Table 5 Summary of HBV DNA in serum samples and liver tissues

Case no.	Serum samples		Liver tissues							
	HBc Ab	Surface	HCC tissues				Non-cancerous tissues			
			Surface	X	Core	cccDNA	Surface	X	Core	cccDNA
1	−	−	−	+	+	−	−	+	−	−
2	+	−	−	+	−	−	−	+	−	−
3	+	−	+	−	−	−	+	+	+	+
4	−	−	−	−	−	−	−	−	−	−
5	−	−	+	−	−	−	+	+	+	−
6	−	−	−	+	−	−	+	+	−	−
7	−	−	−	−	−	−	−	−	+	−
8	−	+	−	+	−	−	−	−	−	−
9	−	−	−	−	−	−	+	−	−	−
10	−	−	+	+	+	−	+	+	+	+
11	−	−	−	+	−	−	+	+	+	−
12	−	−	+	+	+	−	+	+	−	−
13	−	−	+	+	−	−	+	+	−	−
14	−	−	+	+	−	−	+	−	−	−
15	+	−	+	−	−	+	+	+	+	−
16	−	−	+	+	+	−	−	−	+	−
17	−	−	−	−	−	−	−	−	−	−
18	−	−	−	−	−	−	+	+	−	−
19	−	−	+	+	+	−	−	+	−	−
20	−	−	−	+	−	−	−	−	−	−

Ab, antibody; core, HBV C region; HBc, hepatitis B core; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; surface, HBV S region; X, HBV X region.

diabetes mellitus showed a slight degree of fibrosis. Positivity of serum HBc, alcohol abuse and diabetes mellitus may be an additive factor for liver fibrosis.

In patients with chronic viral hepatitis, HCC mostly occurs within liver cirrhosis. Liver cirrhosis results in liver failure, portal hypertension and increased risk of carcinogenesis.[21] In this study, non-cancerous liver tissues of 129 patients with non-B, non-C HCC examined by the LCSK showed mild fibrosis and inflammation. We consider that patients without chronic viral hepatitis may have another risk factor for HCC apart from liver fibrosis.

It has been suggested that NASH may account for a substantial portion of cryptogenic HCC cases.[22] In this study, 129 patients with non-B, non-C HCC examined by the LCSK included 28 patients with NAFLD. Of these, diabetes mellitus was present in 18 (64%) patients. NAFLD has been widely accepted as a possible etiological factor in the development of non-B, non-C HCC; because of the fact that diabetes mellitus was common in patients with NAFLD, insulin resistance may further facilitate the development of HCC.

In addition, there were 96 patients (74%) with LCC of the 129 patients with non-B, non-C HCC examined by the LCSK in this study. LCC was observed in 52 of the 61 patients (85%) with serum HBc antibody, and 44 of the 68 patients (65%) without serum HBc antibody. LCC is characterized by individually scattered or clusters of hepatocytes with atypia, measuring less than 1 mm in diameter, which do not form circumscribed nodules, and have been often found in chronic liver disease.[23,24] LCC is recognized under the microscope as the foci of cellular enlargement and nuclear pleomorphism, hyperchromasia and multinucleation. Although LCC is frequently found in various liver diseases and easily recognized even under low-power magnification due to the characteristic cytological features, its pathological significance is still under debate. LCC has been reported to be observed in various liver diseases such as autoimmune hepatitis, alcoholic cirrhosis and cholestatic liver, although it is more prevalent in HBV-related chronic liver disease.[24–26] It was reported that LCC in HBV-related chronic liver disease demonstrated molecular characteristics different from that in chronic

cholestasis.[27] We also confirmed in this study that HBV DNA was detected in 15 HCC tissues (75%) and 16 non-cancerous liver tissues (80%) obtained from 20 patients with non-B, non-C HCC, although only three of the 20 patients were positive for serum HBc antibody. Based on these findings, we consider that occult HBV infection may be present in a considerable number of patients without serum HBc antibody and occult HBV infection is the major risk factor for the development of HCC in patients without chronic viral hepatitis in the northern area of Kyushu, Japan. Recently, a relationship between the incidence of non-B, non-C HCC and occult HBV infection has been reported.[28–30] The HBV genome persists in a high percentage of HBs antigen negative patients, with and without antibodies against the virus, who develop HCC.[28] It was reported that HBV DNA was detectable in a high proportion of HCC patients without HBs antigen in Japan.[29,30]

In conclusion, the results obtained in the present study indicate that non-cancerous liver tissues of patients with non-B, non-C HCC showed mild fibrosis and inflammation, although serum HBc antibody, alcohol abuse and diabetes mellitus may be additional factors for liver fibrosis. In addition, HBV DNA was detectable in a high proportion of patients with non-B, non-C HCC. Further study of the interaction between HBV DNA in liver tissues and cellular protein will contribute to understanding the involvement of HBV in the HCC of unknown etiology.

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Clinicopathologic Analysis of Combined Hepatocellular-Cholangiocarcinoma According to the Latest WHO Classification

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Abstract: Combined hepatocellular-cholangiocarcinoma comprises < 1% of all liver carcinomas. The histogenesis of combined hepatocellular-cholangiocarcinoma has remained unclear for many years. However, recent advances in hepatic progenitor cell (HPC) investigations have provided new insights. The concept that combined hepatocellular-cholangiocarcinoma originates from HPCs is adopted in the chapter "combined hepatocellular-cholangiocarcinoma" of the latest World Health Organization (WHO) classification. In this study, we conducted clinicopathologic analysis of combined hepatocellular-cholangiocarcinoma according to the latest WHO classification. Fifty-four cases were included in this study. Pathologic diagnosis was made according to the WHO classification. When a tumor contained plural histologic patterns, predominant histologic pattern (≥ 50%) was defined. Minor histologic patterns were also appended. Immunohistochemical staining with biliary markers (CK7, CK19, and EMA), hepatocyte paraffin (HepPar)-1, HPC markers (CD56, c-kit, CD133, and EpCAM), and vimentin was performed. Forty-five and 50 patients were analyzed for progression-free survival and overall survival, respectively. Ten, 1, 32, and 11 cases were diagnosed as: combined hepatocellular-cholangiocarcinoma, classical type; combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype; combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype; and combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular type, respectively. Combined hepatocellular-cholangiocarcinomas usually have high expression of biliary markers. CD56, c-kit, and EpCAM were expressed to various degrees in all combined hepatocellular-cholangiocarcinomas apart from the hepatocellular carcinoma component of combined hepatocellular-cholangiocarcinoma, classical type. The expression of CD133 and vimentin was observed only in combined hepatocellular-cholangiocarcinoma, stem cell features of intermediate cell subtype and cholangiolocellular subtype. The expression of CD133, EpCAM, and vimentin was significantly high in combined hepatocellular-cholangiocarcinoma, subtypes with stem cell features, especially cholangiolocellular subtype. Minor histologic patterns were significantly frequent in combined hepatocellular-cholangiocarcinoma, subtypes with stem cell features, compared with combined hepatocellular-cholangiocarcinoma, classical type. There was no significant difference in clinical outcome between each subtype. Combined hepatocellular-cholangiocarcinoma has wide histologic diversity and shows immunophenotypic expression of not only biliary markers but also HPC markers to various degrees, suggesting that the histogenesis of combined hepatocellular-cholangiocarcinoma could be strongly associated with HPCs. Our results pathologically validate the latest WHO classification of combined hepatocellular-cholangiocarcinoma. However, the complex mixture of histologic subtypes has presented a challenge to the classification of combined hepatocellular-cholangiocarcinoma. Further study should be conducted using a large cohort to support this classification.

Key Words: combined hepatocellular-cholangiocarcinoma, WHO classification, immunohistochemical stain

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With the recent advances in molecular biology, cancer stem cell theory has been widely accepted in not only hematopoietic neoplasms but also solid neoplasms.[1–3] Cancer stem cells are generally defined on the basis of 4 criteria: high efficiency self-renewal, differentiation along at least 2 independent lineages, resistance to conventional genotoxic therapy, and capacity to establish and recapitulate the original tumor.[4] Liver cancers, including hepatocellular carcinoma (HCC), combined

hepatocellular-cholangiocarcinoma, and cholangiocellular carcinoma, are thought to originate from hepatic progenitor cells (HPCs; also referred to as oval cells, tumor-initiating stem-like cells).[5–8] HPCs are liver-specific adult stem cells that are activated when mature hepatocytes and/or cholangiocytes are damaged. HPCs have bipotential: they are capable of differentiation into either hepatocytes or cholangiocytes.[9–11] Although no markers for putative HPCs have yet been generally accepted, CD133, CD90, CD44, epithelial cell adhesion molecule (EpCAM), OV6, CD13, and c-kit are thought to be candidates for HPC markers.[12–23]

The vast majority of malignant primary liver cancer is HCC. Combined hepatocellular-cholangiocarcinoma is relatively rare, comprising <1% of all liver carcinomas.[24] The concept that combined hepatocellular-cholangiocarcinoma originates from HPCs is adopted in the chapter "combined hepatocellular-cholangiocarcinoma" of the latest World Health Organization (WHO) classification of the digestive system. According to the WHO classification, combined hepatocellular-cholangiocarcinoma is divided into classical type and subtypes with stem cell features. Moreover, the latter is subdivided into typical subtype, intermediate cell subtype, and cholangiolocellular subtype. The histologic features of each subtype are shown in Table 1. In our present study, we reassessed 54 cases previously diagnosed as combined hepatocellular-cholangiocarcinoma or cholangiolocellular carcinoma according to the latest WHO classification, by morphologic observation and using immunohistochemical (IHC) stains of hepatocellular, biliary/HPC markers and vimentin.

