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Incidence of and risk factors for hepatocellular carcinoma in primary biliary cirrhosis: National data from Japan

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Short title: HCC in PBC

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ABSTRACT

Primary biliary cirrhosis (PBC) primarily affects females and is rarely complicated by hepatocellular carcinoma (HCC). Although HCC incidence in PBC patients is low, several characteristics and risk factors associated with its development have been reported. In this study, national data concerning the current status of carcinogenesis in PBC patients in Japan are reviewed. Using data from two national questionnaire surveys, we investigated the clinicopathological findings associated with HCC in PBC patients. According to the data of all reviewed PBC patients, HCC incidence was 2.4% (71/2946). HCC incidence by gender was 5.1% (19/370) in males and 2.0% (52/2576) in females, and the proportion of males was 26.7%. Prognosis was significantly poorer in the PBC patients with HCC than in those without. Multivariate analysis of risk factors associated with HCC by gender revealed histological stage at the time of PBC diagnosis as an independent risk factor associated with the development of HCC in females, but not in males. Furthermore, data from another national survey of 178 PBC patients with HCC (male/female = 49/129; proportion of males 27.5%) revealed that the duration between the diagnosis of PBC and that of HCC was significantly shorter in males than in females. In addition, histological stage at the time of HCC diagnosis was an independent risk factor for HCC in females, whereas no risk factors were identified in males. In conclusion, these data indicate that males are at risk of developing HCC at any histological stage of PBC. Therefore, male PBC patients in particular should be carefully screened for HCC from the early stages of PBC.

INTRODUCTION

Primary biliary cirrhosis (PBC) primarily affects middle-aged females. Histologically, the interlobular bile ducts are primarily damaged and show characteristic findings such as chronic nonsuppurative destructive cholangitis (CNSDC) followed by progressive bile duct loss.[1,2] A terminal feature of PBC is irreversible biliary cirrhosis, and liver transplantation is the sole treatment for hepatic failure.[3] Although hepatic failure defines the prognosis in most PBC patients, hepatocellular carcinoma (HCC) is also reported to occur in 0.76%–5.9% PBC patients.[4-9] Recently, however, the incidence of PBC complicated by HCC has been gradually increasing with improvements in PBC treatment and survival.

In general, HCC is typically encountered in the terminal stage, when irreversible biliary cirrhosis sets in. Moreover, the hepatitis virus is a major risk factor for HCC development in patients with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections. In PBC patients, however, no carcinogenic factors directly associated with HCC have been identified. The proposed risk factors for HCC arising from PBC-affected livers include the hepatitis virus, cirrhosis, older age, diabetic mellitus, and male gender.[4,5,10-13] However, epidemiologic studies are limited and provide conflicting results, perhaps because of the low prevalence of the disease and geographical and environmental differences.

In the present study, we evaluated data from two nationwide surveys performed in Japan. Our aim was to clarify the current status of carcinogenesis in PBC patients, identify the associated clinicopathological risk factors, and understand how the pathogenesis of PBC is directly associated with HCC.

MATERIALS and METHODS

Setting and patient selection

A survey of PBC in Japan (national survey by the Intractable Hepato-Biliary Diseases Study Group)

National surveys of PBC patients in Japan have been performed 14 times biennially or triennially by the Intractable Hepato-Biliary Diseases Study Group for Research on Measures for Intractable Disease, which is supported by Health Labour Sciences Research Grants in Japan. The subjects included 7376 patients registered in the 1st–14th surveys performed between 1980 and 2009.[9,14] Of the 7376 patients, the absence or presence of HCC was confirmed during follow-up in 2946 (70 males, 2576 females), who were then investigated in the current study. HBV carriers and HB antigen- and anti-HCV antibody-positive patients were excluded.

A survey of PBC patients with HCC in Japan (national survey by the Liver Cancer Study Group of Japan)

This project was set up at the 47th Annual Meeting of the Liver Cancer Study Group of Japan (President, Professor Ichida), and it was executed in 2011. Questionnaires were sent to 340 hospitals or institutions included in the Liver Cancer Study Group of Japan. Eighty-six of the 340 hospitals responded, and data from 178 PBC patients with HCC from 39 hospitals or institutions were eventually included. HBV carriers and HB antigen- and anti-HCV antibody-positive patients were excluded. The cooperating institutions are listed in the appendix.

