

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

| | |
|------------------------------|--|
| 著者 | Harada Kenichi, Hirohara Junko, Ueno Yoshiyuki, Nakano Toshiaki, Kakuda Yuko, Tsubouchi Hirohito, Ichida Takafumi, Nakanuma Yasuni |
| journal or publication title | Hepatology |
| volume | 57 |
| number | 5 |
| page range | 1942-1949 |
| year | 2013-05-01 |
| URL | http://hdl.handle.net/2297/34774 |

doi: 10.1002/hep.26176

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

Kenichi Harada¹⁾, Junko Hirohara²⁾, Yoshiyuki Ueno³⁾, Toshiaki Nakano⁴⁾, Yuko Kakuda¹⁾, Hirohito Tsubouchi⁵⁾, Takafumi Ichida⁶⁾, Yasuni Nakanuma¹⁾

Kenichi Harada and Junko Hirohara contributed equally to this work.

- 1) Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan
- 2) Third Department of Internal Medicine, Kansai Medical University, Osaka, Japan
- 3) Department of Gastroenterology, Yamagata University Faculty of Medicine, Yamagata, Japan
- 4) University Information Center, Kansai Medical University, Osaka, Japan
- 5) Digestive and Lifestyle Diseases, Department of Human and Environmental Sciences, Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan
- 6) Division of Gastroenterology and Hepatology, Juntendo University School of Medicine, Shizuoka Hospital, Izunokuni, Japan

Short title: HCC in PBC

Address correspondence to:

Kenichi Harada, MD

Department of Human Pathology

Kanazawa University Graduate School of Medicine

Kanazawa 920-8640, Japan

FAX : (0)76-234-4229 (Japan)

TEL : (0)76-265-2199 (Japan)

E-mail: kenichih@med.kanazawa-u.ac.jp

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

1
2
3
4
5
6 Diseases Study Group of Japan. Patients whose condition met one of the following criteria were
7
8 diagnosed as having PBC: 1) histologically confirmed CNSDC with laboratory findings positive for
9
10 PBC; 2) positivity for antimitochondrial (AMA) and/or anti-pyruvate dehydrogenase (PDH)
11
12 antibodies, absence of histological findings of CNSDC, and presence of histological findings
13
14 compatible with PBC; and 3) no histological examination, but positivity for AMA and/or anti-PDH
15
16 dehydrogenase antibodies and clinical findings and course indicative of PBC. PBC symptoms were
17
18 defined as pruritus, overt jaundice, esophageal varices, ascites, and hepatic encephalopathy.¹⁵
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3
4
5
6 for the observation period were 80.1 ± 70.8 (range, 1–443) and 58 months, respectively. The mean
7
8 value for males was 65.1 ± 57.2 (range, 1–237; median, 45) months, while that for females was 82.2
9
10 ± 72.2 (range, 1–443; median, 60) months.

11
12 A comparative analysis of PBC patients with and without HCC revealed male gender, old
13
14 age, low serum albumin levels, low serum total cholesterol levels, advanced histological stage, and
15
16 symptomatic status at the time of PBC diagnosis as significant risk factors for HCC (Table 1). There
17
18 was no difference in total bilirubin levels and the presence or absence of ursodeoxycholic acid
19
20 (UDCA) treatment between the two groups (Table 1). Prognosis was significantly poorer in the PBC
21
22 patients with HCC than in those without (Fig. 1). The cumulative incidence of carcinogenesis was
23
24 6.5% in males and 2.0% in females during the 10 years after PBC diagnosis; the difference between
25
26 males and females was statistically significant ($p < 0.0001$) (Fig. 2). In particular, analyses of HCC
27
28 incidence in patients aged 10–80 years revealed that male PBC patients in their 40s and 50s had an
29
30 increased risk of HCC compared with female PBC patients in the same age groups (data not shown).
31
32 In multivariate analysis for risk factors of HCC, gender and histological stage were selected as
33
34 significant factors ($p < 0.00001$) (Table 2). There was no difference in the proportion of males and
35
36 females who underwent histological staging at PBC diagnosis. The incidence of histological stages 3
37
38 and 4 was approximately 16.0% in both male and female PBC patients without HCC (Table 2),
39
40 whereas that was 14.2% and 57.1% in male and female PBC patients with HCC, respectively.
41
42 Advanced histological stage was a risk factor for HCC in females ($p < 0.0001$; Fig. 3 and Table 2).
43
44 Multivariate analysis for risk factors of HCC by gender revealed that histological stage at the time of
45
46 PBC diagnosis was an independent risk factor for HCC in females (supplementary Table 1), whereas
47
48 no significant independent factors were revealed for males (supplementary Table 2). Moreover,
49
50 although we assessed PBC patients with HCC according to histological stage, we found no
51
52 difference in any clinical or biological characteristics between patients with and without cirrhosis at
53
54
55
56
57
58
59
60