MATERIALS AND METHODS

Patients

Fifty-four cases previously diagnosed as combined hepatocellular-cholangiocarcinoma or cholangiolocellular carcinoma were reevaluated in this study. For diagnosis, we used the classification by Goodman et al[25] and Kim et al[26] for combined hepatocellular-cholangiocarcinoma and that by Stainer et al[27] for cholangiolocellular carcinoma. All cases were resected at Kurume University Hospital between 1993 and 2010. Two previously reported cases were included in this study.[6,9] Liver specimens were fixed in 10% buffered formalin, followed by paraffin embedment. We cut consecutive 4-μm-thick sections and stained them with hematoxylin and eosin and mucicarmine.

Pathologic diagnosis was performed according to the 2010 WHO classification as described before. When a tumor contained plural histologic patterns, predominant histologic pattern (≥ 50%) was defined. Minor histologic components were also appended. All slides were evaluated by 2 pathologists (J.A. and O.N.). Clinical follow-up data were available for progression-free survival (PFS) (554 ± 814 d) in 45 patients and overall survival (OS) (757 ± 828 d) in 50 patients.

This study was approved by the ethical committee of Kurume University (approved #10294).

IHC Stain

We performed IHC analysis on paraffin-embedded sections using the following antibodies: cytokeratin (CK) 7 (OV-TL 12/30; Dako, Glostrup, Denmark), CK19 (BA1; Dako), epithelial membrane antigen (EMA) (E29; Dako), hepatocyte paraffin (HepPar)-1 (OCH1E5; Dako), CD56 (1B6; Novocastra, Newcastle, UK), c-kit (K963; IBL, Fujioka, Japan), CD133 (AC133, Mileni Biotec, Auburn, CA), EpCAM (HEA125, abcam, Cambridge, UK), and vimentin (V-9; Immunon, Pittsburgh, PA). IHC staining was performed using the streptavidin-biotin-peroxidase method. Immunoreactivity was evaluated with grading from 0 to 4 as follows according to the distribution area of positive cells regardless of the staining intensity: 0, no staining; 1, 1% to 5% positive cells; 2, 6% to 25% positive cells; 3, 26% to 50% positive cells; and 4, >50% positive cells. Equivocal reaction was regarded as negative. The HCC component and the cholangiocarcinoma (ChC) component of combined hepatocellular-cholangiocarcinoma, classical type, were evaluated separately.

TABLE 1. Histologic Features of Combined Hepatocellular-Cholangiocarcinomas According to WHO Classification

Subtypes	Mucin Production	Stroma	Histologic Findings
CHC-classical			
HCC component	None	Scarce	Typical HCC, well to poorly differentiated type
ChC component	Observed	Intermediate-abundant	Typical adenocarcinoma, well to poorly differentiated type
CHC-SC-typical	None	Abundant	Nests of mature looking hepatocytes with peripheral clusters of small cells that have a high nucleus:cytoplasm ratio and hyperchromatic nuclei
CHC-SC-int	None	Intermediate-abundant	Tumor cells show features intermediate between hepatocytes and cholangiocytes. These tumor cells show strands, solid nests and/or trabeculae of small, uniform cells with scant cytoplasm and hyperchromatc nuclei
CHC-SC-CLC	None	Abundant	Tumor is composed of admixtures of small monotonous glands, antler-like anastomosing patterns. Each tumor cell is cuboidal, smaller in size than normal hepatocytes, with high nucleus:cytoplasm ratio, and distinct nucleoli

CHC indicates combined hepatocellular-cholangiocarcinoma; CHC-SC-typical, combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype; CHC-SC-int, combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype; CHC-SC-CLC, combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype.

Statistical Analysis

The association between the HCC or ChC component of combined hepatocellular-cholangiocarcinoma, classical type, and combined hepatocellular-cholangiocarcinoma, subtypes with stem cell features, was examined by the Fisher exact test. PFS was defined as the period from the date of resection to that of progressive disease or death. OS was defined as the period from the date of resection to that of death due to any cause. Kaplan-Meier analysis and the log rank test were used to demonstrate and compare survival curves. Differences were considered significant at $P < 0.05$. Analysis was performed using SAS version 9.2 (SAS Institute Inc., NC) and R version 2.9.0 (R Project Team).

RESULTS

Clinicopathologic Analysis

Of 54 cases, 10, 1, 32, and 11 were diagnosed as: combined hepatocellular-cholangiocarcinoma, classical type; combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype; combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype; and combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular type, respectively. Combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype, was excluded from statistical analysis because of the limited number of cases. Clinicopathologic findings of each histologic type are summarized in Table 2. There was no statistical difference in the age of the patients or tumor size among all histologic types. Men were dominant in each histologic type. Patients with hepatitis B virus (HBV) or/and hepatitis C virus (HCV) infection were frequently observed in combined hepatocellular-cholangiocarcinoma. No significant difference was found in the ratio of hepatitis virus infection among all histologic types. The ratio of cases presenting vascular invasion was not significant between combined hepatocellular-cholangiocarcinoma, classical type, and combined hepatocellular-cholangiocarcinoma, subtypes of stem cell features. The ratio of cases with minor histologic components was significantly high in combined hepatocellular-cholangiocarcinoma, subtypes with stem cell features, compared with combined hepatocellular-cholangiocarcinoma, classical type ($P < 0.01$).

Histologic and IHC Findings

Representative morphologic and IHC features of each histologic type are described in Figures 1A–D. Comparisons of IHC scores among HCC or ChC components of combined hepatocellular-cholangiocarcinoma, classical type, combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype, and combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype, are shown in Tables 3–5. Combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype, was excluded from statistical analysis because of the limited number of cases. The HCC component of combined hepatocellular-cholangiocarcinoma, classical type, had significantly higher expression of HerPar-1 compared with that of combined hepatocellular-cholangiocarcinoma, subtypes with stem cell features. In contrast, this component had statistically lower expression compared with the others, apart from c-kit (Table 3). The IHC score of CD133, EpCAM, and vimentin in combined

TABLE 2. Clinicopathologic Findings of Each Histologic Type of Combined Hepatocellular-Cholangiocarcinomas

	CHC-classical	CHC-SC-typical	CHC-SC-int	CHC-SC-CLC
Number	10	1	32	11
Age	67.6 ± 11.0	57	65.3 ± 10.8	67.8 ± 8.3
Sex (M/F)	9/1	0/1	27/5	9/2
Etiology				
NBNC	2	1	9	8
HBV	3	0	9	2
HCV	6	0	14	1
Background liver tissue				
Chronic hepatitis	9	1	18	10
Liver cirrhosis	1	—	13	—
Others	—	—	1	1
Tumor size (mm)	45.6 ± 35.3	30	41.2 ± 23.5	38.3 ± 17.0
Vascular invasion				
Portal vein	9	1	22	6
Hepatic vein	0	0	2	2
Minor component				
HCC-like	—	1	15	5
ChC	—	1	6	1
int-like	1	—	—	—
CLC-like	1	—	1	—

One patient with coinfection of HBV and HCV was included in CHC-classical.
The ratio of cases presenting vascular invasion was not significant between CHC-classical and CHC-SC subtypes (χ^2 test: NS).
The ratio of the cases with minor components was significantly high in CHC-SC subtypes compared with CHC-classical (χ^2 test: $P < 0.01$).
CHC-classical indicates combined hepatocellular-cholangiocarcinoma, classical type; CHC-SC-typical, combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype; CHC-SC-int, combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype; CHC-SC-CLC, combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype; NBNC, non-HBV non-HCV.

hepatocellular-cholangiocarcinoma, subtypes with stem cell features, was significantly high compared with the ChC component of combined hepatocellular-cholangiocarcinoma, classical type (Table 4). Combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype, had a significantly high expression of CK19 compared with the ChC component of combined hepatocellular-cholangiocarcinoma, classical type (Table 4). Moreover, the IHC score of CD133, EpCAM, and vimentin in combined hepatocellular-cholangiocarcinoma,