PBC diagnosis

PBC was diagnosed according to criteria established by the Intractable Hepato-Biliary

Diseases Study Group of Japan. Patients whose condition met one of the following criteria were diagnosed as having PBC: 1) histologically confirmed CNSDC with laboratory findings positive for PBC; 2) positivity for antimitochondrial (AMA) and/or anti-pyruvate dehydrogenase (PDH) antibodies, absence of histological findings of CNSDC, and presence of histological findings compatible with PBC; and 3) no histological examination, but positivity for AMA and/or anti-PDH dehydrogenase antibodies and clinical findings and course indicative of PBC. PBC symptoms were defined as pruritus, overt jaundice, esophageal varices, ascites, and hepatic encephalopathy.[15] Histological findings were classified according to Scheuer's system.[16]

Statistical analysis

The Mann–Whitney U and chi-square tests test were used as nonparametric and independence tests, respectively. Logistic regression analysis was used for the multivariate analysis of prognostic factors. Survival rate was obtained by the Kaplan–Meier method. A p value of <0.05 was considered statistically significant.

RESULTS

HCC incidence in the Japanese PBC population

The current status of and risk factors for HCC in PBC patients in Japan were analyzed on the basis of data from the national survey conducted by the Intractable Hepato-Biliary Diseases Study Group. The total number of PBC patients was 2946. Of these, 2100 cases available for analysis of histological stage of PBC at diagnosis underwent liver biopsy. HCC incidence during follow up was 2.4% (71/ 2946). This incidence was 5.1% (19/370) in males and 2.0% (52/2576) in females, and the proportion of males was 26.7%. The mean ± standard deviation and median values

for the observation period were 80.1 ± 70.8 (range, 1–443) and 58 months, respectively. The mean

value for males was 65.1 ± 57.2 (range, 1–237; median, 45) months, while that for females was 82.2

± 72.2 (range, 1–443; median, 60) months.

A comparative analysis of PBC patients with and without HCC revealed male gender, old

age, low serum albumin levels, low serum total cholesterol levels, advanced histological stage, and

symptomatic status at the time of PBC diagnosis as significant risk factors for HCC (Table 1). There

was no difference in total bilirubin levels and the presence or absence of ursodeoxycholic acid

(UDCA) treatment between the two groups (Table 1). Prognosis was significantly poorer in the PBC

patients with HCC than in those without (Fig. 1). The cumulative incidence of carcinogenesis was

6.5% in males and 2.0% in females during the 10 years after PBC diagnosis; the difference between

males and females was statistically significant (p < 0.0001) (Fig. 2). In particular, analyses of HCC

incidence in patients aged 10–80 years revealed that male PBC patients in their 40s and 50s had an

increased risk of HCC compared with female PBC patients in the same age groups (data not shown).

In multivariate analysis for risk factors of HCC, gender and histological stage were selected as

significant factors (p < 0.00001) (Table 2). There was no difference in the proportion of males and

females who underwent histological staging at PBC diagnosis. The incidence of histological stages 3

and 4 was approximately 16.0% in both male and female PBC patients without HCC (Table 2),

whereas that was 14.2% and 57.1% in male and female PBC patients with HCC, respectively.

Advanced histological stage was a risk factor for HCC in females (p < 0.0001; Fig. 3 and Table 2).

Multivariate analysis for risk factors of HCC by gender revealed that histological stage at the time of

PBC diagnosis was an independent risk factor for HCC in females (supplementary Table 1), whereas

no significant independent factors were revealed for males (supplementary Table 2). Moreover,

although we assessed PBC patients with HCC according to histological stage, we found no

difference in any clinical or biological characteristics between patients with and without cirrhosis at

PBC diagnosis (supplementary Table 3).

PBC patients with HCC in Japan

From the data of the national survey specially set up at the 47th Annual Meeting of the Liver Cancer Study Group of Japan, we collected and investigated those for 178 PBC patients with HCC from a total of 39 hospitals included in the study group. These cases included 100 fatalities in the past years as well as 78 patients followed up from each hospital or institute as of June, 2011. Among the followed-up patients, four underwent liver transplantation, which was performed at the time of HCC discovery in three and 3 years after HCC discovery in one. There were 49 male and 129 female PBC patients with HCC, and the proportion of males was 27.5%, which was similar to that from the previously described national survey of PBC. Although the average age at the time of for PBC diagnosis was slightly higher for males (68 years) than for females (62 years), that at the time of HCC diagnosis was similar between males (73 years) and females (72 years; Fig. 4). Moreover, the duration between the diagnosis of PBC and that of HCC was shorter in males than in females. HCC was diagnosed simultaneously with or prior to the diagnosis of PBC in 32.7% (16/49) males and 14.7% (19/129) females.

