mm^3.

Imaging methods for hepatocellular carcinoma

Before angiographic study, conventional abdominal US, contrast enhanced-CT and DSA were performed in all patients. Two or more physicians carried out US studies using convex-array real-time scanners (LOGIQ 700, General Electric Yokogawa Medical Systems, Tokyo, Japan, and model SSA270A, Toshiba, Tokyo, Japan). Plain CT and contrast-enhanced CT were performed by a CT apparatus (HiSpeed Advantage SG, General Electric, Fairfield, CT, USA), with a scan time of 1 s and a thickness of 3–5 mm.

All 11 patients underwent CT-AP, CT-HA and CO_2-US at the time of DSA study. Selective hepatic intra-arterial DSA was performed by a DSA apparatus (Integris V3000, Philips, Netherlands) with a 0.7 mm focal spot, a 30 cm diameter image intensifier, and a 1024×1024 matrix. DSA with a selective catheterization distal to the common hepatic artery was performed using 5–15 mL of contrast medium (iohexol, Ominipaque, Daiichi Seiyaku, Tokyo, Japan) at an injection rate of 1.5–3.0 mL/s. When accessory or replaced hepatic arteries were found, we examined each hepatic artery through a super selective catheter at a rate of 0.7–1.5 mL/s. A sufficient amount of contrast medium and a carefully measured injection rate were used, avoiding laminar flow and uneven distribution of the contrast medium during each DSA study. Oxygen was administered to patients during the study to enable them to hold their breath for a period of 20 s. Tumor images obtained from DSA films were classified into two categories: a 'hypervascular' stain with sharply demarcated dense lesion adjacent to the site of a tumor, or an 'isovascular' stain with no apparent delineated lesion, which was considered to be of the same vascularity as the surrounding liver parenchyma.

To detect existence of the portal blood flow into the liver mass, CT-AP was carried out by injecting contrast medium through a catheter into the superior mesenteric artery. After 90 mL of diluted contrast medium was injected at a rate of 2.5–3 mL/s, CT were taken with a thickness of 3–5 mm throughout the liver. When a low-density area appeared at the location of the liver mass, suggesting paucity of portal blood flow, it was regarded as a characteristic finding for HCC rather than a regenerative nodule, and was judged to be a positive finding for HCC.

CT-HA was also performed using diluted contrast medium through a catheter placed in the common or proper hepatic artery. A total of 25–30 mL of diluted contrast medium was slowly infused at a rate of 0.8 mL/s. To detect a tiny difference in hepatic arterial blood flow to the tumor, the density of the site of the tumor nodule was judged compared with the surrounding liver tissue.

A dynamic US study was performed by injecting carbon dioxide gas through a catheter placed in the common hepatic or more distal arteries. Carbon dioxide was injected slowly, at a rate of approximately 0.5 mL/s, while monitoring US pictures. The total volume of injected gas varied from 2 mL to 5 mL. After an injection of carbon dioxide, if a tumor became hyperechoic

compared with the surrounding liver tissue, it was judged as a positive enhancement, indicating relative abundance of arterial blood flow. Conversely, if a tumor became hypoechoic compared with the surrounding liver, it was described as negative enhancement or a decreased arterial blood flow in the tumor nodule. As a rule, CT-AP study was performed prior to CO_2-US.

Characteristics of hepatocellular carcinoma

A solitary tumor nodule was found in each of the 11 patients on resected specimens and through ordinary diagnostic techniques, including US, CT, DSA, CT-AP and CT-HA. The median size of each primary tumor was 15 mm in diameter (range 10–22 mm). All the resected specimens were analyzed pathologically for the presence of satellite nodules and tumor extension to distal portal branches. Portal vein invasion was not found in all the resected specimens using macroscopic and histological examination. According to a published histological classification of HCC by Edmondson and Steiner,[17] all the tumors belonged to grade I HCC.

Histological diagnosis and immunohistochemical staining

At least two pathologists and one hepatologist made the diagnosis of HCC. Criteria and description by the International Working Party[16] for the features of well-differentiated HCC were taken into account in the establishment of diagnosis of liver cancer with an ordinary characterization of small HCC.

A part of each liver specimen was soaked in neutral formalin solution immediately after resection and fixed for 24–48 h. Paraffin-embedded fixed specimens were sliced into a thickness of 3 μm. A set of 400 serial thin sections of individual resected HCC specimen was made in each tumor nodule, which analyzed approximately 1.2 mm thickness of HCC tissue. After deparaffinization, immunohistological staining using α-SMA monoclonal antibody (N-terminal decapeptide of α-SMA, clone 1A4, Dako, Glostrup, Denmark) was performed with an indirect avidin-biotin complex method. Light counter staining for hepatocyte nuclei was made with hematoxylin.

When an α-SMA-positive tubular or ring-shaped structure was found unrelated to portal area on the immunohistological specimens, it was regarded as abnormal neovascularity, irrespective of the site in the liver. We therefore regarded such a ring-shaped structure shown by α-SMA staining as a non-triadal vessel and defined it as positive neovascularity. When this positive staining was found exclusively in portal areas, or an irregular, non ring-shaped sinusoidal lining was demonstrated in the liver specimens, they were regarded as non-specific findings for neovasculature.