1
2
3
4
5
6 PBC diagnosis (supplementary Table 3).
7
8
9

10 **PBC patients with HCC in Japan**

11
12 From the data of the national survey specially set up at the 47th Annual Meeting of the
13 Liver Cancer Study Group of Japan, we collected and investigated those for 178 PBC patients with
14 HCC from a total of 39 hospitals included in the study group. These cases included 100 fatalities in
15 the past years as well as 78 patients followed up from each hospital or institute as of June, 2011.
16
17 Among the followed-up patients, four underwent liver transplantation, which was performed at the
18 time of HCC discovery in three and 3 years after HCC discovery in one. There were 49 male and
19 129 female PBC patients with HCC, and the proportion of males was 27.5%, which was similar to
20 that from the previously described national survey of PBC. Although the average age at the time of
21 for PBC diagnosis was slightly higher for males (68 years) than for females (62 years), that at the
22 time of HCC diagnosis was similar between males (73 years) and females (72 years; Fig. 4).
23
24 Moreover, the duration between the diagnosis of PBC and that of HCC was shorter in males than in
25 females. HCC was diagnosed simultaneously with or prior to the diagnosis of PBC in 32.7% (16/49)
26 males and 14.7% (19/129) females.
27
28
29
30
31
32
33
34
35
36
37
38
39

40 Pathological examination for HCC and background liver tissue assessment by biopsy or
41 hepatectomy was conducted for 66 and 82 patients, respectively. Clinicopathological data at the time
42 of HCC diagnosis are shown in Table 3. There were more males with prior HBV infection and a
43 history of alcohol consumption compared with females. There were no differences in the history of
44 blood transfusion, diabetes mellitus, AMA levels, anti-nuclear antibody levels, body mass index,
45 serum triglyceride levels, serum total cholesterol levels associated with nonalcoholic fatty liver
46 disease (including nonalcoholic steatohepatitis), and use of UDCA (Table 3) between males and
47 females. However, an analysis excluding patients with past HBV infection and a history of alcohol
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6 consumption revealed that there was no difference in other clinical findings, although the proportion
7
8 of males (male/female = 24/104, 18.5%) remained higher than that of the total PBC male patients
9
10 (male/female = 370/2576, 12.6%; $p < 0.05$; supplementary Table 4). Moreover, in females, HCC
11
12 incidence gradually increased with histologic stage, while the incidence in males showed no trend or
13
14 statistical significance. There was a significant difference in the distribution of histological stage
15
16 between males and females (Fig. 5). An analysis of PBC patients with HCC according to histological
17
18 stage revealed no clinical findings (including past HBV infection and alcohol consumption) that
19
20 were significantly different between patients with and without cirrhosis at HCC diagnosis
21
22 (supplementary Table 5). There was also no significant difference in tumor number and
23
24 differentiation between males and females (supplementary Table 6).
25
26
27
28
29

30 DISCUSSION

31
32
33
34 Recently, we encountered PBC patients with HCC during routine pathological assessments,
35
36 and the number of these patients appears to have increased according to reports from other
37
38 institutes.^{11,17,18} In most patients, HCC is detected during follow-up for PBC, whereas some patients
39
40 are simultaneously diagnosed with PBC and HCC or diagnosed with HCC prior to PBC. Although
41
42 prognosis has improved with advances in treatment for PBC, the precise reason for the increased
43
44 number of PBC patients with HCC in recent decades remains unknown. Therefore, we analyzed data
45
46 from Japanese PBC patients and those with PBC and HCC who were independently surveyed by two
47
48 different study groups. One set of data was from a national survey of PBC patients performed 14
49
50 times between 1980 and 2009, while the other was from PBC patients with HCC who were
51
52 evaluated as a special project of the Annual Meeting of the Liver Cancer Study Group of Japan in
53
54 2011. Both surveys collected data through questionnaires administered to foundation hospitals or
55
56
57
58
59
60

1
2
3
4
5
6 specialized hospitals for hepatology in Japan. Therefore, although the investigative
7
8 hospitals/institutions and objectives did not match, it is speculated that most PBC patients with HCC
9
10 overlapped. Moreover, the proportion of males among PBC patients with HCC almost coincided in
11
12 these two independent studies (26.7% vs 27.5%), validating the use of these studies together as
13
14 representative of the situation in Japan.
15

16
17 Although some studies have reported that PBC patients do not have an increased risk of
18
19 developing HCC,¹⁹ others showed that HCC incidence was high in PBC patients.^{5,7,20} HCC incidence
20
21 among PBC patients is reportedly low at 0.76%–5.9% according to previous reports.⁴⁻⁹ In this study,
22
23 we investigated the incidence of and risk factors for HCC in Japanese PBC patients. According to
24
25 data from the nationwide survey by the Intractable Hepato-Biliary Diseases Study Group, HCC
26
27 incidence was 2.4%. As for risk factors associated with HCC in PBC patients, several conflicting
28
29 results have been reported.^{4,5,11-13} In general, male gender, advanced stage, HCV infection, and a
30
31 history of blood transfusion were reported to be associated with HCC in PBC patients.^{5,7,20} In a
32
33 proportional hazards analysis of patients with PBC in Japan, Shibuya et al.⁵ reported three factors to
34
35 be independently associated with HCC development: age at the time of diagnosis, male gender, and
36
37 history of blood transfusion. While autoimmune liver disease, including PBC, is more common in
38
39 females than in males, HCC incidence in PBC patients was higher in males than in females. In
40
41 agreement with previous reports from Japan, Europe, and USA,^{4,5,11-13} gender was identified as a risk
42
43 factor associated with HCC in the nationwide survey of PBC patients conducted by the Intractable
44
45 Hepato-Biliary Diseases Study Group. HCC incidence was 5.1% in males and 2.0% in females
46
47 (proportion of males, 26.7%), indicating that male PBC patients had a 2.1-fold higher risk of HCC
48
49 compared with female PBC patients. The proportion of males among the PBC patients with HCC
50
51 was consistent with that in the nationwide survey by the Liver Cancer Study Group of Japan (27.5%).
52
53 Moreover, cumulative HCC incidence was 6.5% in males and 2.0% in females during the 10 years
54
55
56
57
58
59
60