Pathological examination for HCC and background liver tissue assessment by biopsy or hepatectomy was conducted for 66 and 82 patients, respectively. Clinicopathological data at the time of HCC diagnosis are shown in Table 3. There were more males with prior HBV infection and a history of alcohol consumption compared with females. There were no differences in the history of blood transfusion, diabetes mellitus, AMA levels, anti-nuclear antibody levels, body mass index, serum triglyceride levels, serum total cholesterol levels associated with nonalcoholic fatty liver disease (including nonalcoholic steatohepatitis), and use of UDCA (Table 3) between males and females. However, an analysis excluding patients with past HBV infection and a history of alcohol

consumption revealed that there was no difference in other clinical findings, although the proportion

of males (male/female = 24/104, 18.5%) remained higher than that of the total PBC male patients

(male/female = 370/2576, 12.6%; $p < 0.05$; supplementary Table 4). Moreover, in females, HCC

incidence gradually increased with histologic stage, while the incidence in males showed no trend or

statistical significance. There was a significant difference in the distribution of histological stage

between males and females (Fig. 5). An analysis of PBC patients with HCC according to histological

stage revealed no clinical findings (including past HBV infection and alcohol consumption) that

were significantly different between patients with and without cirrhosis at HCC diagnosis

(supplementary Table 5). There was also no significant difference in tumor number and

differentiation between males and females (supplementary Table 6).

DISCUSSION

Recently, we encountered PBC patients with HCC during routine pathological assessments,

and the number of these patients appears to have increased according to reports from other

institutes.[11,17,18] In most patients, HCC is detected during follow-up for PBC, whereas some patients

are simultaneously diagnosed with PBC and HCC or diagnosed with HCC prior to PBC. Although

prognosis has improved with advances in treatment for PBC, the precise reason for the increased

number of PBC patients with HCC in recent decades remains unknown. Therefore, we analyzed data

from Japanese PBC patients and those with PBC and HCC who were independently surveyed by two

different study groups. One set of data was from a national survey of PBC patients performed 14

times between 1980 and 2009, while the other was from PBC patients with HCC who were

evaluated as a special project of the Annual Meeting of the Liver Cancer Study Group of Japan in

2011. Both surveys collected data through questionnaires administered to foundation hospitals or

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specialized hospitals for hepatology in Japan. Therefore, although the investigative hospitals/institutions and objectives did not match, it is speculated that most PBC patients with HCC overlapped. Moreover, the proportion of males among PBC patients with HCC almost coincided in these two independent studies (26.7% vs 27.5%), validating the use of these studies together as representative of the situation in Japan.

Although some studies have reported that PBC patients do not have an increased risk of developing HCC,[19] others showed that HCC incidence was high in PBC patients.[5,7,20] HCC incidence among PBC patients is reportedly low at 0.76%–5.9% according to previous reports.[4-9] In this study, we investigated the incidence of and risk factors for HCC in Japanese PBC patients. According to data from the nationwide survey by the Intractable Hepato-Biliary Diseases Study Group, HCC incidence was 2.4%. As for risk factors associated with HCC in PBC patients, several conflicting results have been reported.[4,5,11-13] In general, male gender, advanced stage, HCV infection, and a history of blood transfusion were reported to be associated with HCC in PBC patients.[5,7,20] In a proportional hazards analysis of patients with PBC in Japan, Shibuya et al.[5] reported three factors to be independently associated with HCC development: age at the time of diagnosis, male gender, and history of blood transfusion. While autoimmune liver disease, including PBC, is more common in females than in males, HCC incidence in PBC patients was higher in males than in females. In agreement with previous reports from Japan, Europe, and USA,[4,5,11-13] gender was identified as a risk factor associated with HCC in the nationwide survey of PBC patients conducted by the Intractable Hepato-Biliary Diseases Study Group. HCC incidence was 5.1% in males and 2.0% in females (proportion of males, 26.7%), indicating that male PBC patients had a 2.1-fold higher risk of HCC compared with female PBC patients. The proportion of males among the PBC patients with HCC was consistent with that in the nationwide survey by the Liver Cancer Study Group of Japan (27.5%). Moreover, cumulative HCC incidence was 6.5% in males and 2.0% in females during the 10 years