The shapes of the non-triadal vessels were classified into two categories described previously.[14] Briefly, a type I vessel was characterized by a thin-walled, nuclei-poor and oval-shaped vessel, and type II as a thick-walled, nuclei-rich and slender-shaped vessel. The ratio of the major axis over the minor axis of an α-SMA positive vessel was usually two or less in type I, and three or more in type II.

RESULTS

Radiological characteristics of hepatocellular carcinoma (Table 1)

On intra-arterial DSA images, all patients showed an isovascular staining at the site of HCC detected by US and/or CT. Hypervascular staining was not found in the other parts of the liver. Although three patients showed a distinct low-density area, the other eight patients (72.7%) did not demonstrate any explicit low-density area on CT-AP study, suggesting no paucity of portal

Table 1 Radiological and immunohistochemical findings of 11 nodules of hepatocellular carcinoma

Case	Age/sex	Tumor size (mm)	Number of tumors	Digital subtraction angiography	Portal flow	Arterial flow	Thin-walled oval non-triadal vessel	Connection of neovascularity with pre-existing portal vein
1.	57M	10	solitary	isovascular	isovascular	decrease	positive	yes
2.	56M	12	solitary	isovascular	decrease	isovascular	positive	yes
3.	57M	14	solitary	isovascular	isovascular	decrease	positive	not detected
4.	61M	15	solitary	isovascular	isovascular	isovascular	positive	not detected
5.	53M	15	solitary	isovascular	isovascular	decrease	positive	yes
6.	63M	15	solitary	isovascular	decrease	isovascular	positive	yes
7.	62M	15	solitary	isovascular	isovascular	decrease	positive	yes
8.	70M	15	solitary	isovascular	decrease	isovascular	positive	yes
9.	59M	17	solitary	isovascular	isovascular	isovascular	positive	yes
10.	56M	20	solitary	isovascular	isovascular	isovascular	positive	yes
11.	54M	22	solitary	isovascular	isovascular	isovascular	positive	yes

blood flow in the tumor nodules. On CO_2-US study, isovascular enhancement was found in seven patients and negative enhancement (relative arterial paucity) was demonstrated in four patients. No patient showed positive enhancement on CO_2-US or CT-HA. These four tumors were 15 mm or less in size and showed a retained portal blood flow on CT-AP.

All 11 tumors showed isovascular staining on ordinary digital subtraction angiography, and four of the tumors showed negative enhancement on intra-arterial CO_2-US or CT-HA, suggesting arterial blood paucity.

Because entire tumor nodules did not show a feature of classical or advanced HCC with arterial hypervascularity, fine needle biopsy was required for establishment of diagnosis of HCC before surgical resection.

Histology of hepatocellular carcinoma and alpha-smooth muscle actin positive vessels

When the degrees of histological differentiation of HCC were classified according to Edmondson and Steiner,[17] all the resected HCC were classified as grade I. According to the criteria of histological classification by the International Working Party,[16] the findings of all tumor nodules coincided with the features of well-differentiated HCC. Each tumor in all 11 patients had increased cell density with a concentration more than twice as high as surrounding liver tissue. Fatty metamorphosis was found in three tumor nodules, pseudoglandular formation in four, interstitial tumor cell infiltration in six, enhancement of eosinophilic staining in cytoplasm in seven, and basophilic enhancement in nuclei in five. Mild to moderate degrees of nuclear atypism were also found in six tumors. Every tumor had a structure of portal triad within the nodule, consisting of portal vein, artery and bile duct. No tumors had fibrous capsular formation around them.

Alpha-SMA positive vascular structure was found in the cancerous parts of specimens in all 11 patients. All the positively stained vessels showed discrete, round or oval-shaped circular configuration. They also showed thin-walled vessels lined with scattered squamous nuclei, indicating type I vessels.[14] The number of the type I vessels found in a slice of the HCC specimens ranged from six to 83, with a median of 13. None of the HCC nodules had slender-shaped and thick-walled type II vessels with abundant round nuclei. Almost all of these immunohistochemically detected vessels were hardly identified as vascular or even ring-shaped structures on ordinary hematoxylin and eosin (HE) staining. The numbers of neovascular structure were few per high power field in eight patients and 5–10 in three patients.

Alpha-smooth muscle actin positive vessels in serial thin sections

In nine (81.8%) out of 11 HCC nodules, serial thin sections of the specimens showed that the thin-walled, oval non-triadal vessels were connected to portal veins in portal triads within well-differentiated cancer (Figs 1,2). This type of neovascular structure found in a well-differentiated HCC looked like a surviving portal vein among diminishing and disappearing arteries and bile ducts. We could not demonstrate the connection between unpaired vessels and portal triads within the tumor in the serial thin sections of the remaining two patients. Both of the latter two HCC nodules had a less number of α-SMA positive non-triadal vessels in the tumor tissues, and the origin of the existing non-triadal vessels remained unexplained from the limited number of tissues with a slice thickness of 1.2 mm.

In case 6 (Table 1), a small hepatic nodule 15 mm in diameter was detected in a routine check-up with US in a 63-year-old patient with HCV-related cirrhosis. Although DSA, CT-HA and CO_2-US could not demarcate arterial hypervascularity of the tumor, a relative decrease in portal blood flow was demonstrated by CT-AP. A histological finding from fine needle biopsy for the nodule showed a well-differentiated HCC. Surgically resected specimen also showed histology of a uniform well-differentiated variety of cancer with structures of portal triads within the HCC nodule. Alpha-SMA staining showed sparse unpaired vessels. Figure 1a–d illustrates microscopic findings of serial thin section of the resected HCC, showing that the unpaired vessels joined portal veins in original portal triads within the tumor nodule.