1
2
3
4
5
6 after PBC diagnosis, and male PBC patients had a 3.3-fold higher risk of HCC compared with
7
8 females. In general, during the carcinogenesis of HCC, estrogen can protect hepatocytes from
9
10 malignant transformation via downregulation of IL-6 release from Kupffer cells, indicating that
11
12 estrogen-mediated inhibition of IL-6 production by Kupffer cells potentially decreased the risk of
13
14 HCC in females.^{21,22} Therefore, although PBC primarily affects females, HCC may be more
15
16 common in male PBC patients because of a lack of estrogen-mediated prevention. The national
17
18 survey by the Liver Cancer Study Group of Japan revealed that the duration between the diagnosis of
19
20 PBC and that of HCC was shorter in males than in females and that the diagnosis of HCC was
21
22 performed simultaneously at or prior to the diagnosis of PBC in 32.7% males and 14.7% females.
23
24 Several reasons may be responsible for the delayed diagnosis of PBC and carcinogenesis in the early
25
26 stage in males, but the details remain unspecified. Moreover, the rate of past HBV infection and
27
28 alcohol consumption was significantly higher in males than in females, indicating that these factors
29
30 also possibly affect the increased HCC incidence in male PBC patients. Watanabe et al. reported that
31
32 past HBV infection is an important factor in the association of HCC with PBC.¹⁸ In a patient with
33
34 HBV infection, HBV-DNA possibly integrates into the human genome, but the frequency of this
35
36 integration in prior HBV-infected PBC patients with HCC remains unknown. Moreover, because the
37
38 distribution of past HBV infection by gender in the whole PBC population could not be obtained, the
39
40 extent to which previous infection with HBV is directly associated with HCC carcinogenesis in male
41
42 PBC patients remains debatable. However, analysis excluding cases with past HBV infection and a
43
44 history of alcohol consumption revealed that the proportion of males with HCC in PBC patients with
45
46 HCC remained high compared with that of all PBC male patients. In addition, analysis according to
47
48 histological stage (non-cirrhosis vs. cirrhosis) suggested that past HBV infection and alcohol
49
50 consumption were not directly associated with progression to cirrhosis in PBC patients with HCC.
51
52
53
54

55
56 In addition to male gender, the national survey by the Intractable Hepato-Biliary Diseases
57
58
59
60

1
2
3
4
5
6 Study Group demonstrated that old age, low serum albumin levels, low total cholesterol levels,
7
8 advanced histological stage, and symptomatic status at the time of PBC diagnosis were statistically
9
10 significant in PBC patients with HCC compared to those without HCC. However, multivariate
11
12 analysis by gender revealed that histological stage at the time of diagnosis of PBC was an
13
14 independent risk factor for HCC in females, but not in males. In addition to at the time of diagnosis
15
16 of PBC, at that of HCC, histological stage is associated with HCC by national survey for PBC with
17
18 HCC patients. However, there was no difference in any clinical or biological characteristics between
19
20 PBC patients with HCC with or without cirrhosis at HCC diagnosis. In females, HCC incidence
21
22 gradually increased according to histological stage, indicating that the terminal stage of PBC, which
23
24 is a cirrhotic state, may be a risk factor for HCC development in females, whereas males are likely to
25
26 develop HCC at any stage. The carcinogenesis of HCC in PBC patients should be further clarified.
27
28 PBC is pathologically characterized by CNSDC, and the main inflammatory lesions associated with
29
30 PBC are not hepatocytes but cholangiocytes, which may be one of the reasons why HCC incidence
31
32 in PBC patients is relatively low compared with the incidence of sustained hepatic diseases such as
33
34 chronic viral hepatitis and autoimmune hepatitis. Male PBC patients with HCC are thought to be a
35
36 good model because they lack estrogen-mediated prevention of HCC. Unlike that in hepatic
37
38 diseases, intrahepatic cholestasis is found from the early stage in PBC,^{1,23} and some mitogenic
39
40 factors in the bile of PBC patients presumably participate in the carcinogenesis of HCC from an
41
42 early stage.^{17,24} However, this hypothesis remains a matter of speculation, and further study is
43
44 required to clarify the molecular mechanism involved in the carcinogenesis of HCC in PBC patients.
45
46
47
48

49 In conclusion, we investigated the risk factors for HCC using data from two nationwide
50
51 surveys of PBC patients in Japan. Because male PBC patients are at risk of developing HCC at any
52
53 histologic stage, they should be carefully screened for HCC from an early stage of PBC, irrespective
54
55 of histological stage.
56
57
58
59
60