after PBC diagnosis, and male PBC patients had a 3.3-fold higher risk of HCC compared with females. In general, during the carcinogenesis of HCC, estrogen can protect hepatocytes from malignant transformation via downregulation of IL-6 release from Kupffer cells, indicating that estrogen-mediated inhibition of IL-6 production by Kupffer cells potentially decreased the risk of HCC in females.[21,22] Therefore, although PBC primarily affects females, HCC may be more common in male PBC patients because of a lack of estrogen-mediated prevention. The national survey by the Liver Cancer Study Group of Japan revealed that the duration between the diagnosis of PBC and that of HCC was shorter in males than in females and that the diagnosis of HCC was performed simultaneously at or prior to the diagnosis of PBC in 32.7% males and 14.7% females. Several reasons may be responsible for the delayed diagnosis of PBC and carcinogenesis in the early stage in males, but the details remain unspecified. Moreover, the rate of past HBV infection and alcohol consumption was significantly higher in males than in females, indicating that these factors also possibly affect the increased HCC incidence in male PBC patients. Watanabe et al. reported that past HBV infection is an important factor in the association of HCC with PBC.[18] In a patient with HBV infection, HBV-DNA possibly integrates into the human genome, but the frequency of this integration in prior HBV-infected PBC patients with HCC remains unknown. Moreover, because the distribution of past HBV infection by gender in the whole PBC population could not be obtained, the extent to which previous infection with HBV is directly associated with HCC carcinogenesis in male PBC patients remains debatable. However, analysis excluding cases with past HBV infection and a history of alcohol consumption revealed that the proportion of males with HCC in PBC patients with HCC remained high compared with that of all PBC male patients. In addition, analysis according to histological stage (non-cirrhosis *vs.* cirrhosis) suggested that past HBV infection and alcohol consumption were not directly associated with progression to cirrhosis in PBC patients with HCC.

In addition to male gender, the national survey by the Intractable Hepato-Biliary Diseases

Study Group demonstrated that old age, low serum albumin levels, low total cholesterol levels, advanced histological stage, and symptomatic status at the time of PBC diagnosis were statistically significant in PBC patients with HCC compared to those without HCC. However, multivariate analysis by gender revealed that histological stage at the time of diagnosis of PBC was an independent risk factor for HCC in females, but not in males. In addition to at the time of diagnosis of PBC, at that of HCC, histological stage is associated with HCC by national survey for PBC with HCC patients. However, there was no difference in any clinical or biological characteristics between PBC patients with HCC with or without cirrhosis at HCC diagnosis. In females, HCC incidence gradually increased according to histological stage, indicating that the terminal stage of PBC, which is a cirrhotic state, may be a risk factor for HCC development in females, whereas males are likely to develop HCC at any stage. The carcinogenesis of HCC in PBC patients should be further clarified. PBC is pathologically characterized by CNSDC, and the main inflammatory lesions associated with PBC are not hepatocytes but cholangiocytes, which may be one of the reasons why HCC incidence in PBC patients is relatively low compared with the incidence of sustained hepatitic diseases such as chronic viral hepatitis and autoimmune hepatitis. Male PBC patients with HCC are thought to be a good model because they lack estrogen-mediated prevention of HCC. Unlike that in hepatitic diseases, intrahepatic cholestasis is found from the early stage in PBC,[1,23] and some mitogenic factors in the bile of PBC patients presumably participate in the carcinogenesis of HCC from an early stage.[17,24] However, this hypothesis remains a matter of speculation, and further study is required to clarify the molecular mechanism involved in the carcinogenesis of HCC in PBC patients.

In conclusion, we investigated the risk factors for HCC using data from two nationwide surveys of PBC patients in Japan. Because male PBC patients are at risk of developing HCC at any histologic stage, they should be carefully screened for HCC from an early stage of PBC, irrespective of histological stage.

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FIGURE LEGENDS

Fig. 1   Kaplan–Meier curve for survival in patients with primary biliary cirrhosis with (+) or without (−) hepatocellular carcinoma. There is a statistically significant difference between the curves ($p < 0.05$).

Fig. 2 Cumulative appearance rates of hepatocellular carcinomsa in patients with primary biliary cirrhosis by gender. There is a statistically significant difference between males and females.