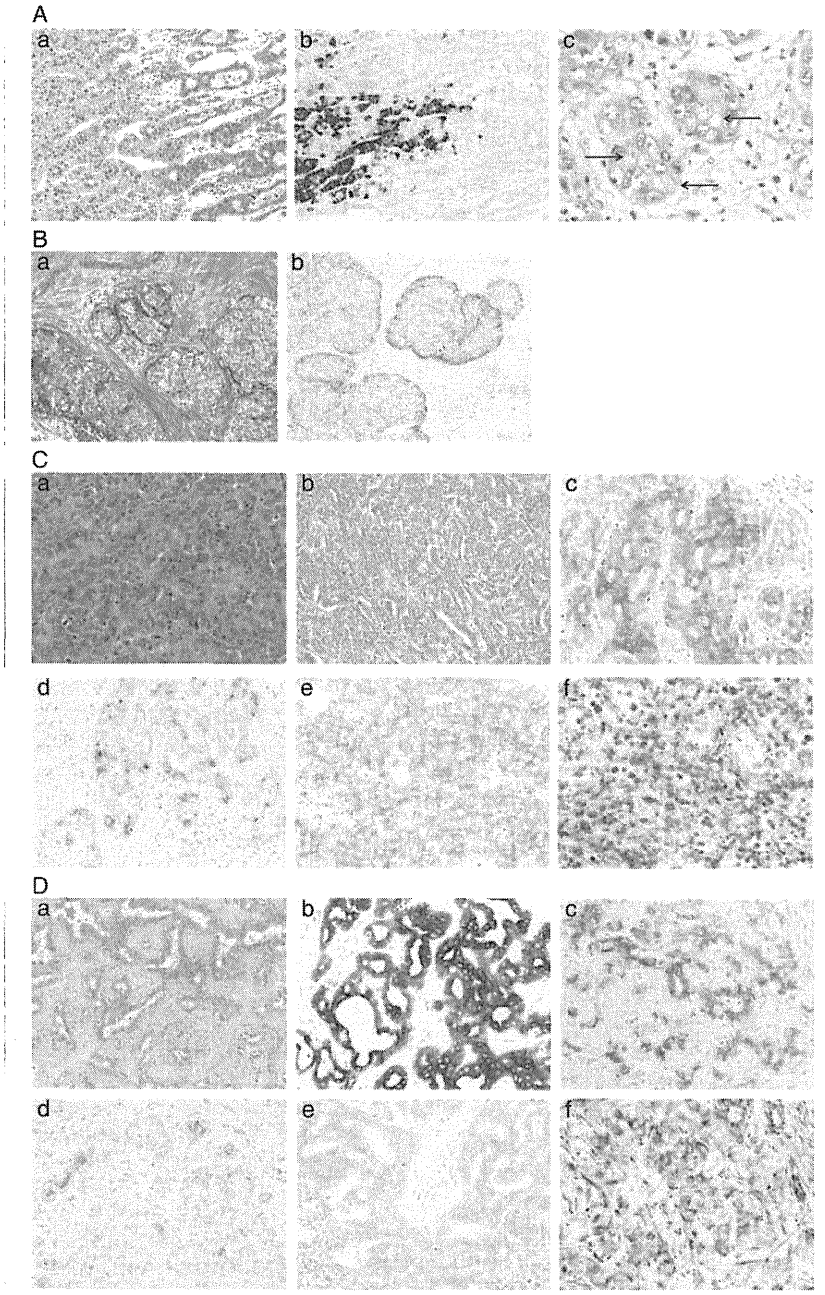

FIGURE 1. (*continued*)

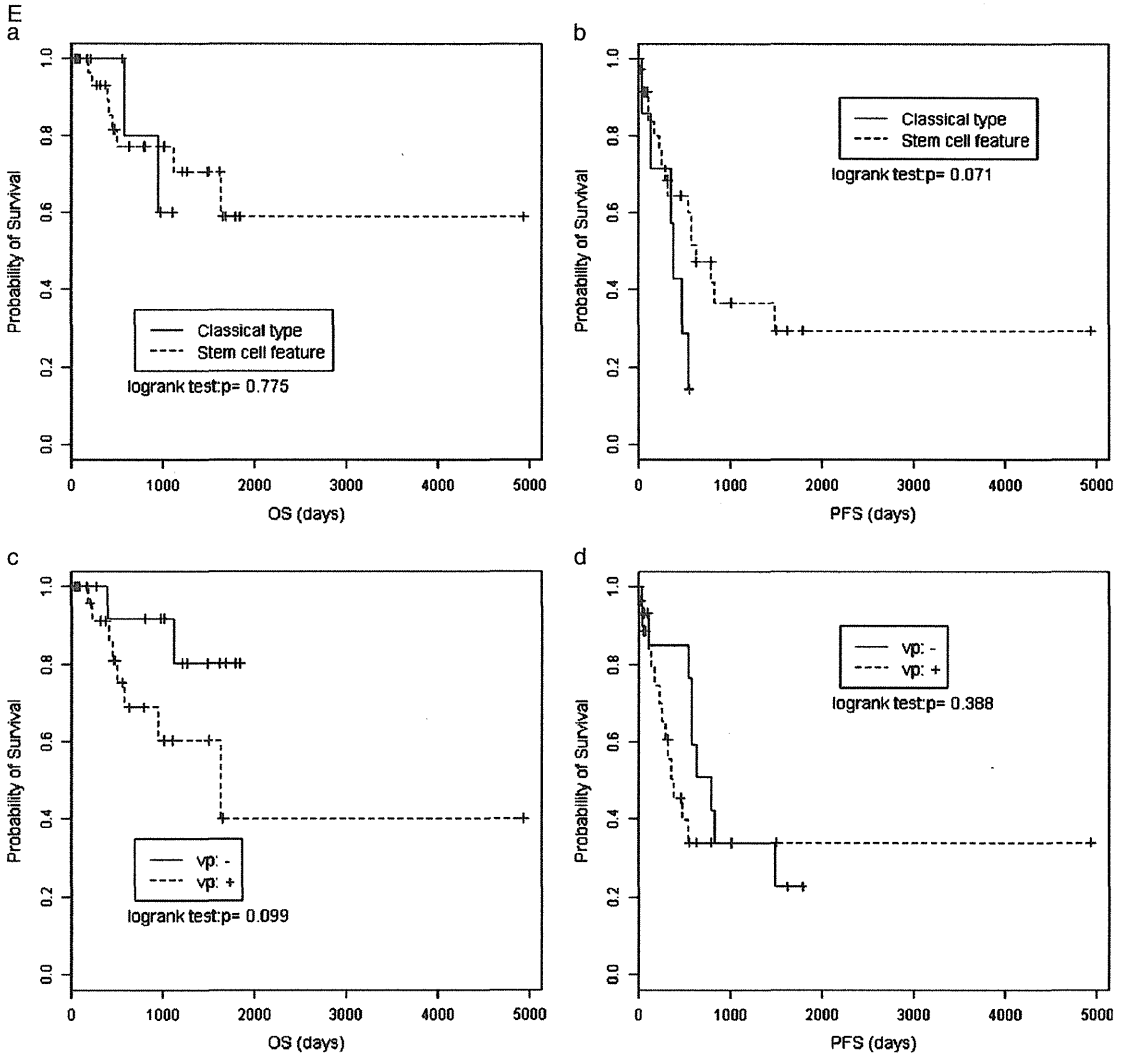

FIGURE 1. A, Representative microscopic findings of combined hepatocellular-cholangiocarcinoma, classical type. The HCC (left) and ChC (right) components were contiguous with transitional features at the boundary (a). Immunochemical stain for HerPar-1 was positive only in the HCC component (b). Mucin production was observed in the lumina surface of small glands of the ChC component (c: arrows, mucicarmine stain). B, Microscopic findings of combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype. The tumor showed a nested growth pattern with peripheral clusters of small cells that had a high nucleus:cytoplasm ratio in the sclerotic stroma (a). EpCAM showed a predominantly circumferential staining pattern (b). C, Representative microscopic findings of combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype. The tumor was composed of small, oval-shaped cells with a trabecular, solid nested (a) or ill-defined glandular proliferative (b) pattern. CK19 was diffusely positive for tumor cells (c). Tumor cells showed variable staining for CD133 (d), EpCAM (e), and vimentin (f). D, Representative microscopic findings of combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype. The tumor cells showed a tubular structure with marked fibrous stroma (a). Immunostains with CK19 (b) and vimentin (f) showed positive reaction in the cytoplasm at various degrees. CD133 (d) was positive for the lumina surface of glandular structures. The expression of CD56 (c) and EpCAM (e) was observed in the cell surface of tumor cells. E, Kaplan-Meier curve demonstrating that OS and PFS of patients with combined hepatocellular-cholangiocarcinoma, classical type, were not statistically different from those of patients with combined hepatocellular-cholangiocarcinoma, subtypes with stem cell features (a and b). However, combined hepatocellular-cholangiocarcinoma, classical type, tended to show shorter PFS compared with combined hepatocellular-cholangiocarcinoma, subtypes with stem cell features (b). There was no significant difference in OS and PFS between the patients with and without portal vein permeation (c and d). However, cases with portal vein permeation had a tendency to shorter OS compared with cases without portal vein permeation (d).

TABLE 3. HCC Component of Combined Hepatocellular-Cholangiocarcinoma Classical Type, Versus Combined Hepatocellular-Cholangiocarcinoma, Subtypes With Stem Cell Features

		IHC Score					*P*	*P* (Classical
Antibody	WHO	0	1	2	3	4	(Overall)	vs. SCs)
CK7	HCC-comp-classical	3	1	4	0	2	0.001	—
	SC-int	1	2	3	7	19		0.0033
	SC-CLC	0	0	0	0	11		< 0.001
CK19	HCC-comp-classical	9	0	1	0	0	< 0.001	—
	SC-int	3	2	5	5	17		< 0.001
	SC-CLC	2	0	0	0	9		< 0.001
EMA	HCC-comp-classical	10	0	0	0	0	< 0.001	—
	SC-int	1	0	7	3	21		< 0.001
	SC-CLC	0	0	1	1	9		< 0.001
HepPar-1	HCC-comp-classical	0	3	1	1	5	< 0.001	—
	SC-int	23	6	3	0	0		< 0.001
	SC-CLC	11	0	0	0	0		< 0.001
CD56	HCC-comp-classical	10	0	0	0	0	0.0191	—
	SC-int	21	6	3	1	1		0.4228
	SC-CLC	3	2	1	2	3		0.0018
c-kit	HCC-comp-classical	10	0	0	0	0	0.2065	—
	SC-int	3	2	5	5	17		1
	SC-CLC	9	2	0	0	0		0.4762
CD133	HCC-comp-classical	10	0	0	0	0	0.0052	—
	SC-int	27	3	2	0	0		0.7464
	SC-CLC	4	3	1	0	3		0.0067
EpCAM	HCC-comp-classical	10	0	0	0	0	0.0042	—
	SC-int	17	1	4	4	6		0.1772
	SC-CLC	2	0	2	0	7		< 0.001
Vimentin	HCC-comp-classical	10	0	0	0	0	0.0071	—
	SC-int	23	2	2	2	3		0.9214
	SC-CLC	2	2	4	2	1		< 0.001

P-value (overall) was calculated by the Fisher exact test for comparison among HCC component of CHC-classical, SC-int, and SC-CLC.

P-value was for pairwise comparison between HCC component of CHC-classical and SC-int or SC-CLC.

HCC-comp-classical indicates HCC component of combined hepatocellular-cholangiocarcinoma, classical type; SC-int, combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype; SC-CLC, combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype.

stem cell features, cholangiolocellular subtype, was significantly high compared with the ChC component of combined hepatocellular-cholangiocarcinoma, classical type, and combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype (Tables 4, 5).