ACKNOWLEDGEMENTS

The authors thank for cooperating questionnaire investigation of PBC with HCC projected at 47th Annual Meeting of the Liver Cancer Study Group of Japan (President, Professor Ichida); Dr. Komori and Dr. Ishibashi (National Hospital Organization Nagasaki Medical Center, Gastroenterology), Dr. Ueda and Dr. Kaneko (Kanazawa University Graduate School of Medicine, Internal Medicine), Dr. Taniai and Dr. Hashimoto (Tokyo Women's Medical University, Internal Medicine and Gastroenterology), Dr. Tateishi and Dr. Koike (The University of Tokyo, Gastroenterology), Dr. Takahashi, Dr. Abe, and Dr. Ohira (Fukushima Medical University, Internal Medicine), Dr. Korenaga, Dr. Tomiyama, and Dr. Hino (Kawasaki Medical School, Internal Medicine), Dr. Shida and Dr. Maguchi (Teine Keijinkai Hospital, Gastroenterology), Dr. Kita and Dr. Osaki (Osaka Red Cross Hospital, Gastroenterology), Dr. Makino and Dr. Fukuda (Ikeda Municipal Hospital, Gastroenterology), Dr. Yoshihara and Dr. Yoshimatsu (Oita University, Internal Medicine 1 Faculty of Medicine), Dr. Inoue and Dr. Enomoto (University of Yamanashi, First Department of Internal Medicine), Dr. Shinomura and Dr. Sasaki (Sapporo Medical University, Internal Medicine), Dr. Sakamoto (Hirosaki University, Graduate School of Medicine, Internal Medicine), Dr. Mukai (Ohta Nishinouchi Hospital, Gastroenterology), Dr. Sugimoto (Nagoya University, Surgery), Dr. Fujiyama, Dr. Nishimura and Dr. Fukumoto (Shiga University of Medical Science, Internal Medicine), Dr. Kon (Kansai Medical University, Surgery), Dr. Kaibori (Kansai Medical University, Hirakata Hospital, Surgery), Dr. Okazaki (Kansai Medical University, Internal Medicine), Dr. Seki (Kansai Medical University, Takii Hospital, Internal Medicine), Dr. Katayama (Osaka Medical Center for Cancer and Cardiovascular Diseases, Internal Medicine), Dr. Nakamura (Okayama University, Internal Medicine), Dr. Fujioka (Okayama Saiseikai General Hospital, Hepatology), Dr. Taura (Nagasaki University, Internal Medicine), Dr. Koga and Dr. Katafuchi (Kurume University, Internal Medicine), Dr. Sakaguchi (Hamamatsu University School of Medicine, Surgery), Dr. Inui (Fujita Health

1
2
3
4
5
6 University School of Medicine, Banbuntane Houtokukai Hospital, Internal Medicine), Dr. Yoshioka
7
8 (Fujita Health University School of Medicine, Internal Medicine), Dr. Yoshikawa (Sanraku Hospital,
9
10 Internal Medicine), Dr. Misawa (Sapporo City General Hospital, Surgery), Dr. Kakizoe (Kakizoe
11
12 Hospital, Surgery), Dr. Tsuchiya, Dr. Kurosaki and Dr. Izumi (Musashino Red Cross Hospital,
13
14 Gastroenterology), Dr. Ogawa (Nishinomiya Municipal Central Hospital, Internal Medicine), Dr.
15
16 Kaiho (Kimitsu Chuo Hospital, Surgery), Dr. Horigome (Iida Municipal Hospital, Surgery), Dr.
17
18 Shibata and Dr. Ariyoshi (University of Occupational and Environmental Health, Japan, Internal
19
20 Medicine), Dr. Muro (National Hospital Organization Ohita Medical Center, Internal Medicine), Dr.
21
22 Ikeda (Yokosuka Kyosai Hospital, Internal Medicine), Dr. Takahashi and Dr. Nakamura (Hokkaido
23
24 Cancer Center, Internal Medicine), Dr. Matsunami (Matsunami General Hospital, Surgery), Dr. Doi
25
26 (Otemae Hospital, Internal Medicine), Dr. Itamoto and Dr. Nakahara (Hiroshima Prefectural
27
28 Hospital, Surgery), Dr. Kitamoto (Hiroshima Prefectural Hospital, Internal Medicine), Dr. Miyazaki
29
30 and Dr. Yakushijin (Osaka University, Graduate School of Medicine, Internal Medicine), Dr.
31
32 Watanabe (Yame General Hospital, Surgery), Dr. Kawada and Dr. Iwai (Osaka City University
33
34 Graduate School of Medicine, Internal Medicine), Dr. Ishihara (Yokkaichi-Shoukaki Center, Internal
35
36 Medicine), Dr. Inoue (Osaka General Medical Center, Internal Medicine), Dr. Hayashi and Dr.
37
38 Ishizu (Kinki Central Hospital of the Mutual Aid Association of Public School Teachers, Internal
39
40 Medicine), Dr. Aoki (Kinki Central Hospital of the Mutual Aid Association of Public School
41
42 Teachers, Surgery), Dr. Usuda (Aizawa Hospital, Gastroenterology), Dr. Nakagome (Shirakawa
43
44 Kosei General Hospital, Internal Medicine), Dr. Onji and Dr. Abe (Ehime University Graduate
45
46 School of Medicine, Internal Medicine), Dr. Chayama and Dr. Aikata (Hiroshima University,
47
48 Graduate School of Biomedical Sciences, Internal Medicine), Dr. Ishibashi (National Hospital
49
50 Organization Nagasaki Medical Center, Clinical Research Center), Dr. Seki (Daito Central Hospital),
51
52 Dr. Itsubo and Dr. Koike (The Jikei University School of Medicine, Internal Medicine), Dr. Hijikata
53
54
55
56
57
58
59
60