Fig. 3 Histological stage at the diagnosis of primary biliary cirrhosis (PBC) in patients with or without hepatocellular carcinoma (HCC) by gender. The proportion of patients with histological stages 3 and 4 at the time of PBC diagnosis is approximately 16.0% for both male and female PBC patients without HCC. However, the proportion of patients with histological stages 3 and 4 is 14.2% in male and 57.1% in female PBC patients with HCC. Moreover, there is a significant difference in the proportion of female PBC patients with HCC and that without. The parentheses identify the number of patients examined.

Fig. 4 Average age at the time of diagnosis of primary biliary cirrhosis (PBC) and hepatocellular carcinoma (HCC), and the duration between the diagnosis of PBC and that of HCC. The duration between the diagnosis of PBC and that of HCC is shorter in males than in females ($p < 0.05$). The parentheses identify the number of patients examined.

Fig. 5 Histologic stage by gender at the time of hepatocellular carcinoma (HCC) diagnosis in patients with primary biliary cirrhosis. In females, HCC incidence gradually increases

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6   according to histological stage, with a statistically significant difference ($p < 0.05$). The
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8   parentheses indicate the number of patients examined.
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Table 1

Clinical and biological characteristics of PBC patients with or without HCC at PBC diagnosis

24	HCC (+)	HCC (−)	p
Number	71	2875	
Sex (M:F)	19:52	351:2524	0.0003
Age (Mean ± SD)	60.5 ± 10.4	56.4 ± 11.2	0.0023
T-Bilirubin (Mean ± SD)	1.37 ± 1.63	0.99 ± 1.52	0.1061
Albumin (Mean ± SD)	3.81 ± 0.58	4.05 ± 0.51	0.0002
T-cholesterol (Mean ± SD)	201.3 ± 60.5	217.4 ± 86.7	0.0397
Histological stage (I/II/III/IV)	10/17/14/8	1060/662/263/66	<0.0001
Use of UDCA (%)	89.7	91.8	0.5291
Clinical stage (asymptomatic:symptomatic)	38:33	2775/100	<0.0001

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Table 2

Factors associated with increased risk of HCC in PBC patients (multivariate analysis)

	regression coefficient	standard deviation	χ^2	odds ratio	P value
Sex (M:F)	−0.5646	0.1737	10.56	3.0932	0.0012
Age	−0.0242	0.0149	2.63	0.9760	0.1050
T-Bilirubin	0.0302	0.0880	0.12	1.0307	0.7313
Albumin	0.0274	0.3087	0.01	1.0277	0.9292
T-cholesterol	0.0021	0.0026	0.65	1.0021	0.4210
Histological stage (I/II/III/IV)	−0.7294	0.1661	19.27	0.4821	<0.0001
Use of UDCA (%)	−0.2823	0.2473	1.3	1.7590	0.2537
Clinical stage (asymptomatic:symptomatic)	0.2990	0.1674	3.19	0.5498	0.0741

150x112mm (300 x 300 DPI)

Table 3

Clinical and biological characteristics of male and female PBC patients at HCC diagnosis

	Male (n = 49)	Female (n = 129)	Total (n = 178)
Blood transfusion	9%	8%	9%
past HBV infection*	33%	18%	22%
Alcohol intake*	27%	2%	9%
Diabetes mellitus	24%	23%	24%
AMA levels	86%	82%	83%
ANA levels	41%	49%	47%
BMI (>25%)	25%	31%	29%
Triglyceride (≥150)	8%	9%	9%
Total cholesterol (>220)	15%	9%	11%
associated with NAFLD	0%	4%	3%
Use of UDCA	84%	84%	84%

(*p < 0.05)

150x112mm (300 x 300 DPI)

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Supplementary Table 1

Factors associated with increased risk of HCC in <u>female</u> PBC patients (multivariate analysis)

	regression coefficient	standard deviation	χ^2	odds ratio	P value
Age	−0.0130	0.0174	0.56	0.9870	0.4531
T-Bilirubin	0.0817	0.1171	0.49	1.0851	0.4852
Albumin	−0.1771	0.3366	0.28	0.8376	0.5987
T-cholesterol	0.0038	0.0033	1.32	1.0038	0.2512
Histological stage (I/II/III/IV)	−1.0255	0.1964	27.25	0.3586	<0.0001
Use of UDCA (%)	−0.1607	0.3151	0.26	1.3791	0.6100
Clinical stage (asymptomatic:symptomatic)	0.4252	0.1913	4.94	0.4271	0.0263

150x112mm (300 x 300 DPI)