Case 7 (Table 1) was a 62-year-old man. HE staining for the resected specimen of the tumor showed a well-differentiated HCC with crowded nuclei, nuclear deviation to sinusoid, and interstitial invasion of the tumor cells. Immunohistochemistry with α-SMA disclosed the scattered existence of unaccompanied vessels in the tumor nodules. Figure 2a–d shows microscopic findings of serial thin sections of the α-SMA positive vessels, which were connected to normal portal veins in portal triad. Portal blood therefore fed the entire part of the tumor nodule, but arterial blood flow partly diminished with development of the non-triadal vessels.

Relationship between radiological findings and non-triadal vessels

Immunohistochemical study using α-SMA monoclonal antibody showed unaccompanied vessels in the all tumor tissues. Every tumor also had the structure of a portal triad within the tumor nodules. All but two HCC specimens showed a connection between the unaccompanied vessels and portal veins within portal triads. When the diagnosis of well-differentiated HCC was established, the joint between the unpaired vessels and original portal vein was found irrespective of the detailed findings of imagings.

Case 7 (Table 1), a 62-year-old man with HCV-positive cirrhosis, developed a solitary hepatic mass with a diameter of 15 mm. Routine check-up of his liver with US demonstrated a round hypoechoic mass in the left lobe of the liver (Fig. 3a). The nodule was isovascular on DSA, but slightly hypovascular on CO_2-US (Fig. 3b), which suggested relative paucity of arterial blood flow in the tumor nodule. CT-AP demonstrated

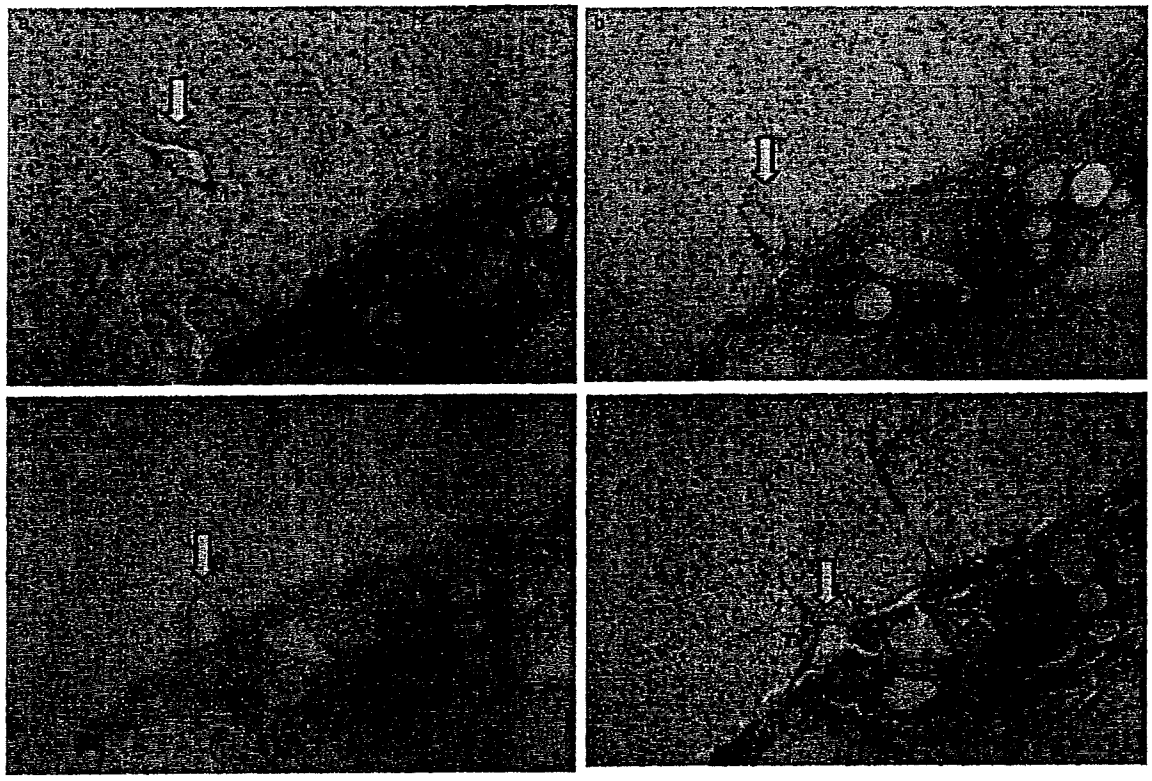

Figure 1 (a–d) Immunohistochemical staining of alpha-smooth muscle actin for serial thin sections of well-differentiated hepatocellular carcinoma in case 6. A non-triadal vessel joined a portal vein in an original portal tract. Arrows indicate a non-triadal vessel.

that there was a sufficient portal blood supply in the tumor nodule.

Alpha-smooth muscle actin in non-cancerous parts of liver

Unaccompanied vessel with positive α-SMA staining was not found in all the non-cancerous parts of the resected specimens. Alpha-SMA positive vascular structure was only found in the portal area as normal arterial and portal components of portal triad.