The HCC component and the ChC component of combined hepatocellular-cholangiocarcinoma, classical type, were evaluated separately. The former component consisted of tumor cells with trabecular and/or pseudoglandular structures, suggesting well-differentiated or moderately differentiated HCC (Fig. 1Aa, left). This component showed significantly high expression of HerPar-1 (Fig. 1Ab), followed by CK7 and CK19. There were no expressions of the other markers. The latter component had an irregular tubular pattern with small-sized to medium-sized lumina (Fig. 1Aa, right). Mucin production was confirmed with mucicarmine stain at the lumina surface (Fig. 1Ac). This component showed high expression of biliary markers and low expression of HerPar-1 (Fig. 1Ab), CD56, c-kit, and EpCAM. No expressions of CD133 and vimentin were observed. In 9 of 10 cases, portal vein permeation was found around the tumor. Tumor casts of the intraportal vein were composed solely of the HCC component in 2 cases and both HCC and ChC components in 7 cases. Two of 10 cases had minor components, each of which was a combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype–like, and combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype–like component.

Only 1 case was classified into combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype. This case was accompanied by the HCC component and the ChC component adjacent to combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype. This tumor showed a nested pattern with peripheral clusters of small cells that had a high N/C ratio and hyperchromatic nuclei. Tumor cells located inside nests mimicked mature hepatocytes and had a

TABLE 4. ChC Component of Combined Hepatocellular-Cholangiocarcinoma Classical Type, Versus Combined Hepatocellular-Cholangiocarcinoma, Subtypes With Stem Cell Features

		IHC Score					*P*	*P* (Classical
Antibody	WHO	0	1	2	3	4	(Overall)	vs. SCs)
CK7	ChC-comp-classical	1	0	1	1	7	0.4029	—
	SC-int	1	2	3	7	19		0.7811
	SC-CLC	0	0	0	0	11		0.0902
CK19	ChC-comp-classical	3	0	1	3	3	0.182	—
	SC-int	3	2	5	5	17		0.3419
	SC-CLC	2	0	0	0	9		0.0418
EMA	ChC-comp-classical	2	1	3	0	4	0.1327	—
	SC-int	1	0	7	3	21		0.0868
	SC-CLC	0	0	1	1	9		0.1081
HepPar-1	ChC-comp-classical	9	1	0	0	0	0.3226	—
	SC-int	23	6	3	0	0		0.5557
	SC-CLC	11	0	0	0	0		0.4762
CD56	ChC-comp-classical	7	2	1	0	0	0.1154	—
	SC-int	21	6	3	1	1		1
	SC-CLC	3	2	1	2	3		0.1702
c-kit	ChC-comp-classical	9	0	1	0	0	0.1327	—
	SC-int	3	2	5	5	17		0.4572
	SC-CLC	9	2	0	0	0		0.4762
CD133	ChC-comp-classical	10	0	0	0	0	0.0052	—
	SC-int	27	3	2	0	0		0.7464
	SC-CLC	4	3	1	0	3		0.0067
EpCAM	ChC-comp-classical	9	0	0	1	0	0.0092	—
	SC-int	17	1	4	4	6		0.3558
	SC-CLC	2	0	2	0	7		< 0.001
Vimentin	ChC-comp-classical	10	0	0	0	0	0.0071	—
	SC-int	23	2	2	2	3		0.9214
	SC-CLC	2	2	4	2	1		< 0.001

P-value (overall) was calculated by the Fisher exact test for comparison among ChC component of CHC-classical, SC-int, and SC-CLC.

P-value was for pairwise comparison between ChC component of CHC-classical and SC-int or SC-CLC.

ChC-comp-classical indicates ChC component of combined hepatocellular-cholangiocarcinoma, classical type; SC-int, combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype; SC-CLC, combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype.

TABLE 5. Combined Hepatocellular-Cholangiocarcinoma Stem Cell Features, Intermediate Cell Subtype Versus Cholangiolocellular Subtype

		IHC Score					
Antibody	**WHO**	**0**	**1**	**2**	**3**	**4**	***P***
CK7	SC-int	1	2	3	7	19	0.2014
	SC-CLC	0	0	0	0	11	
CK19	SC-int	3	2	5	5	17	0.2802
	SC-CLC	2	0	0	0	9	
EMA	SC-int	1	0	7	3	21	0.868
	SC-CLC	0	0	1	1	9	
HepPar-1	SC-int	23	6	3	0	0	0.1758
	SC-CLC	11	0	0	0	0	
CD56	SC-int	21	6	3	1	1	0.0284
	SC-CLC	3	2	1	2	3	
c-kit	SC-int	3	2	5	5	17	0.1191
	SC-CLC	9	2	0	0	0	
CD133	SC-int	27	3	2	0	0	0.0023
	SC-CLC	4	3	1	0	3	
EpCAM	SC-int	17	1	4	4	6	0.0362
	SC-CLC	2	0	2	0	7	
vimentin	SC-int	23	2	2	2	3	0.0069
	SC-CLC	2	2	4	2	1	

P-value was calculated by the Fisher exact test for comparison between SC-int and SC-CLC.

SC-int indicates combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype; SC-CLC, combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype.

partial clear cell change (Fig. 1Ba). Immunohistochemically, biliary markers (CK7, CK19, and EMA), CD56, c-kit, and EpCAM (Fig. 1Bb) were positive for both cells mimicking hepatocytes and peripheral small cells. However, EpCAM showed a predominantly circumferential staining pattern. HepPar-1 was positive only inside the nests.

Thirty-two cases were classified into combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype. This type of tumor showed strands/trabeculae of small, uniform, round to oval cells with scanty cytoplasm and hyperchromatic nuclei (Figs. 1Ca, b). Mucin production was not observed in tumor cells. Most cases had expressions of biliary markers, such as CK7, CK19 (Fig. 1Cc), and EMA. The cases with HepPar-1 and CD56 expression were relatively limited in number. CD133 (Fig. 1Cd) and EpCAM (Fig. 1Ce) expressions were observed in some cases. Vimentin, which is a representative marker of mesenchymal cells, was also positive in some cases (Figs. 1Cf). The tumor cells with vimentin expression showed an epithelial growth pattern and lacked a mesenchymal growth pattern. Fifteen, 6, and 1 case was accompanied by HCC-like, ChC-like, and combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype–like components, respectively, as minor components.

Eleven cases were classified into combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype. These cases demonstrated admixtures of small monotonous glands and antler-like anastomosing patterns embedded within a thick desmoplastic stroma (Fig. 1Da). Mucin production was not observed in tumor cells. All cases had plural biliary marker expressions [CK7, CK19 (Fig. 1Db), and EMA]. HepPar-1 was not positive for tumor cells with combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype. c-kit expression was observed in a relatively limited area. CD56 (Fig. 1Dc), CD133 (Fig. 1Dd), and EpCAM (Fig. 1De) expressions were observed in several cases. Vimentin expression was also observed in some cases (Fig. 1Df). Six cases had minor components. Five of them were accompanied by an HCC-like component, and 1 of them was accompanied by a ChC-like component.

Clinical and Follow-up Data

There was no significant difference in clinical outcome between combined hepatocellular-cholangiocarcinoma of classical type and subtypes with stem cell features (Figs. 1Ea, b) and between combined hepatocellular-cholangiocarcinoma, stem cell features of intermediate cell subtype and cholangiolocellular subtype. However, combined hepatocellular-cholangiocarcinoma, classical type, tended to show shorter PFS compared with combined hepatocellular-cholangiocarcinoma, subtypes with stem cell features (Fig. 1Eb). There was no statistical difference in OS and PFS between the patients with and without portal vein permeation (Fig. 1Ec, d). However, cases with portal vein permeation had a tendency to shorter OS compared with cases without portal vein permeation (Fig. 1Ec).

DISCUSSION

Combined hepatocellular-cholangiocarcinoma was reported to be a primary malignant liver tumor by Wells in 1903. Allen and Lisa[10] subclassified combined hepatocellular-cholangiocarcinoma into mixed and combined type. In 1985, Goodman et al[25] classified combined hepatocellular-cholangiocarcinoma into collision type (type I), transitional tumor (type II), and fibrolamellar tumor (type III). Moreover, Taguchi et al[28] classified combined hepatocellular-cholangiocarcinoma into 3 histologic types according to the combination pattern of HCC and ChC elements and also the presence of the transitional features of both elements. Although these classifications have been proposed, the histogenesis of combined hepatocellular-cholangiocarcinoma had remained unclear for many years. However, recent advances of HPC investigations have provided new insight. It has been shown that murine mature polypoid hepatocytes have a stem cell–like regenerative capacity and that even human hepatocytes are highly regenerative.[29] The adult liver harbors facultative biopotential progenitors that give rise to an intermediary cell type, described as oval cells, which are thought to differentiate into both biliary epithelium and hepatocytes.[30] Oval cells have been linked to the subsequent development of hepatic malignancies in animal models.[5,8] The existence of human cell populations that have similar characteristics to animal oval cells has been confirmed, and they have been termed HPCs.[7] HPCs have been identified in several pathologic liver conditions, such as

hepatitis, cirrhosis, focal nodular hyperplasia, and hepatocellular adenoma.[31–34] As HPCs have bipotential—that is, being capable of differentiation into either hepatocytes or cholangiocytes[31,35,36]—the hypothesis that combined hepatocellular-cholangiocarcinoma is derived from HPCs is easily acceptable.[37]

Combined hepatocellular-cholangiocarcinoma, classical type, corresponds with type I of Goodman's classification.[25] Ten cases were classified into this type of tumor in our serial cases. Combined hepatocellular-cholangiocarcinoma, classical type, is composed of a typical HCC area and a typical ChC area. The possible histogenesis is as follows: (i) HCC and ChC arise independently and separately; (ii) HCC or ChC arises first and transforms to ChC or HCC, respectively, in various degrees; and (iii) malignant transformation of HPCs occurs, and they differentiate completely and incompletely to HCC and ChC.

Combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype, is newly adopted in the latest WHO classification. Originally, this type of tumor was reported by Theise et al.[11] Subsequently, Fujii et al[38] reported 21 cases of scirrhous HCCs, stem cell features. They described small tumor cells located at peripheral tumor nests that had a similar characteristic with side population cells isolated from HCC cell lines Huh7 and PLC5. Moreover, Ki-67 labeling index was higher in small tumor cells located at the periphery of tumor cell nests than in the central part of tumor cells mimicking HCC cells.[34] Although tumor cells of HCC or combined hepatocellular-cholangiocarcinoma adjacent to sclerotic stroma sometimes show small ductular proliferation, the Ki-67 labeling index of the reactive ductular epithelium under a pathogenic condition is generally much less than that of small tumor cells located at the periphery of tumor cell nests.[38,39] However, only 1 case was classified into combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype, in our present study. A case with typical features showing combined hepatocellular-cholangiocarcinoma, stem cell features, typical subtype, might be rare. Further studies should be conducted to clarify the clinicopathologic significance of this tumor.

The subtype of combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype, corresponds to cases previously reported as liver carcinoma of the intermediate (hepatocyte-cholangiocyte) phenotype.[26] Tumor cells are morphologically and immunophenotypically composed of intermediate cells between HCC and ChC. The histogenesis is thought to be transformed in HPCs. In our present study, 32 cases were classified into combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype. Tumor cells immunochemically show the positive reaction of not only biliary markers but also HPC markers. Moreover, vimentin, which is a representative mesenchymal marker, was also positive in some cases. Nakanuma et al[40] reported that peripheral ChC is divided into ductular type and duct type. The former had histologic resemblance to reactive bile ductules and showed expression of CD56 and vimentin.[40,41] Furthermore, they mentioned that the ChC component of combined hepatocellular-cholangiocarcinoma shared the features of the ductular type of ChC. These findings strongly support the hypothesis that combined hepatocellular-cholangiocarcinoma originates from HPCs. However, the precise mechanism and significance of the expression of the mesenchymal marker vimentin are still elusive.

Cholangiolocellular carcinoma was categorized as a subtype of ChC on the basis of the previous WHO classification. In the latest WHO classification, cholangiolocellular carcinoma is classified into combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype. Cholangiolocellular carcinoma was originally reported by Steiner and Higginson in 1959.[27] As cholangiolocellular carcinoma is a rare malignant liver tumor accounting for 0.56% to 1% of all primary liver cancer cases, serial reports are limited in number.[6,9,27] Although cholangiolocellular carcinoma is thought to originate from canals of Hering, Komuta et al[6] provided reliable evidence that cholangiolocellular carcinoma originates from HPCs. IHC findings in our study were similar to those in previous reports. The expression of vimentin was also confirmed in combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype, and in combined hepatocellular-cholangiocarcinoma, stem cell features, intermediate cell subtype. Collectively, these results strongly suggest that combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype, originates from HPCs.

Thirty-four (63%) of 54 cases were associated with HBV and/or HCV infection. Previous reports on combined hepatocellular-cholangiocarcinoma also showed that 58% to 93% cases were affected by HBV and/or HCV infection.[26,28,37,42] Komuta and colleagues reported that 30% of cases of cholangiolocellular carcinoma were infected with HBV and/or HCV.[17] The findings of previous reports and our present study support the hypothesis that chronic hepatitis virus infection might be associated with various types of combined hepatocellular-cholangiocarcinoma. However, currently the precise mechanisms of the carcinogenesis of combined hepatocellular-cholangiocarcinoma and chronic hepatic damage due to HBV and/or HCV remain unclear.

Several reports document combined hepatocellular-cholangiocarcinoma as an aggressive tumor with poor outcome, compared with HCC.[28,43–47] In this study, no significant differences were found in patient outcome between combined hepatocellular-cholangiocarcinoma, classical type, and combined hepatocellular-cholangiocarcinoma, subtypes with stem cell features. Although vascular invasion, satellite lesion, number of tumors, and tumor size were listed as worse prognostic factors in combined hepatocellular-cholangiocarcinoma,[43,44,47] there was no significant difference in patient outcome between the cases with and without portal vein permeation. The insufficient number of cases and the diversity of stages at diagnosis or postoperative treatment made it difficult to make any

generalizations about the clinical outcome. Moreover, as we did not compare the outcome among combined hepatocellular-cholangiocarcinoma and other primary liver tumors, it might be difficult to address the aggressiveness of combined hepatocellular-cholangiocarcinoma in this study.

Our results validate the latest WHO classification of combined hepatocellular-cholangiocarcinoma with regard to morphologic features and immunophenotypes. However, clinical aspects, including the prognosis, of each subtype remain unknown as described in the WHO classification.[24] To enforce this classification, combined hepatocellular-cholangiocarcinomas should be strictly categorized. In the present study, we defined the predominant histologic pattern ($\geq 50\%$) as its pathologic diagnosis for the sake of expedience when a tumor contained plural histologic patterns. However, there is no definitive description in the WHO classification that the tumor amount should be reflected in pathologic diagnosis and handled as a minor histologic component. The description that HCC-like and/or ChC-like areas are frequently present at the periphery of combined hepatocellular-cholangiocarcinoma, stem cell features, cholangiolocellular subtype, is seen in the WHO classification. In fact, minor histologic components were observed in 31 (57.4%) of 54 cases of combined hepatocellular-cholangiocarcinoma. The ratio of having minor histologic components was significantly higher in combined hepatocellular-cholangiocarcinoma, subtypes with stem cell features, compared with combined hepatocellular-cholangiocarcinoma, classical type, in this study (Table 2). At present, the clinicopathologic significance of the minor component is unclear. However, recently Komuta et al[48] reported that intrahepatic cholangiocarcinoma (ICC) with histologic diversity (hepatic differentiation area and/or ductular areas) tended to show better prognosis compared with ICC without histologic diversity. In addition, they mentioned that ICC with histologic diversity had a similar molecular profile to cholangiolocellular carcinoma. We showed that combined hepatocellular-cholangiocarcinoma was usually composed of a complex heterogenous mixture of histologic subtypes in this study. Therefore, describing all components that may be present might be a tentative method at present while the significance of the minor component remains elusive.

In conclusion, combined hepatocellular-cholangiocarcinoma is a neoplasm with wide histologic diversity and shows immunophenotypic expression of not only biliary markers but also HPC markers to various degrees, indicating that the histogenesis of combined hepatocellular-cholangiocarcinoma could be strongly associated with HPCs. Our results practically validate the latest WHO classification of combined hepatocellular-cholangiocarcinoma with regard to pathologic features. However, the complex mixture of histologic subtypes has presented a challenge to the classification of combined hepatocellular-cholangiocarcinoma. Further expanded studies using a large cohort should be conducted to confirm the utility of this classification.

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Prediction of early HBeAg seroconversion by decreased titers of HBeAg in the serum combined with increased grades of lobular inflammation in the liver

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
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Summary

Background: Hepatitis B e antigen (HBeAg) seroconversion is an important hallmark in the natural course of chronic hepatitis B. This study was designed to predict early HBeAg seroconversion within 1 year, by not only biochemical and virological markers, but also pathological parameters in patients with chronic hepatitis B.

Material/Methods: In a retrospective cohort study, 234 patients with HBeAg were reviewed for demographic, biochemical, virological and pathological data at the time of liver biopsy. Then, the patients who accomplished HBeAg seroconversion within 1 year thereafter were compared with those who did not, for sorting out factors predictive of early HBeAg seroconversion.

Results: Early HBeAg seroconversion occurred in 58 (24.8%) patients. In univariate analysis, factors predictive of early HBeAg seroconversion were: alanine aminotransferase (ALT) (p=0.002), IP-10 (p=0.029), HBsAg (p=0.003), HBeAg (p<0.001), HBV DNA (p=0.001), HBcrAg (p=0.001), core-promoter mutations (p=0.040), fibrosis (p=0.033) and lobular inflammation (p=0.002). In multivariate analysis, only serum HBeAg levels <100 Paul Ehrlich Institute (PEI) U/ml and grades of lobular inflammation >2 were independent factors for early HBeAg seroconversion (odds ratio 8.430 [95% confidence interval 4.173–17.032], p<0.001; and 4.330 [2.009–9.331], p<0.001; respectively).

Conclusions: HBeAg levels < 100 PEIU/ml combined with grades of lobular inflammation >2 are useful for predicting early HBeAg seroconversion. In patients without liver biopsies, high ALT levels (>200 IU/L) can substitute for lobular inflammation (grades >2).

key words: alanine aminotransferase • chronic hepatitis • hepatitis B virus • hepatitis B e antigen • lobular inflammation • seroconversion

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Background

Worldwide, an estimated 350 million people are infected with hepatitis B virus (HBV) persistently [1,2]. HBV infection is a major global concern, because up to 40% of patients can develop grave complications, such as decompensated cirrhosis and hepatocellular carcinoma (HCC) [3]. In the natural course of chronic hepatitis B, HBeAg seroconversion, defined by the loss of HBeAg and development of the corresponding antibody (anti-HBe), is an important hallmark, because it is highly correlated with a favorable long-term outcome. Seroconversion is usually followed by sustained suppression of HBV DNA, normalization of alanine aminotransferase (ALT) levels, and clinical remission accompanied by ameliorated necro-inflammatory activities in the liver [4–6].