Supplementary Table 2

Factors associated with increased risk of HCC in <u>male</u> PBC patients (multivariate analysis)

	regression coefficient	standard deviation	χ^2	odds ratio	P value
Age	−0.0542	0.0319	2.89	0.9472	0.0893
T-Bilirubin	−0.1018	0.1790	0.32	0.9032	0.5697
Albumin	0.5884	0.5591	1.11	1.8011	0.2926
T-cholesterol	0.0001	0.0020	0.00	1.0001	0.9511
Histological stage (I/II/III/IV)	0.2484	0.4096	0.37	1.2819	0.5443
Use of UDCA (%)	−0.5367	0.4254	1.59	2.9258	0.2071
Clinical stage (asymptomatic:symptomatic)	−0.3590	0.4635	0.60	2.0506	0.4385

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Supplementary Table 3

Clinical and biological characteristics of PBC patients with or without cirrhosis at PBC diagnosis

	Non-cirrhosis	Cirrhosis	
Gender (M:F)	13:28	1:7	NS
Age (yrs)	59.8 ± 9.7	57.3±10.5	NS
T-Bilirubin	1.15±0.52	1.42±1.13	NS
Albumin	3.89±0.56	3.77±0.79	NS
T-cholesterol	201.0±59.4	229.4±77.9	NS
Duration of the diagnosis from PBC to HCC (years)	9.5±5.8	7.9±4.2	NS
Use of UDCA (No:Yes)	5:36	0:8	NS
Clinical stage (asymptomatic:symptomatic)	25:16	4:4	NS

Noncirrhosis, Scheuer's stage 1-3; Cirrhosis, Scheuer's stage 4; NS, not significant

150x112mm (300 x 300 DPI)

Supplementary Table 4

Clinical and biological characteristics of male and female PBC patients with HCC at HCC diagnosis
(excluding cases with past HBV infection or history of alcohol intake)

	Male (n = 24)	Female (n = 104)	Total (n = 128)
Blood transfusion	8%	7%	7%
Diabetes mellitus	18%	22%	22%
AMA levels	89%	93%	92%
ANA levels	41%	59%	56%
BMI(≥25%)	20%	33%	31%
Triglyceride (≥150)	11%	9%	10%
Total cholesterol (>220)	9%	11%	11%
associated with NAFLD	0%	6%	4%
Use of UDCA	83%	90%	89%

150x112mm (300 x 300 DPI)

Supplementary Table 5

Clinical and biological characteristics of PBC patients with or without cirrhosis at HCC diagnosis

	Non-cirrhosis (n = 43)	Cirrhosis (n = 39)	
Blood transfusion	9 %	10 %	NS
Past HBV infection	26 %	23 %	NS
Alcohol intake	9 %	10 %	NS
Diabetes mellitus	23 %	28 %	NS
AMA levels	93 %	97 %	NS
ANA levels	48 %	50 %	NS
BMI(>25%)	19 %	39 %	NS
Triglyceride ($\geq$150)	13 %	10 %	NS
Total cholesterol (>220)	15 %	12 %	NS
associated with NAFLD	0 %	3 %	NS
Use of UDCA	81 %	82%	NS

Non-cirrhosis, Scheuer's stage 1-3; Cirrhosis, Scheuer's stage 4; NS, not significant

150x112mm (300 x 300 DPI)

Supplementary Table 6

Characteristics of HCC in male and female PBC patients with HCC

	Male	Female	Total
Number of HCC	(n = 49)	(n = 128)	(n = 178)
Solitary	65 %	60 %	62 %
Multiple	35 %	38 %	37 %
Unknown	0 %	2 %	2 %
Differentiation of HCC	(n = 25)	(n = 41)	(n = 66)
Well differentiated	44 %	37 %	39 %
Moderately differentiated	48 %	56 %	53 %
Poorly differentiated	8 %	7 %	8 %