1
2
3
4
5
6 (The Jikei University School of Medicine, Daisan Hospital, Internal Medicine), Dr. Sakisaka and Dr.
7
8 Takeyama (School of Medicine, Fukuoka University, Internal Medicine), Dr. Iijima and Dr. Tanaka
9
10 (Hyogo College of Medicine, Internal Medicine), Dr. Yamamoto (Hyogo College of Medicine,
11
12 Internal Medicine), Dr. Takamori (Teikyo University School of Medicine, Internal Medicine), Dr.
13
14 Kikuchi (Teikyo University School of Medicine University Hospital, Mizonokuchi, Internal
15
16 Medicine), Dr. Shinohara (Yokkaichi Municipal Hospital, Surgery), Dr. Nagao (Matsushita
17
18 Memorial Hospital, Gastroenterology), Dr. Kirikoshi and Dr. Morimoto (Yokohama City University
19
20 Medical Center, Internal Medicine), Dr. Okumoto and Dr. Saito (Yamagata University, Internal
21
22 Medicine), Dr. Saito and Dr. Nikami (Nishisaitama-Chuo National Hospital, Gastroenterology), Dr.
23
24 Makita (National Hospital Organization Nishigunma Hospital, Digestive Organ Internal Medicine
25
26 and Surgery), Dr. Nakanishi, Dr. Todo and Dr. Chuma (Hokkaido University Hospital, Internal
27
28 Medicine), Dr. Kawamura (Sapporo Hokuyu Hospital, Institute for artificial organs transplanation
29
30 and gene therapy), Dr. Yamamoto and Dr. Katagiri (Chiba Cancer Center, Surgery), Dr. Watanabe
31
32 and Dr. Shimizu (Gifu Prefectural General Medical Center, Internal Medicine), Dr. Nishimura
33
34 (Toyota Kosei Hospital, Internal Medicine), Dr. Taoka (Suzuka General Hospital, Surgery), Dr.
35
36 Okano (Suzuka General Hospital, Internal Medicine), Dr. Fujita (Tango Central Hospital, Surgery),
37
38 Dr. Monden (NTT WEST Osaka Hospital, Surgery), Dr. Kaneko (NTT WEST Osaka Hospital,
39
40 Internal Medicine), Dr. Nishihara and Dr. Iwasaki (Kochi Medical School, Internal Medicine), Dr.
41
42 Yamaguchi (Kochi Medical School, Surgery), Dr. Saito and Dr. Takami (Kyusyu Medical Center,
43
44 Surgery), Dr. Kajiwara (Steel Memorial Yawata Hospital, Gastroenterology), Dr. Deguchi and Dr.
45
46 Suzuki (Faculty of Medicine, Kagawa University, Gastroenterology and Neurology), Dr. Ogura and
47
48 Dr. Kuroda (Matsuzaka City Hospital, Surgery), Dr. Kamiike (National Hospital Organization Kure
49
50 Medical Center, Surgery), Dr. Takano (National Hospital Organization Kure Medical Center, Internal
51
52 Medicine), Dr. Takeuchi (National Hospital Organization Iwakuni Clinical Center, Surgery), Dr.
53
54
55
56
57
58
59
60

1
2
3
4
5
6 Miyashita (National Hospital Organization Iwakuni Clinical Center, Internal Medicine), Dr.
7
8 Kobayashi (Tokyo Kyosai Hospital, Internal Medicine), Dr. Mizusawa (Yasugi Municipal Hospital,
9
10 Surgery), Dr. Watanabe and Dr. Adachi (Yasugi Municipal Hospital, Internal Medicine), Dr. Tawada
11
12 and Dr. Okabe (Graduate School of Medicine, Chiba University, Internal Medicine), Dr. Yoshida and
13
14 Dr. Kamimura (Saiseikai Niigata Daini Hospital, Internal Medicine), Dr. Hayashi (Hayashi Hospital,
15
16 Surgery), Dr. Yoshikawa (Kokuho Central Hospital, Surgery), Dr. Sato and Dr. Fukuda (Kokuho
17
18 Central Hospital, Internal Medicine), Dr. Kumagai (Showa University School of Medicine, Toyosu
19
20 Hospital, Surgery), Dr. Nomura (Showa University School of Medicine, Toyosu Hospital, Internal
21
22 Medicine), Dr. Sasaki (Showa University School of Medicine, Internal Medicine), Dr. Inoue and Dr.
23
24 Watanabe (Showa University Fujigaoka Hospital, Internal Medicine), Dr. Shimamura (Kurashiki
25
26 Riverside Hospital, Internal Medicine), Dr. Mima (Tokushima Kensei Hospital, Surgery), Dr. Kadota
27
28 (Tokushima Kensei Hospital, Internal Medicine), Dr. Yamazaki (Osaka Koseinenkin Hospital,
29
30 Surgery), Dr. Naito (Osaka Koseinenkin Hospital, Internal Medicine), Dr. Sakamoto and Dr. Sakurai
31
32 (Tokyo Medical and Dental University, Internal Medicine), Dr. Nishida and Dr. Sakamoto (Aiseikai
33
34 Yamashina Hospital, Internal Medicine), Dr. Makuuchi, Dr. Takamoto, and Dr. Sano (Japan Red
35
36 Cross Medical Center, Surgery), Dr. Kobayashi and Dr. Omori (Kameda Medical Center, Internal
37
38 Medicine), Dr. Hirose (Japan Red Cross Otsu Hospital, Internal Medicine), Dr. Kawanami, Dr.
39
40 Takenaka, Dr. Kondo, and Dr. Yamada (Japan Red Cross Otsu Hospital, Gastroenterology), Dr.
41
42 Omura (Hokkaido P.W.F.A.C Sapporo-Kosei General Hospital, Gastroenterology), Dr. Ishizu
43
44 (Hokkaido P.W.F.A.C Sapporo-Kosei General Hospital, Surgery), and Health and Labour Sciences
45
46 Research Grants for Research on Measures for Intractable Diseases for a national survey of PBC
47
48 patients. Finally, the authors would like to thank Enago (www.enago.jp) for the English language
49
50 review.
51
52
53
54
55
56
57
58
59
60