To date, a number of factors have been found to predispose patients to spontaneous HBeAg seroconversion [7–19]. However, few studies have evaluated pathological factors for predicting early HBeAg seroconversion. In a small series of patients from Spain, the Knodell's index of histological activity was one of the independent predictors of early HBeAg seroconversion [14]. Recently, novel markers of the replication of HBV were introduced, such as levels of HBsAg, HBeAg and HBcrAg (HBV core-related antigen), which can replace HBV DNA levels. These serological markers of HBV replication have been evaluated for sensitive and reliable prediction of early HBeAg seroconversion [20–23]. In the present study, an attempt was made to select factors predictive of early HBeAg seroconversion, from among many biochemical, virological and pathological parameters, based on the data of 234 HBeAg-positive patients with chronic hepatitis B.

Material and Methods

Patients and study design

This is a retrospective cohort study with use of stored sera and liver biopsy specimens from patients with chronic hepatitis B who were taken care of in the Hepatology Department, Nagasaki Medical Center, Japan, during 1991 through 2005. The clinical database was reviewed to identify consecutive patients who underwent liver biopsies and had been followed for longer than 1 year. The inclusion criteria were presence of hepatitis B surface antigen (HBsAg) for 6 months or longer, positivity for HBeAg at the time of liver biopsy, and lack of antiviral treatments before receiving liver biopsies. The exclusion criteria were co-infection with hepatitis C virus (HCV) or human immunodeficiency virus type-1, serological markers suggestive of autoimmune disease, daily intake of alcohol >50 g, recent exposure to hepatotoxic drugs, and no stored sera available. They were followed every 3 months or more frequently, if indicated clinically, and their serum samples were monitored for liver biochemistry and serologic markers of HBV infection, including HBsAg, HBeAg, anti-HBe, HBV DNA and HBcrAg. Serum samples had been stored at –20°C until use.

Antiviral therapy was commenced immediately in the patients with: (1) significant fibrosis/cirrhosis detected by liver biopsy; and (2) evidence of decompensation, such as ascites, varices and hepatic encephalopathy.

To identify predictors of early HBeAg seroconversion, clinical, biological, virological and pathological data at the time of liver biopsy were compared between patients who did and who did not achieve early HBeAg seroconversion, within 1 year after receiving liver biopsies, by univariate and multivariate analyses. Further, patients were stratified by independent factors for HBeAg seroconversion, and the cumulative incidence of HBeAg seroconversion was compared between groups using the Kaplan-Meier method. The study protocol complied with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki, and was approved by the review board of the institution. Each patient gave a written informed consent before participating in this study.

Routine laboratory tests for HBV markers

Quantitative measurements of HBsAg and HBeAg were carried out using commercial enzyme-linked immunosorbent assay (ELISA) kits in the ARCHITECT ANALYSER i2000 (Abbott Japan Co., Ltd., Tokyo, Japan) in accordance with the manufactures' instructions in Nagasaki Medical Center. The sensitivity of HBsAg assay ranged from 0.05 to 250 IU/ml. Sera with HBsAg >250 IU/ml were serially diluted 100-fold so as to include them within the dynamic range. HBeAg was quantified by a two-step immunoassay with use of chemiluminescence microparticles. Briefly, undiluted samples were mixed with paramagnetic beads coated with anti-HBe. After a washing step, conjugate and reactants were added for exciting emission of the light that is proportional to the concentration of HBeAg. The result was expressed by the ratio of relative light unit (RLU) of the sample to the cut-off RLU (S/CO). Samples with S/CO values >1.0 were regarded positive for HBeAg. Then, serial dilutions of the reference standard of PE HBeAg (Paul-Ehrlich Institute, Langen, Germany) were used to define the linear range of the assay and create a reference curve for linear regression. The linear range was 0.024–100 PEIU/ml. A standard curve was produced, and linear regression was used to convert assay results into appropriate units (PEIU/ml). For samples that fell outside the linear range of the assay, the assay was performed on serial dilutions to ensure the linearity.

HBV DNA and HBcrAg

HBV DNA was determined by the COBAS Taqman HBV test (Roche Diagnostics K.K., Tokyo, Japan). Values under or over the detection range were recorded as 2.1 or 9.1 log copies/ml. HBcrAg was measured by the CLEIA HBcrAg assay kit (Fujirebio, Inc., Tokyo, Japan) in a fully automated analyzer (Lumipulse system, Fujirebio, Inc.). Values under or over the detection range were recorded as 3.0 or 7.0 log copies/ml. Assays for HBV DNA and HBcrAg were performed in a commercial clinical laboratory (SRL, Inc., Tokyo, Japan). Sera with values over the detection range were diluted to include them within the dynamic range.

Interferon-inducible protein 10 (IP-10)

IP-10 was quantified by the Invitrogen Human IP-10 ELISA (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol in Nagasaki Medical Center.

HBV genotyping

HBV DNA was extracted from serum (100 µl) with use of the SMITEST EX R&D extraction kit (MBL Co., Ltd., Nagoya, Japan). It was amplified for determination of genotypes by

Table 1. Histological evaluation of liver biopsy specimens.

(A) Fibrosis staging	
Stage	**Fibrosis**
0	None
1	Enlarged, fibrotic portal tracts
2	Periportal or portal-portal septa but intact architecture
3	Fibrosis with architectural distortion without obvious cirrhosis
4	Probable or definite cirrhosis

(B) Inflammation grading			
Grade	**Portal/periportal activity**		**Lobular inflammation**
	Piecemeal necrosis	**Lymphocyte aggregation**	
0	None or minimal	None	None
1	Inflammation only	< 1/3 in portal triad	Inflammation alone
2	Mild	1/3–2/3 in portal areas	Focal necrosis or acidphil bodies
3	Moderate	> 2/3 in portal areas	Severe focal cell damages
4	Severe	Entire portal triad	Damage with bridging necrosis

the SMITEST HBV Genotyping Kit (MBL Co., Ltd.) based on hybridization with type-specific probes immobilized on a solid-phase support [24].

Precore stop codon (G1896A) and core promoter (A1762T/G1764A) mutations

A1896 mutation in the precore (PreC) region was detected by the enzyme-linked minisequence assay (SMITEST HBV PreC ELMA, Roche Diagnostics, Tokyo, Japan), and mutations in the core promoter (CP) region for T1762/A1764 by the enzyme-linked specific probe assay (SMITEST HBV Core Promoter Mutation Detection Kit, Roche Diagnostics K.K.). The results were recorded as "the wild-type" and "mutant types" dominantly expressed by HBV isolates [25].

Histological examination

Liver biopsy was taken by fine-needle aspiration (16G sonopsy) guided by ultrasonography. Biopsy specimens were fixed in 10% neutral formalin, cut at 3- to 4-μm thickness, and stained with Hematoxyline-Eosin and Azan-Mallory, as well as for silver to visualize reticuline fibers. Tissue sections were examined independently by two senior liver pathologists. For each biopsy specimen, a protocol was filled out for grading necro-inflammation and staging fibrosis by the criteria of Desmet et al. [26] and Scheuer [27] (Table 1). As for the portal activity, not only piecemeal necrosis, but also lymphocytic aggregation was categorized into 5 (0–4) grades in the respective area involved.

Statistical analysis

Continuous variables were compared between groups by the Mann-Whitney *U* test, and categorical variables by χ^2 and Fisher's exact tests. The cumulative incidence of HBeAg seroconversion was calculated using the Kaplan-Meier method, and the difference was evaluated by the log-rank test. Multiple logistic regression analysis was performed to identify independent factors in significant association with early HBeAg seroconversion. A p value <0.05 was considered significant. Statistical analyses were performed using the SPSS version 17.0 software package (SPSS Inc., Chicago, IL, USA).

Results

Baseline characteristics of patients

Among the 673 patients with HBsAg who had received liver biopsies in our hospital during 1991 through 2005, 234 (34.8%) patients who met the inclusion criteria were enrolled in this study. Demographic and laboratory characteristics at the time of liver biopsy are listed in Table 2. They had a median age of 37 years (range: 12–74), and 161 (69%) were men. Of them, 231 (99%) were infected with HBV of genotype C. The median serum ALT level at the baseline was 141 IU/l (range: 13–2644 IU/l), and the median duration of follow-up was 86.5 months (range: 12.0–213.0 months). During the follow-up, 91 (39%) received antiviral treatment, with interferon (IFN) or lamividine, or the combination thereof.

Comparison of clinical features between patients with and without early HBeAg seroconversion

Early HBeAg seroconversion, within 1 year after receiving liver biopsies, was achieved by 58 of the 234 (24.8%) patients. In univariate analysis, factors predictive of early HBeAg seroconversion were: ALT (p=0.002), IP-10 (p=0.029), HBsAg (p=0.003), HBeAg (p<0.001), HBV DNA (p=0.001), HBcrAg (p<0.001), CP mutations (p=0.040), fibrosis (p=0.033) and lobular inflammation (p=0.002). Other factors including age, albumin, platelets, AFP, PreC mutation, cell infiltration and

Table 2. Baseline characteristics of patients.