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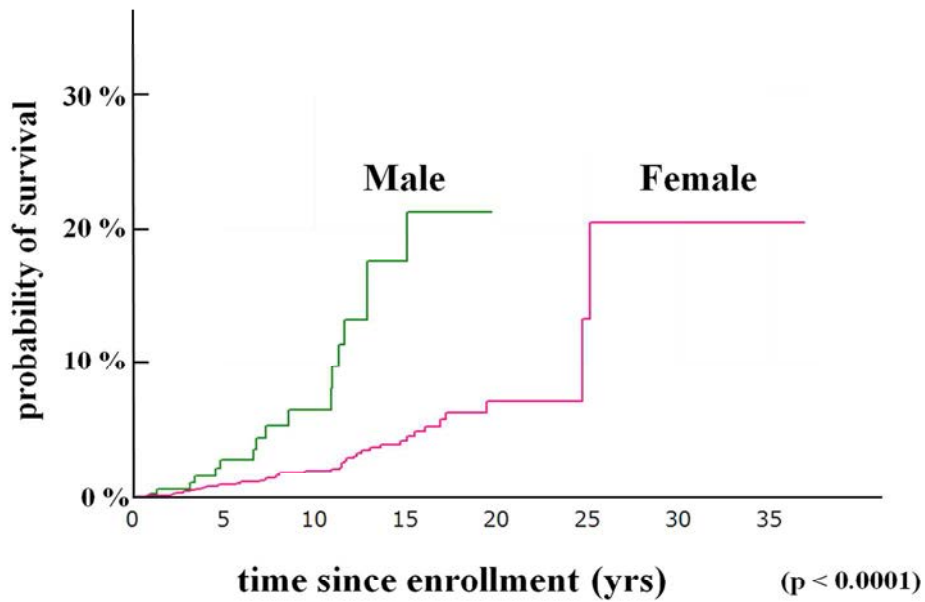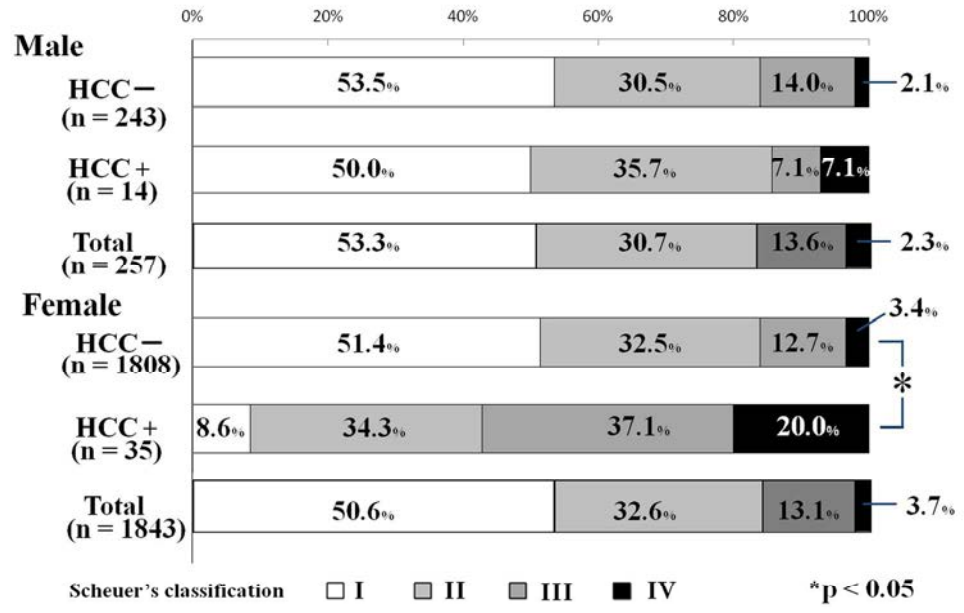55
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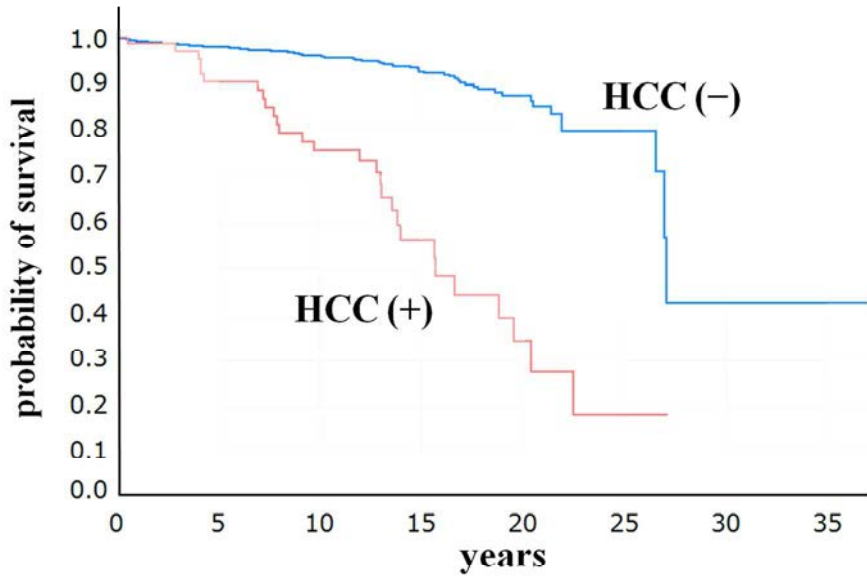
Fig. 1