DISCUSSION

Murine monoclonal antibody α-SMA can recognize an actin isoform typical of smooth muscle cells and prevalent vascular smooth muscle cells.[18] Because α-SMA can delineate intrahepatic vessels including artery, portal vein, perisinusoidal cells and neovascular structure in HCC tissue,[15,19–24] we characterized specific features of neovascular structures in well-differentiated and moderately differentiated liver tumors, separately.[14] Briefly, non-triadal vessels found in HCC tissues proved to consist of two different varieties: type I vessels with round- or oval-shaped, thin-walled and sparse nuclear arrangement along the wall; and type II vessels with thick-walled, abundant nuclear arrangement and a much more slender and long-shaped structure (ratio of five times or more of the longest to the shortest diameter). Type I vessels were usually found in a well-differentiated HCC without hypervascularity, while type II non-triadal vessels were firmly associated with angiographic hypervascularity in moderately to poorly differentiated HCC.

In this study, we attempted to explore the origin of the non-triadal or unaccompanied vessels found in the early stage of HCC, through an immunohistochemical study of serial thin sections of HCC, using anti-α-SMA antibody. Anti-α-SMA antibody could successfully demonstrate the intrahepatic vascular structures containing smooth muscle: normal hepatic arteries, portal veins and intratumoral, non-triadal vessels. Although α-SMA staining was sometimes found lightly and irregularly in perisinusoidal parts of both cancerous and non-cancerous tissue, various forms of ring-shaped stains were only found in HCC. The non-triadal vessels found in a well-differentiated HCC were usually type I vessels with oval-shaped, thin-walled staining lined by scattered squamous nuclei. The current study, using serial thin sections of resected HCC specimens, disclosed that the oval-shaped vessels proved to be

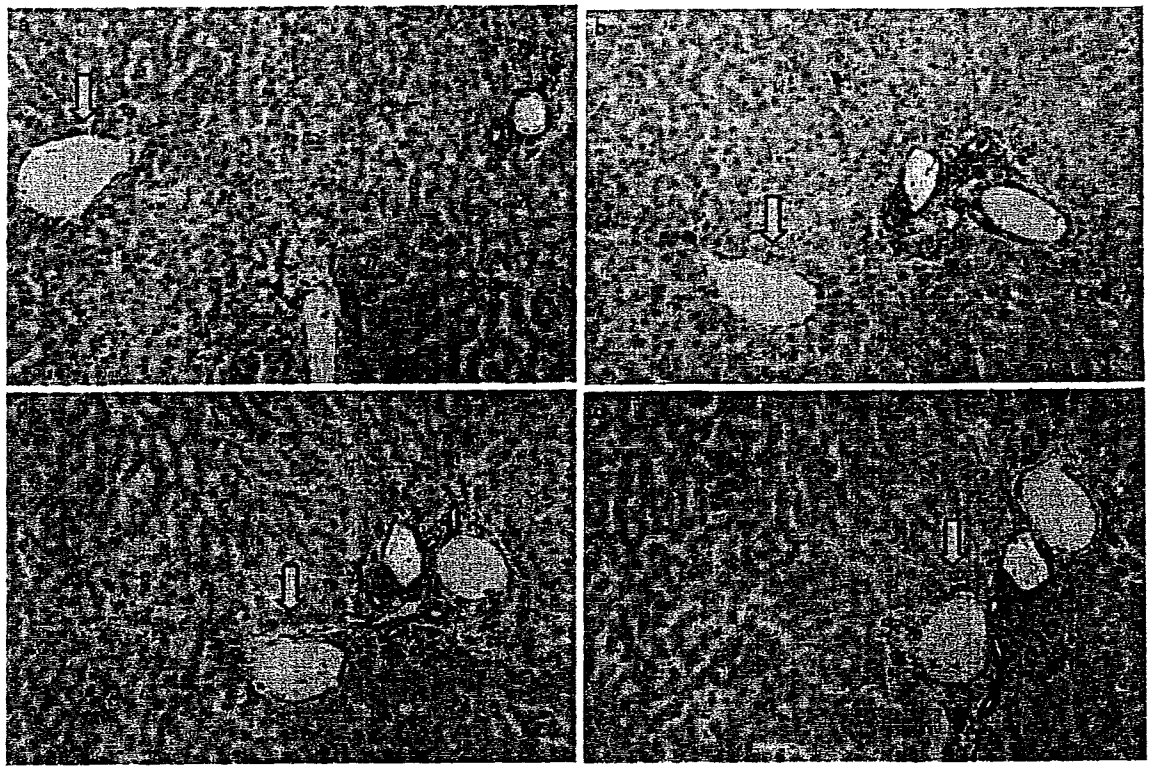

Figure 2 (a–d) Immunohistochemical staining of alpha-smooth muscle actin for serial thin sections of well-differentiated hepatocellular carcinoma in case 7. A non-triadal vessel is connected to a portal vein in a portal triad. Arrows indicate a non-triadal vessel.