Features	Total (n=234)
Demographic data	
Age (years)	37 (12–74)
Men (%)	161 (69)
Biolochemical markers	
Albumin (g/dl)	4.1 (2.5–5.0)
Platelets ($\times 10^3$/mm^3)	179 (43–338)
ALT (IU/l)	141 (13–2644)
AFP (ng/ml)	7 (0–1863)
IP-10 (ng/ml)	214 (66–3253)
Virological markers	
HBV genotypes: A/B/C (%)	1/2/231 (0/1/ 99)
HBsAg (IU/ml)	8039 (2–261647)
HBeAg (PEIU/ml)	245.3 (0.01–3179.7)
HBV DNA (log copies/ml)	7.7 (3.6–8.9)
HBcrAg (log U/ml)	7.8 (5.4–9.2)
PC mutations: wild/mix/ mutant (%)	132/100/2 (56/43/1)
CP mutations: wild/mix/ mutant/others (%)	55/50/126/3 (24/21/54/1)
Pathological features	
Fibrosis stages: 0/1/2/3/4 (%)	15/73/54/38/54 (7/31/23/16/ 23)
Lymphocytic aggregation: 0/1/2/3/4 (%)	6/65/107/45/11 (2/28/46/19/5)
Piecemeal necrosis: 0/1/2/3/4 (%)	59/52/57/58/8 (25/22/24/25/4)
Lobular inflammation: 0/1/2/3/4 (%)	4/91/104/32/3 (2/39/44/14/1)
Antiviral treatments	
Within 1 year of biopsy (%)	91 (39)
Antiviral agents: 1/2/3/4* (%)	44/33/13/1 (49/36/14/1)
Duration of follow up (months)	86.5 (12.0–213.0)

Qualitative variables are expressed in the number with percentage in parentheses, and quantitative variables are expressed in the median with range in parentheses. ALT – alanine aminotransferase; AFP – alpha-fetoprotein; IP-10 – the interferon-gamma inducible protein-10; HBV – hepatitis B virus; HBsAg – hepatitis B surface antigen; HBeAg – hepatitis B e antigen; HBcrAg – hepatitis B virus core-related antigen; PC – precore; CP – core promoter. * 1, Interferon alpha; 2, lamivudine; 3, lamivudine plus interferon-alpha; 4, entecavir.

piecemeal necrosis in the liver, as well as treatments within 1 year after the entry and type of antiviral agents, were not associated with early HBeAg seroconversion (Table 3).

Evaluation of HBV markers for predicting early HBeAg seroconversion

HBV markers were compared for sensitivity and specificity in predicting early HBeAg seroconversion by the receiver operating characteristic analysis (Figure 1). HBeAg at the time of liver biopsy was the best predictor of early HBeAg seroconversion, with the widest area under the curve of 0.750; it was larger than those of HBcrAg (0.708), HBV DNA (0.650) and HBsAg (0.630). Hence, HBeAg was selected as the best HBV marker predictive of early seroconversion. Based on the receiver operating characteristic curve, HBeAg titers were dichotomized by 100 PEIU/ml in the immunoassay.

Independent predictors for early HBeAg seroconversion

A multivariate logistic regression analysis was performed to select independent predictors of early HBeAg seroconversion from among variables significant in the univariate analysis (Table 4). Of all factors, including histological characteristics, HBeAg <100 PEIU/ml and grades ≥2 lobular inflammation remained as independent factors predictive of early HBeAg seroconversion (Table 4A). Of factors exclusive of histological parameters, HBeAg <100 PEIU/ml and ALT ≥200 IU/ml remained as independent factors for early HBeAg seroconversion (Table 4B).

Combinations of two independent factors for predicting early HBeAg seroconversion

Two combinations of independent factors were evaluated for the performance in predicting early HBeAg seroconversion. The patients who had two predictors in combination, HBeAg <100 PEIU/ml and grades ≥2 lobular inflammation, achieved early HBeAg seroconversion in the highest frequency at 66.0% (31/47). In a remarkable contrast, merely 6.9% (4/58) of the patients without either of these predictors achieved early HBeAg seroconversion (Figure 2A).

Likewise, early seroconversion was achieved by 18 of the 30 (60.0%) patients with the other combination of independent factors, exclusive of pathological parameters, HBeAg <100 PEIU/ml and ALT ≥200 IU/l. By contrast, only 6 of the 99 (6.1%) patients without either of them achieved early HBeAg seroconversion (Figure 2B).

Sensitivity, specificity, positive predictive value and negative predictive value of predicting early HBeAg seroconversion are: 74.5% (31/58), 90.9% (160/176), 66.0% (31/47) and 85.6% (160/187), respectively, for the combination of HBeAg <100 PEIU/ml and grades ≥2 lobular inflammation; and 31.0% (18/58), 93.2% (164/176), 60.0% (18/30) and 80.4% (164/204), respectively, for the combination of HBeAg <100 PEIU/ml and ALT ≥200 IU/l.

Long-term clinical outcomes

Besides the 58 patients with early HBeAg seroconversion, an additional 97 patients achieved HBeAg seroconversion during a median follow-up period of 86.5 months. Cumulative

Table 3. Univariate analysis of risk factors for early HBeAg seroconversion.

Variables	Early HBeAg seroconversion		p value
	Achieved (n=58)	Not achieved (n=176)	
Demographic data			
Age (years)	36 (17–69)	37 (12–74)	0.303
Men (%)	41 (71)	120 (68)	0.721
Biolochemical markers			
Albumin (g/dl)	4.1 (2.8–4.8)	4.1 (2.5–5.0)	0.877
Platelets ($\times 10^3/mm^3$)	171 (43–291)	186 (57–338)	0.487
ALT (IU/l)	227 (18–2072)	121 (13–2644)	0.002
AFP (ng/ml)	12 (1–1863)	6 (0–683)	0.070
IP-10 (ng/ml)	259 (77–1743)	204 (66–3253)	0.029
Virological markers			
HBV genotypes A/B/C (%)	0/0/58 (0/0/100)	1/2/173 (1/1/98)	1
HBsAg (IU/ml)	5127 (8–261647)	9033 (2–128511)	0.003
HBeAg (PEIU/ml)	20.9 (0.01–1985.0)	377.1 (0.01–3179.7)	<0.001
HBV DNA (log copies/ml)	7.2 (3.7–8.7)	7.8 (3.6–8.9)	0.001
HBcrAg (log U/ml)	7.2 (5.7–9.2)	8.0 (5.4–9.1)	<0.001
PC mutations: wild/mix/mutant (%)	26/31/1 (45/53/2)	106/69/1 (60/39/1)	0.075
CP mutations: wild/mix/mutant/others (%)	8/9/40/1 (14/15/69/2)	47/41/86/2 (27/23/49/1)	0.040
Pathological features			
Fibrosis stage: 0/1/2/3/4 (%)	1/12/18/14/13 (2/21/31/24/22)	14/61/36/24/ 41 (8/35/20/14/23)	0.033
Lymphocytic aggregation: 0/1/2/3/4 (%)	0/11/27/17/3 (0/19/47/29/5)	6/54/80/28/8 (3/31/45/16/5)	0.087
Piecemeal necrosis: 0/1/2/3/4 (%)	7/12/18/19/2 (12/21/31/33/3)	52/40/39/39/6 (30/23/22/22/3)	0.068
Lobular inflammation: 0/1/2/3/4 (%)	0/13/29/15/1 (0/22/50/26/2)	4/78/75/17/2 (2/44/43/10/1)	0.002
Antiviral treatments within 1 year after biopsy (%)	28 (48)	63 (36)	0.091
Antiviral agents: 1/2/3/4* (%)	18/5/5/0 (64/18/18/0)	26/28/8/1 (41/44/13/2)	0.051

Qualitative variables are expressed by the number of patients with percentage in parentheses, and quantitative variables are expressed by the median with range in parentheses. ALT – alanine aminotransferase; AFP – alpha-fetoprotein; IP-10 – the interferon-gamma inducible protein-10; HBV – hepatitis B virus; HBsAg – hepatitis B surface antigen; HBeAg – hepatitis B e antigen; HBcrAg – hepatitis B virus core-related antigen; PC – precore; CP – core promoter. * 1, Interferon alpha; 2, lamivudine; 3, lamivudine plus interferon-alpha; 4, entecavir.

rates of HBeAg seroconversion at 1, 3, 5, 7 and 10 years were 24.8%, 50.1%, 66.3%, 71.3% and 73.1%, respectively, during the follow-up >10 years after liver biopsies (Figure 3). Of note, HCC developed in 18 of the 234 (7.7%) patients during the follow-up.

Figure 4A compares cumulative HBeAg seroconversion rates stratified by HBeAg titers and grades of lobular inflammation. The patients, who had the combination of HBeAg <100 PEIU/ml and lobular inflammation grades ≥2, gained an HBeAg seroconversion rate higher than those having 3 other combinations. Likewise, cumulative HBeAg seroconversion rates stratified by HBeAg titers and ALT levels are compared in Figure 4B. HBeAg seroconversion rate of the patients, who had the combination of HBeAg <100 PEIU/ml and ALT ≥200 IU/l, was higher than those with 3

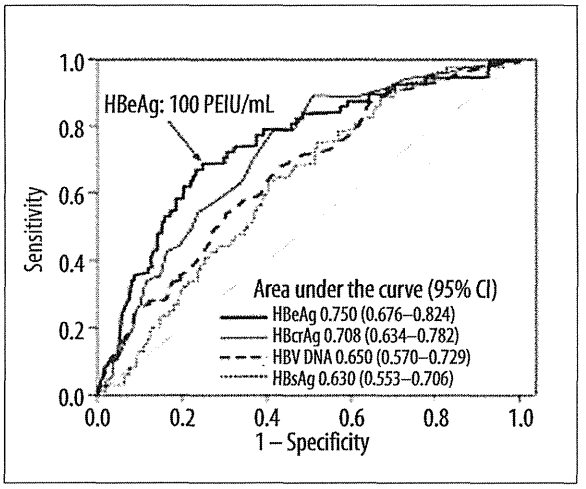


Figure 1. Receiver operating characteristic curves for evaluation of the power of predicting early HBeAg seroconversion.

other combinations, with definitive (p=0.003 and p<0.001) or marginal (p=0.061) significance.