REFERENCE

1. Nakanuma Y, Ohta G. Histometric and serial section observations of the intrahepatic bile ducts in primary biliary cirrhosis. *Gastroenterology* 1979; 76: 1326-1332.
2. Kaplan MM, Gershwin ME. Primary biliary cirrhosis. *N Engl J Med* 2005; 353: 1261-1273.
3. Yamagiwa S, Ichida T. Recurrence of primary biliary cirrhosis and primary sclerosing cholangitis after liver transplantation in Japan. *Hepatology* 2007; 37 Suppl 3: S449-454.
4. Jones DE, Metcalf JV, Collier JD, Bassendine MF, James OF. Hepatocellular carcinoma in primary biliary cirrhosis and its impact on outcomes. *Hepatology* 1997; 26: 1138-1142.
5. Shibuya A, Tanaka K, Miyakawa H, Shibata M, Takatori M, Sekiyama K, Hashimoto N, et al. Hepatocellular carcinoma and survival in patients with primary biliary cirrhosis. *Hepatology* 2002; 35: 1172-1178.
6. Deutsch M, Papatheodoridis GV, Tzakou A, Hadziyannis SJ. Risk of hepatocellular carcinoma and extrahepatic malignancies in primary biliary cirrhosis. *Eur J Gastroenterol Hepatol* 2008; 20: 5-9.
7. Floreani A, Biagini MR, Chiaramonte M, Milani S, Surrenti C, Naccarato R. Incidence of hepatic and extra-hepatic malignancies in primary biliary cirrhosis (PBC). *Ital J Gastroenterol* 1993; 25: 473-476.
8. Miyake Y, Iwasaki Y, Terada R, Okamoto R, Ikeda H, Makino Y, Kobashi H, et al. Persistent elevation of serum alanine aminotransferase levels leads to poor survival and hepatocellular carcinoma development in type 1 autoimmune hepatitis. *Aliment Pharmacol Ther* 2006; 24: 1197-1205.
9. Nakano T, Inoue K, Hirohara J, Arita S, Higuchi K, Omata M, Toda G. Long-term prognosis of primary biliary cirrhosis (PBC) in Japan and analysis of the factors of stage progression in asymptomatic PBC (a-PBC). *Hepatology* 2002; 22: 250-260.
10. Findor J, He XS, Sord J, Terg R, Gershwin ME. Primary biliary cirrhosis and hepatocellular carcinoma. *Autoimmun Rev* 2002; 1: 220-225.
11. Silveira MG, Suzuki A, Lindor KD. Surveillance for hepatocellular carcinoma in patients with primary biliary cirrhosis. *Hepatology* 2008; 48: 1149-1156.
12. Cavazza A, Caballeria L, Floreani A, Farinati F, Bruguera M, Caroli D, Pares A. Incidence, risk factors, and survival of hepatocellular carcinoma in primary biliary cirrhosis: comparative analysis from two centers. *Hepatology* 2009; 50: 1162-1168.
13. Suzuki A, Lymp J, Donlinger J, Mendes F, Angulo P, Lindor K. Clinical predictors for hepatocellular carcinoma in patients with primary biliary cirrhosis. *Clin Gastroenterol Hepatol* 2007; 5: 259-264.
14. Sasaki H, Inoue K, Higuchi K, Yasuyama T, Koyata H, Kuroki T, Yamamoto S, et al. Primary biliary cirrhosis in Japan: national survey by the Subcommittee on Autoimmune hepatitis.

- 1
2
3
4
5 Gastroenterol Jpn 1985; 20: 476-485.
- 6
7 15. Prince MI, Chetwynd A, Craig WL, Metcalf JV, James OF. Asymptomatic primary biliary
8 cirrhosis: clinical features, prognosis, and symptom progression in a large population based
9 cohort. Gut 2004; 53: 865-870.
- 10
11 16. Scheuer P, Lefkowitz JH, editors. Liver biopsy interpretation. 7th ed. Philadelphia: Elsevier
12 Saunders Ltd; 2007.
- 13
14 17. Kadokawa Y, Omagari K, Ohba K, Kitamura S, Ohara H, Takeshima F, Mizuta Y, et al.
15 Hepatocellular carcinoma in a male patient with early stage (stage I) primary biliary cirrhosis.
16 Intern Med 2005; 44: 207-211.
- 17
18 18. Watanabe T, Soga K, Hirono H, Hasegawa K, Shibasaki K, Kawai H, Aoyagi Y. Features of
19 hepatocellular carcinoma in cases with autoimmune hepatitis and primary biliary cirrhosis.
20 World J Gastroenterol 2009; 15: 231-239.
- 21
22 19. Turissini SB, Kaplan MM. Hepatocellular carcinoma in primary biliary cirrhosis. Am J
23 Gastroenterol 1997; 92: 676-678.
- 24
25 20. Caballeria L, Pares A, Castells A, Gines A, Bru C, Rodes J. Hepatocellular carcinoma in
26 primary biliary cirrhosis: similar incidence to that in hepatitis C virus-related cirrhosis. Am J
27 Gastroenterol 2001; 96: 1160-1163.
- 28
29 21. Naugler WE, Sakurai T, Kim S, Maeda S, Kim K, Elsharkawy AM, Karin M. Gender disparity
30 in liver cancer due to sex differences in MyD88-dependent IL-6 production. Science 2007; 317:
31 121-124.
- 32
33 22. Yeh SH, Chen PJ. Gender disparity of hepatocellular carcinoma: the roles of sex hormones.
34 Oncology 2010; 78 Suppl 1: 172-179.
- 35
36 23. Harada K, Ozaki S, Gershwin ME, Nakanuma Y. Enhanced apoptosis relates to bile duct loss in
37 primary biliary cirrhosis. Hepatology 1997; 26: 1399-1405.
- 38
39 24. Nijhawan PK, Therneau TM, Dickson ER, Boynton J, Lindor KD. Incidence of cancer in
40 primary biliary cirrhosis: the Mayo experience. Hepatology 1999; 29: 1396-1398.
- 41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