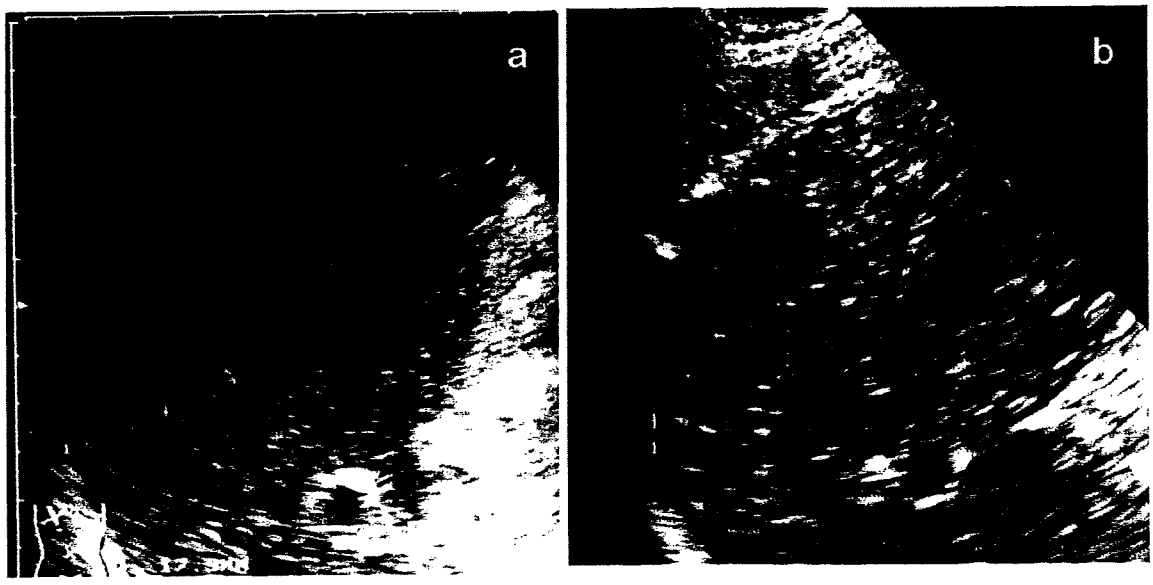

Figure 3 Ultrasonographic findings of a well-differentiated hepatocellular carcinoma (a) before and (b) after intra-arterial enhancement with carbon dioxide gas.

connected to portal veins in portal triad in HCC tissue. We could not demonstrate the origin of the unpaired vessels in well-differentiated HCC in only two of 11 specimens. One of the reasons for the failure of demonstration was scarcity of the number of unaccompanied vessels, and another reason was shortage of the slice thickness of total serial section. Unaccompanied vessels found in well-differentiated HCC were not connected with other vascular structures, including arteries in portal triads or hepatic veins. Although only the structure of portal veins definitely 'survived' from a portal triad, the reason why bile ducts and arteries 'perished' in a well-differentiated tumor was not clear.

Detection of non-triadal vessels using an immunohistological technique greatly helped in establishing diagnosis of well-differentiated HCC. Among various histological findings of well-differentiated HCC, demonstration of interstitial or stromal invasion[25] and detection of unpaired vessels were significantly valuable in the establishment of the diagnosis as HCC. The International Working Party[16] also supported the finding of non-triadal vessels in the diagnosis of HCC, and previous studies[14,23,24] described the value of the existence of specific vascular structure. Therefore, one of the important points of the current study was to explore the origin and pathogenesis of 'abnormal' non-triadal vessels found in a well-differentiated HCC. Type I vessels proved to originate from normal portal veins and this type of vessel did not meet the concept of the 'neovascular structure' of cancer. This type of neovascular structure looks like a surviving and remaining portal vein irrespective of surrounding tissue. Although this morphological study indicates that the 'abnormal' non-triadal vessel seems to be an abnormal portal vein only found in well-differentiated HCC, the possibility of the role of draining the vessel is not excluded completely.

The other significant implications shown in this study was an understanding of the imagings of blood flow at an early stage of HCC. A well-differentiated HCC sometimes shows shortage of hepatic arterial blood flow, which is demonstrated by CT-HA, CO_2-US, or both. During exploration of hepatic arterial dominance for small hepatic nodules in order to establish the diagnosis of HCC, varied modalities of imaging have been performed over the last decade.[8,26,27] Detailed analysis of imaging disclosed that some nodules of well-differentiated HCC proved to have hepatic arterial shortage instead of arterial hypervascularity.[9-11] The current study gave substantial explanation for the reason behind arterial blood paucity at an early stage of HCC. From the viewpoint of origin of non-triadal vessels, only portal veins survive and feed the tissue of well-differentiated HCC, while hepatic arteries and bile ducts diminish and disappear from the portal triads within HCC nodules. The ratio of hepatic arteries and portal veins in tumor nodules seems to result in findings of hepatic arterial paucity in sensitive tomographic imaging. The reason why each of the well-differentiated HCC nodules did not show arterial hypovascularity on imagings might be explained by sensitivity of each imaging modality, and by diversity and irregularity in the distribution of type I vessels. If the percentage of blood supply from pre-existing portal veins to an early stage of HCC was much more dominant than that from hepatic arteries, sensitive tomographic imagings would demonstrate arterial paucity in the tumor nodule. Moreover, actual blood flow per time and functional features of blood circulation in the vessels are still uncertain, and future studies should therefore be aimed at a pathophysiological aspect of hemodynamics of small and early HCC.