Discussion

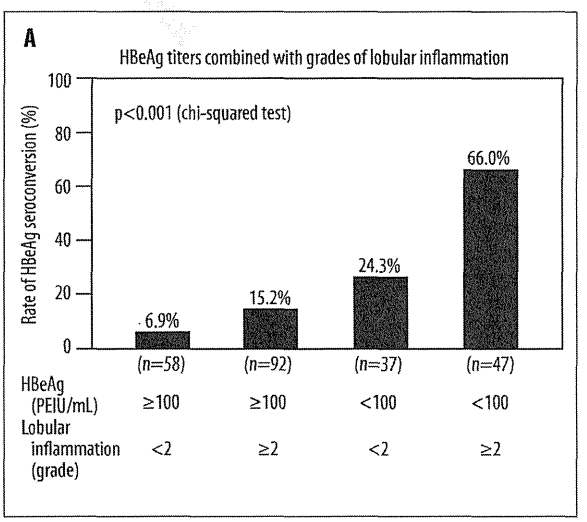
HBeAg seroconversion is important as a clinical target in the management of chronic hepatitis B. In the absence of therapeutic interventions, HBeAg seroconversion occurs spontaneously at a rate of 0.8–15% per year [28]. To date, many factors have been found in association with HBeAg seroconversion, including older age, high ALT levels, genotype B (compared with C), the Knodell's index of histologic activities, the amount of HBV core antigen in the liver, high serum AFP levels, increased immunoglobulin-M anti-HBc titers, increased serum β_2-microglobulin concentrations, enhanced expression of HLA-antigens on the membrane of hepatocytes, non-vertical transmission modes, low HBV DNA levels, and high serum levels of IL-10 as well as IL-12 [7–19].

It would be clinically useful to predict early HBeAg seroconversion, because antiviral treatments can be withheld in the patients in whom HBeAg disappears and anti-HBe develops within a certain time limit, perhaps 1 year. In the present study, the majority of patients (99% of the 234 examined) were infected with HBV of genotype C. Patients with persistent HBV infection in Japan are infected with HBV of either genotype B or C, with an increasing gradient of C toward the south [29,30]. All

Table 4. Multivariate analysis for the risk of early HBeAg seroconversion.

Variables	Odds ratio	95% confidence interval	p value
(A) All factors including histological characteristics			
HBeAg (<100 PEIU/ml)	8.430	4.173–17.032	<0.001
Lobular inflammation (≥2)	4.330	2.009–9.331	<0.001
(B) Factors exclusive of histological characteristics			
HBeAg (<100 PEIU/ml)	7.327	3.703–14.497	<0.001
ALT (≥200 IU/l)	3.093	1.562–6.127	0.001

HBeAg – hepatitis B e antigen; ALT – alanine aminotransferase.



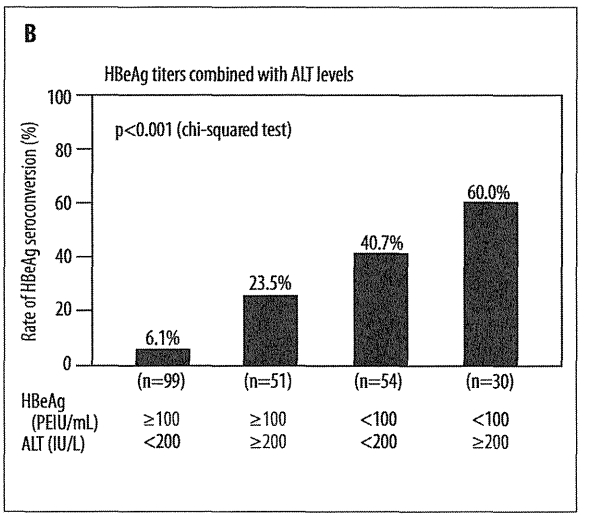


Figure 2. Probability of early HBeAg seroconversion. (**A**) The rate of early HBeAg seroconversion assessed by HBeAg titers and grades of lobular inflammation. (**B**) The rate of early HBeAg seroconversion assessed by HBeAg titers and ALT levels.

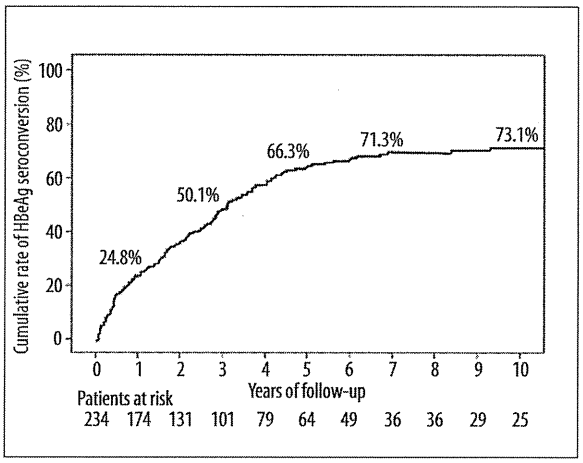


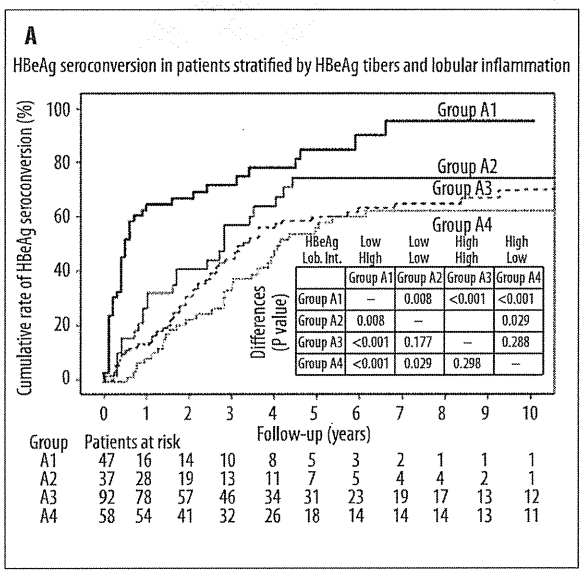
Figure 3. Cumulative rates of HBeAg seroconversion in the 234 patients during 10 years. Cumulative rates of HBeAg seroconversion at 1, 3, 5, 7 and 10 years were 24.8%, 50.1%, 66.3%, 71.3% and 73.1%, respectively, during the follow-up.

the 234 patients had received liver biopsies before they were started to be followed for HBeAg seroconversion. The present study is unique in that, not only serological variables, but also histological parameters were evaluated for the association with early HBeAg seroconversion within 1 year. By univariate analysis, many factors that have been reported in association with HBeAg seroconversion predicted early HBeAg seroconversion. Among them, only HBeAg (<100 PEIU/ml) and lobular inflammation (grades ≥2) remained as independent factors for early HBeAg seroconversion by multivariate analysis.

Previous clinical studies have indicated that serial monitoring of HBsAg, HBeAg and HBV DNA levels during antiviral treatments is useful for predicting HBeAg seroconversion [20–23]. Although the determination of HBV DNA in sera remains as an important tool for monitoring outcomes of patients with chronic hepatitis B, it is technically challenging, costly, and subject to inconsistency. Hence, three serological markers of HBV replication, HBsAg, HBeAg and HBcrAg, were quantitated for evaluating the performance in predicting early HBeAg seroconversion, in comparison with HBV DNA levels. In the receiver operating characteristic analysis, HBeAg levels performed the best amongst these four replication markers, with an area under curve wider than those of the other three. Since the quantitation of HBeAg is relatively easy, fast, and inexpensive, HBeAg would be qualified as a sensitive and practical predictor of early HBeAg seroconversion [20–23].

The histological activity has been reported to predict early HBeAg seroconversion in previous studies [14,31]. Therefore, pathological parameters including the stage of fibrosis, as well as grades of portal inflammation, piecemeal necrosis and lobular inflammation, were evaluated in this study. By multivariate analysis, lobular inflammation of grades ≥2, represented by focal necrosis or acidophil bodies, was identified as an independent factor for early seroconversion. Hence, portal inflammation without necrosis would not be enough, but instead, severe lobular inflammation may be required for predicting early seroconversion.

Many previous studies have identified a variety of factors associated with HBeAg seroconversion [7–19], but a combination of serum markers of HBV with pathological parameters was evaluated rarely. Therefore, the combination of HBeAg <100 PEIU/ml and grades ≥2 lobular inflammation was evaluated for the predictability of early HBeAg seroconversion. Patients with neither HBeAg <100 PEIU/ml nor grades ≥2 lobular inflammation had a minimal chance for early HBeAg seroconversion (6.9% [4/58]), whereas a high proportion of patients with both of these predictors did accomplish early seroconversion (66.0% [31/47]) (Figure 2A). Thus, the combination of histologic activity and serum HBV marker would be very useful for predicting early HBeAg seroconversion, and serve in decision making whether or not



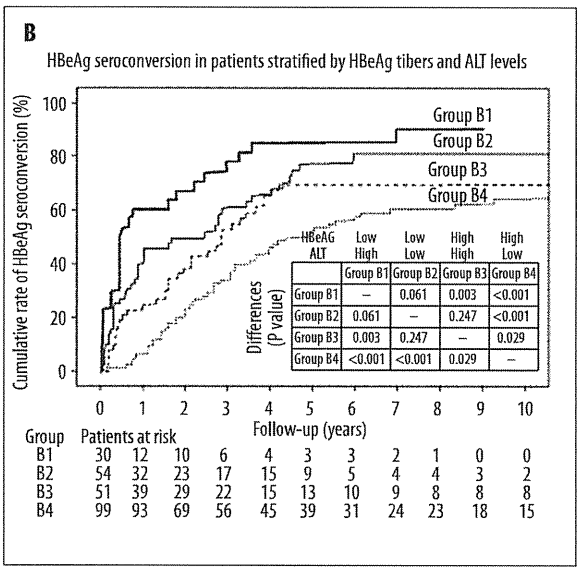


Figure 4. Cumulative rates of HBeAg seroconversion in four groups of patients. (**A**) Cumulative rates of HBeAg seroconversion stratified by HBeAg titers and grades of lobular inflammation. (**B**) Cumulative rates of HBeAg seroconversion stratified by HBeAg titers and ALT levels. HBeAg titers were dichotomized into low (<100 PEIU/ml) or high (≥100 PEIU/ml); lobular inflammation grades into low (<2) or high (≥2); and ALT levels into low (<200 IU/l) or high (≥200 IU/l).