FIGURE LEGENDS

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 carcinoma 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

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

For Peer Review

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 |

150x112mm (300 x 300 DPI)

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 ($\geq 25\%$) | 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)



Supplementary Table 1

**Factors associated with increased risk of HCC
in female 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 male 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 |

150x112mm (300 x 300 DPI)

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($\geq 25\%$) | 19 % | 39 % | NS |
| Triglyceride (≥ 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 % |

150x112mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

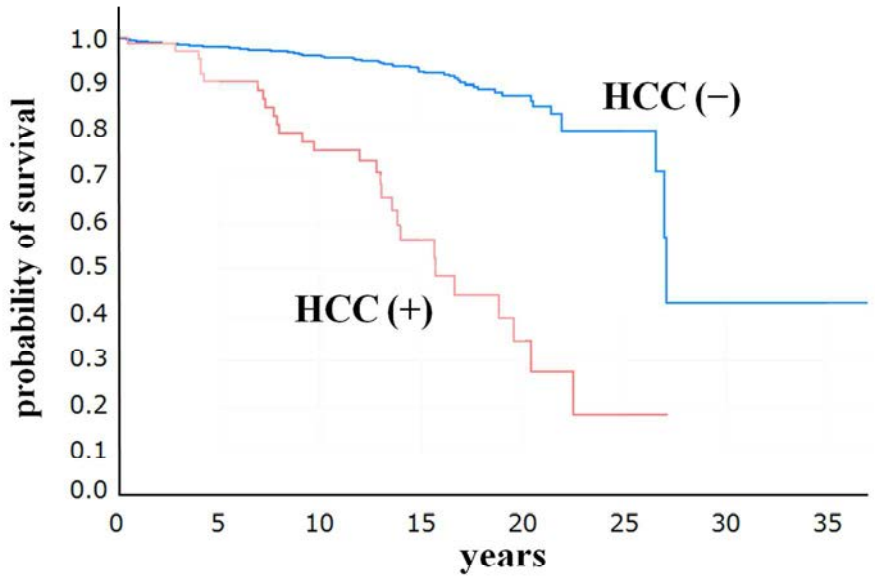


Fig. 1

150x112mm (300 x 300 DPI)

Review

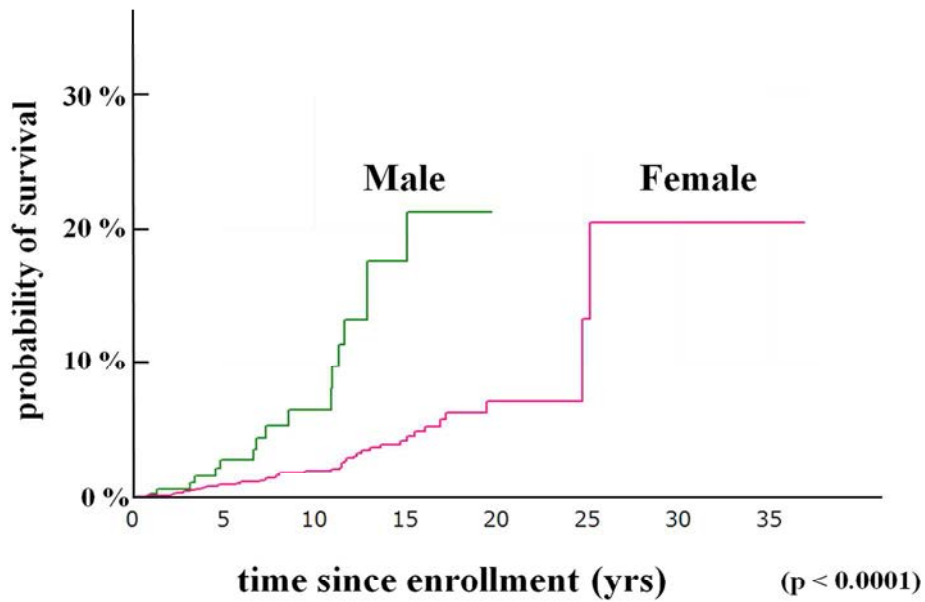


Fig. 2

150x112mm (300 x 300 DPI)