Non-triadal vessels should be evaluated correctly, not only in advanced HCC but also at an early stage of the tumor. The difference in angiographic vascularity might originate from an 'arterial character' in type II vessels and a 'portal character' in type I vessels. From the immunohistological results of vascular structure in HCC, only type II vessels seem to stand for 'neovascular' structure, which usually develops in any noticeable and hypervascular cancer of other organs. Type I vessels, which are almost exclusively found in a well-differentiated HCC, differed from the real definition of 'neovascular structure', because the non-triadal vessels proved to originate from pre-existing portal veins in this study. The realization of non-triadal vessel (unaccompanied or unpaired vessel) is still important in the establishment of diagnosis of early stage HCC, and in recognition of its hemodynamics. Two types of non-triadal vessels should be correctly identified not only for the study of radiological examination of liver cancer but also in the investigation of the angiogenetic process of HCC.

Although the exact reason for the solely surviving portal vein in a well-differentiated HCC tissue remains unknown, pathogenetic mechanisms are considered to differ significantly between type I and type II non-triadal vessels in HCC tissues. Type II angiogenesis is believed to depend on various kinds of angiogenetic factors including vascular endothelial growth factor and platelet derived growth factor. Our previous study[14] disclosed that type II vessels were firmly associated with angiographic hypervascularity, indicating substantial structure for arterial blood flow dominance in moderately to poorly differentiated HCC. Clinicopathological relationship and stimulating factors for angiogenesis have been studied recently,[28-31] and the pathogenesis and chorological process of characteristic vascular pattern should be investigated separately in well-differentiated HCC in the future.

In conclusion, non-triadal vessels found in early stage HCC originate from normal portal veins in portal triads, which constitute a 'pseudo-neovascular structure' in well-differentiated liver cancer. Importance of the unpaired vessels derived from portal veins include: (i) diagnostic help for histological exploration of well-differentiated variety of HCC; and (ii) substantial explanation of arterial blood paucity often found in detailed diagnostic imagings of early stage and small-sized HCC.

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REVIEW

Hepatitis B Virus Genotypes and Response to Antiviral Therapy

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SUMMARY

Hepatitis B virus (HBV) infection is an important health problem worldwide. The virus has been classified according to 8 genotypes (A–H) based on sequence divergence. Most genotypes have specific geographic distributions; genotypes A and D are prevalent in Western Europe and North America, and genotypes B and C are prevalent in East Asia and Oceania. Currently accepted treatment for chronic hepatitis B includes interferon alpha, or the nucleoside/nucleotide analogues lamivudine and adefovir. The impact of HBV genotypes on response to antiviral therapy has been studied. HBV genotypes D and C are associated with a lower rate of favorable response to interferon alpha therapy than genotypes A and B, respectively. A study in Germany suggested that the rate of resistance to lamivudine was higher in patients with HBV genotype A infection than in patients with genotype D infection. No difference in the risk of lamivudine resistance is found between patients with genotype B and patients with genotype C. In patients with genotype C infection, however, virological response is worse during lamivudine therapy, and is also less durable after the discontinuation of therapy than in patients with genotype B infection. Determining the genotype could be helpful for predicting the outcome of antiviral therapy in patients with chronic hepatitis B. (Clin. Lab. 2006;52:43-47)

KEY WORDS

hepatitis B, genotype, adefovir, antiviral therapy, interferon, lamivudine

INTRODUCTION

Hepatitis B virus (HBV) affects more than 350 million people worldwide. It is the major causative agent for chronic hepatitis, cirrhosis, and hepatocellular carcinoma, accounting for 1 million deaths annually [1,2].

Currently available antiviral therapy for chronic hepatitis B includes the immunomodulator interferon alpha, and the nucleoside/nucleotide analogues lamivudine and adefovir dipivoxil [3,4]. In addition, entecavir has recently been licensed in the United States. A number of other nucleotide analogues, such as tenofovir, emtricitabine, telbivudine and clevudine, are now at the stage of clinical trials. Interferon alpha is expensive, and is often poorly tolerated. Long-term treatment with nucleoside/nucleotide analogues may induce the emergence of drug-resistant variants with mutations in the reverse transcriptase (rt) domain of the HBV polymerase gene. For instance, mutations from methionine to isoleucine, valine or serine at amino acid rt204 in the tyrosine-methionine-aspartate-aspartate (YMDD) motif are associated with resistance to some nucleoside/nucleotide analogues including lamivudine [5–8].

HBV was traditionally classified into four subtypes (*adr*, *adw*, *ayr*, and *ayw*) based on antigenic determinants of hepatitis B surface antigen [9]. According to the Paris workshop on hepatitis B surface antigen subtypes, these subtypes were further divided into eight types (*adr*, *ayr*, *ayw1*, *ayw2*, *ayw3*, *ayw4*, *adw2*, and *adw4*) [10]. However, the clinical significance of HBV subtypes has not been established. With recent advances in molecular biology techniques, HBV has been classified according to 8 genotypes (A–H) based on sequence divergence in the entire genome exceeding 8% [11–14]. Most genotypes have specific geographic distributions. Genotype A is prevalent in Northwest Europe (including Germany), North America, and Central Africa. Genotypes B and C are common in Southeast Asia, China, Japan, and Oceania. Genotype B is further divided into two subgroups: "B*j*" (corresponds to "B1" of the classi-

fication of Norder et al. [15]) which is exclusive to Japan, and "B*a*" which is common in other countries in Asia [16]. Genotype D prevails in South Europe, the Middle East, and India, although this type has an almost worldwide distribution.