150x112mm (300 x 300 DPI)

Fig. 2

150x112mm (300 x 300 DPI)

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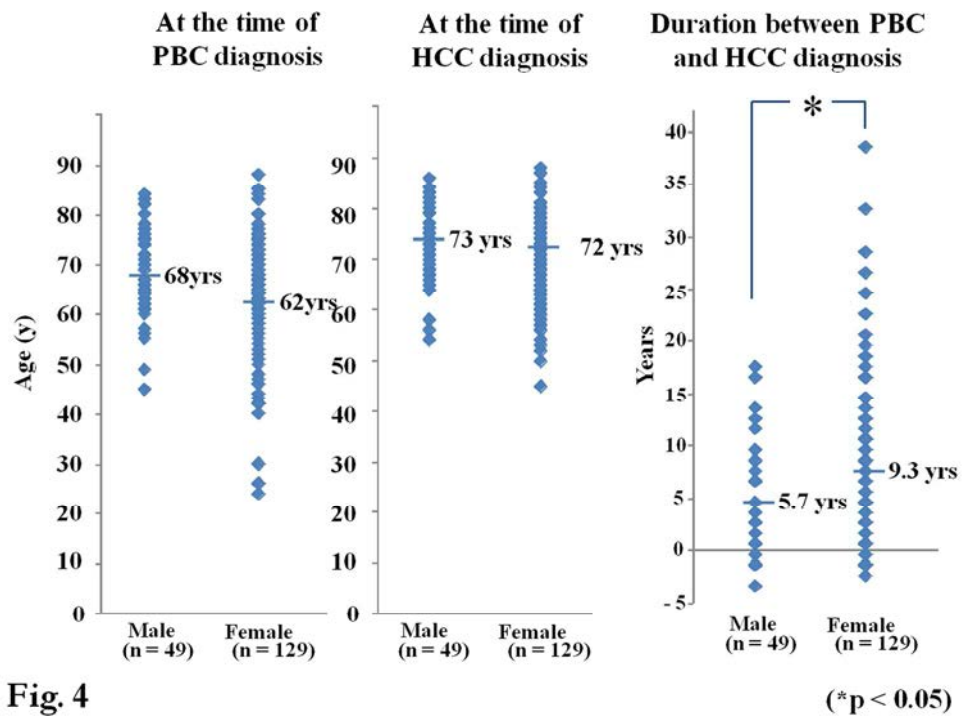60

Fig. 3

150x112mm (300 x 300 DPI)

Fig. 4

150x112mm (300 x 300 DPI)

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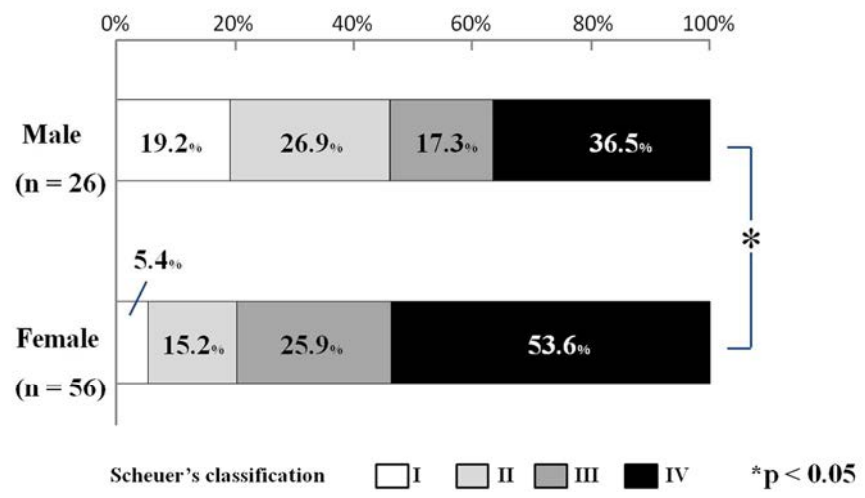31
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Fig. 5

150x112mm (300 x 300 DPI)