Review

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

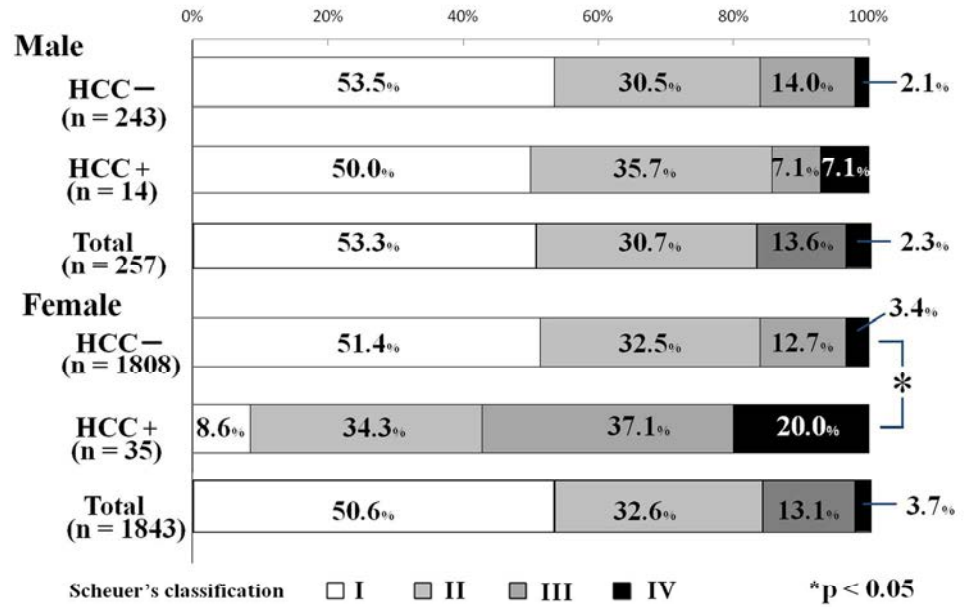


Fig. 3

150x112mm (300 x 300 DPI)

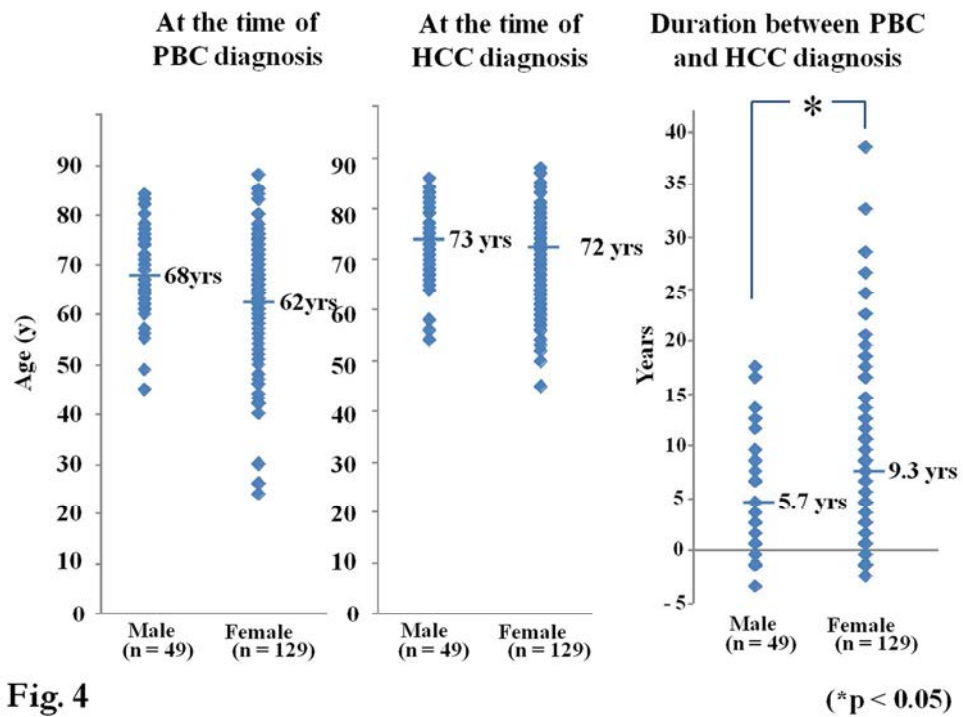


Fig. 4

150x112mm (300 x 300 DPI)

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

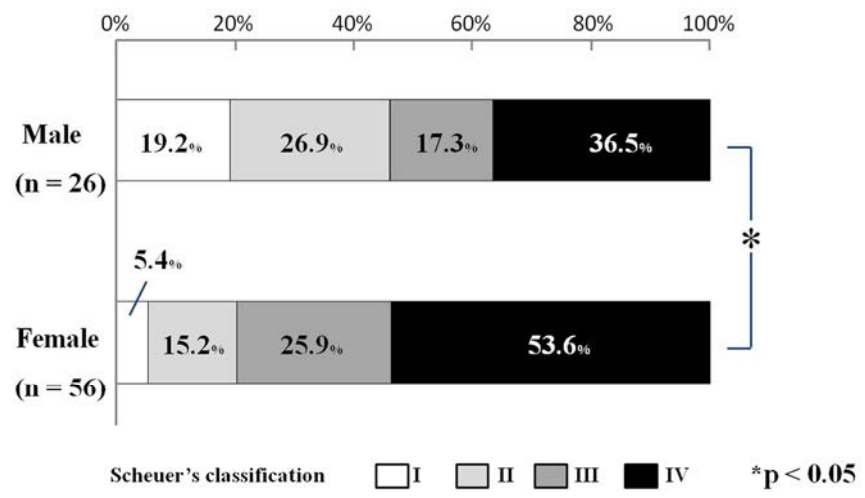


Fig. 5

150x112mm (300 x 300 DPI)