The contribution of HBV genotypes to therapeutic outcome has recently attracted the interest of researchers and clinicians. The clinical outcome of chronic HBV infection has been compared between genotypes A and D in Western countries, and between genotypes B and C in Asia. In general, genotypes D and C are associated with more severe liver disease than genotypes A and B, respectively [17,18]. The impact of HBV genotypes on the response to antiviral therapy has also been studied. In this article, we review the role of HBV genotypes with particular reference to the implications for therapy.

HBV GENOTYPES AND RESPONSE TO INTERFERON ALPHA

Zhang et al. [19] studied HBV genotypes in 35 hepatitis B e antigen (HBeAg)-negative chronic hepatitis B patients in France who had been treated with interferon alpha for a mean duration of 16 months (range, 3–40 months). The response to interferon, defined as sustained normalization of serum alanine aminotransferase (ALT) levels and significant decrease of viremia levels, was found in 7 (70%) of 10 patients with genotype A infection, compared with 10 (40%) of 25 patients with genotype D or E infection ($p = 0.001$). The rate of response to interferon was independent of both initial serum viral DNA levels and interferon doses.

In Germany, Erhardt et al. [20] determined the efficacy of interferon alpha in 165 consecutive patients with chronic replicative hepatitis B with different genotypes. Among them, 119 patients (72%) had HBeAg-positive and 46 (28%) had HBeAg-negative hepatitis B infection. Six months after the end of a 4- to 15-month course of interferon therapy, a sustained response, defined as normal ALT levels and negative HBV DNA in hybridization assay, was found in 38 (49%) of the 78 patients with genotype A infection, compared with 17 (26%) of the 66 patients with genotype D infection ($p < 0.005$). Multivariate logistic regression analysis identified HBV genotypes and pretreatment ALT levels as independent predictive parameters of response to interferon ($p < 0.009$ and $p < 0.02$, respectively).

Kao et al. [21] retrospectively studied 58 Taiwanese HBeAg-positive patients with genotype B or C infection who had been treated with interferon alpha for 24 weeks. The patients with genotype C infection had a higher serum ALT level than patients with genotype B infection. The rate of response to interferon, defined as normalization of serum ALT level and loss of HBeAg and HBV DNA 48 weeks post-treatment was 13/32 (41%) and 4/26 (15%) in genotype B and C patients, respectively. Younger age and genotype B infection were significant and independent variables for predicting the response to interferon alpha treatment ($p = 0.001$ and 0.045, respectively).

Wai et al. [22] performed a retrospective analysis of a previously reported randomized controlled trial to determine the effects of HBV genotype on the response to interferon alpha in ethnic Chinese patients with HBeAg-positive chronic hepatitis. Among the 66 patients with elevated pretreatment ALT level, an antiviral response, defined as sustained clearance of serum HBV DNA by direct spot hybridization and clearance of HBeAg at month 12, was achieved in 57% and 21% of patients treated with interferon alpha for 16 weeks ($p = 0.019$), compared with 25% and 8% of untreated controls ($p = 0.45$) with HBV genotype B and C, respectively. Multivariate analysis showed that genotype B and low pretreatment HBV DNA levels were independent predictors of antiviral response ($p = 0.001$ and 0.029, respectively).

The attachment of a polyethyleneglycol moiety to interferon alpha produces a biologically active molecule, peginterferon alpha, with a long half-life and favorable pharmacokinetics. Janssen et al. [23] reported the results of a multicenter, randomized controlled trial of peginterferon alpha for HBeAg-positive chronic hepatitis B. For 266 patients (90 with genotype A, 23 with genotype B, 39 with genotype C, 103 with genotype D, and 11 with other types), 52-week treatment regimen was started with weekly doses of 100 µg peginterferon alpha-2b, and then during weeks 32 to 52, the dose of peginterferon was reduced to 50 µg per week. Although patients were randomly assigned peginterferon monotherapy or combination therapy with lamivudine, the rate of HBeAg loss 26 weeks post-treatment was similar between the two groups (49/136 [36%] vs 46/130 [35%]; $p = 0.91$). There was a significant difference in the sustained loss of HBeAg according to HBV genotype by univariate analysis ($p = 0.01$); 42 (47%) with genotype A and 10 (44%) with genotype B had sustainedly lost HBeAg, compared with 11 (28%) with genotype C and 26 (25%) with genotype D.

These results indicate that HBV genotypes D and C are associated with a lower rate of favorable response to interferon alpha therapy than genotypes A and B, respectively.

HBV GENOTYPES AND RESPONSE TO LAMIVUDINE

In a study conducted in Germany, Zöllner et al. [24] found that among 26 consecutive patients with chronic hepatitis B (20 were HBeAg-positive and 6 were HBeAg-negative), drug-resistant mutant variants developed in 7 of 13 carriers with *adw* and 1 of 13 carriers with *ayw* during 3 to 31 months of lamivudine treatment; the risk of lamivudine resistance was significantly higher in *adw* carriers than in *ayw* carriers ($p = 0.03$). In Germany, subtype *adw* corresponds to genotype A, and subtype *ayw* corresponds to genotype D. Zöllner et al.

[25] further studied the mutational pattern of the HBV polymerase gene in 41 patients during selection of lamivudine-resistant strains. Twenty-six patients (63%) carried resistant HBV genotype A and 15 patients (37%) carried resistant HBV genotype D. The rate of mutations from methionine to isoleucine at rt204 was significantly higher for genotype D (67%) compared with genotype A (19%), whereas mutations from methionine to valine at rt204 prevailed in genotype A (81% in genotype A vs 33% in genotype D; $p = 0.006$).

Buti et al. [26], however, reported that the proportion of lamivudine-resistant mutations did not differ between HBV genotypes A and D during longer term observation. Among the 27 patients in Spain with chronic hepatitis B (8 HBeAg-positive and 19 anti-HBe-positive) receiving lamivudine for a mean period of 24 months (range, 12–36 months), 16 had genotype A HBV (equivalent to *adw*) and 11 had genotype D (equivalent to *ayw*). At year 1 of therapy, the lamivudine-resistant variants were found more often in genotype A patients than in genotype D patients (7/16 [43%] vs 2/11 [18%]), but the difference was not significant. At year 2, the drug-resistant variants were found in 8 (53%) of the 15 genotype A patients and in 5 (55%) of the 9 genotype D patients, and at year 3, in 3 (75%) of the 4 genotype A and genotype D patients. The Kaplan–Meier estimate for the median time to lamivudine resistance in all 27 patients was 24 months, independent of HBV genotype.

Kao et al. [27] enrolled 31 consecutive Taiwanese patients with HBeAg who had been treated with lamivudine. During the study period, which ranged from 6 to 30 months, 3 (23%) of the 13 patients with genotype B infection and 2 (11%) of the 18 patients with genotype C infection had seroconversion to anti-HBe. Lamivudine-resistant mutant variants occurred in 2 (15%) patients from the genotype B group and 4 (22%) from the genotype C group ($p = 0.9$) after a mean duration of 9 months (range, 8–13 months).

In another study conducted in Taiwan, Chien et al. [28] examined the determinants of sustained HBeAg seroconversion after discontinuation of lamivudine. In a total of 82 HBeAg-positive patients who had achieved a complete response (defined as HBeAg seroconversion with HBV DNA seroclearance by hybrid capture assay and normal ALT levels) to a mean period of 16 months (range, 3–55 months) lamivudine therapy was followed-up. In 38 (61%) of the 62 patients with genotype B and 5 (25%) of the 20 patients with genotype C, complete response was sustained for 12 months after the end of therapy. The categorical analysis showed that patients with genotype B, age ≤ 36 years, and additional treatment over 8 months after HBeAg seroconversion had a higher sustained response to lamivudine.

Akuta et al. [29] determined the cumulative rate of emergence of drug-resistant mutant variants in 213 Japanese patients with chronic hepatitis B (108 were HBeAg-positive and 105 were HBeAg-negative) who had been treated with lamivudine for more than 1 year. Eight (3.8%) patients were infected with genotype A, 20 (9.4%) patients with genotype B, and 185 (86.9%) patients with genotype C. The emergence rate of lamivudine resistance was independent of the genotype, but was significantly higher in the B*a* subgroup of HBV (66.7% at 2 years) than in the B*j* subgroup (7.1% at 2 years, $p < 0.05$). In patients with genotype C infection, lamivudine-resistant variants emerged significantly more often in the HBeAg-positive state than in the HBeAg-negative state ($p < 0.05$). In contrast, the emergence rate was not associated with HBeAg status in patients with genotype B infection.

In summary, a study conducted in Germany suggested that the rate of emergence of resistance to lamivudine was higher in patients with HBV genotype A infection than in patients with genotype D infection. However, this result has not been confirmed by a longer term study. There is no difference between patients with genotype B and C for the risk of lamivudine resistance. In patients with genotype B infection, the virological response is better during lamivudine therapy, and is also more durable after the discontinuation of therapy than in patients with genotype C infection.

HBV GENOTYPES AND RESPONSE TO ADEFOVIR DIPIVOXIL AND NEW NUCLEOTIDE ANALOGUES

Westland et al. [30] examined HBV genotypes in patients with chronic hepatitis B who had been enrolled in 2 multinational phase III studies of adefovir dipivoxil. In the 269 patients (43 with genotype A, 52 with genotype B, 71 with genotype C, 96 with genotype D, and 7 with other types) who received 10 mg adefovir dipivoxil for 48 weeks, potent reductions in serum HBV DNA were observed regardless of HBV genotype, HBeAg status, or race; similarly, there was no statistical difference in HBeAg seroconversion rates between genotypes in these patients.

To date, the impacts of HBV genotype on the therapeutic response and emergence of drug-resistant variants in patients receiving entecavir or other nucleotide analogues at the stage of clinical trials have not yet been reported.

CONCLUSIONS

Differences in response to therapy with antiviral agents, including interferon alpha and lamivudine, do exist among different HBV genotypes. Genotype determination could be helpful for predicting the outcome of therapy in patients with chronic hepatitis B. However, we have to be aware that the genotype is not the only factor that determines whether a patient should be treated. In Asian countries, for example, the most prevalent genotype, genotype C, is associated with progressive liver disease; spontaneous remission of HBV infection occurs rarely in patients who chronically carry this

genotype [18]. Patients with genotype C often need to be treated with an antiviral agent, such as interferon alpha or lamivudine (which are currently available), even though the therapeutic response is expected to be poor. Further studies are needed to clarify the efficacy of new antiviral agents in patients with various genotypes.

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