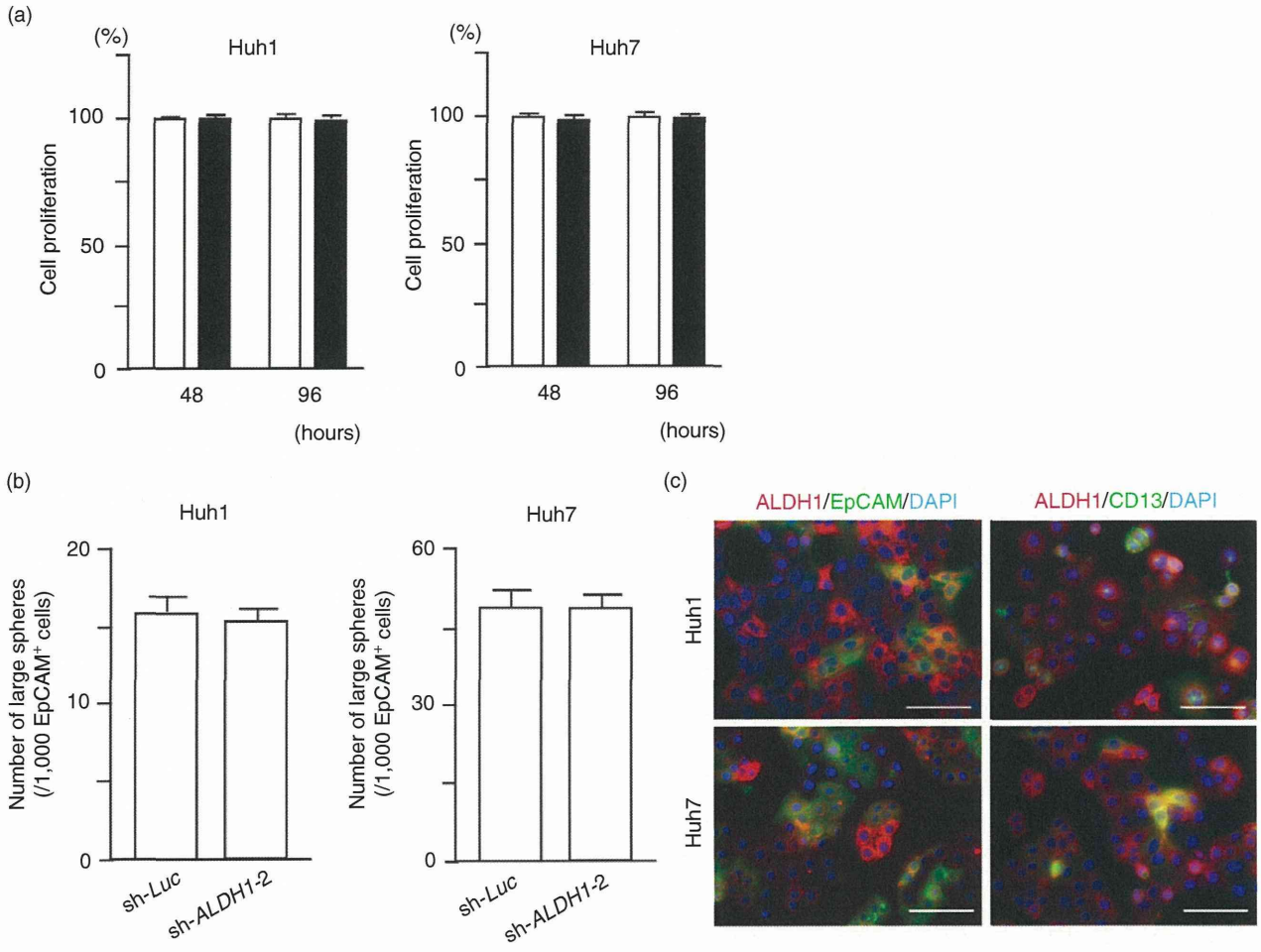

Figure 3 Impact of *ALDH1* depletion on tumor-initiating hepatocellular carcinoma (HCC) cells. (a) Cell proliferation in epithelial cell adhesion molecule (EpCAM)$^+$ HCC cells transduced with indicated viruses. The percentages of Huh1 cells stably expressing sh-*Luc* and sh-*ALDH1*-2 were 100.7 ± 1.1 and 99.0 ± 1.5 at 48 h, and 100.4 ± 1.4 and 97.9 ± 1.8 at 96 h, respectively. The percentages of Huh7 cells stably expressing sh-*Luc* and sh-*ALDH1*-2 were 100.3 ± 1.6 and 98.0 ± 1.8 at 48 h, and 99.4 ± 1.5 and 99.5 ± 1.2 at 96 h, respectively. These results are representative of three independent experiments. □, sh-*Luc*; ■, sh-*ALDH1*-2. (b) Number of large spheres generated from 1000 Huh1 cells stably expressing sh-*Luc*, sh-*ALDH1*-2 were 16.1 ± 1.2 and 15.2 ± 0.9, respectively. Number of large spheres generated from 1000 Huh7 cells stably expressing sh-*Luc*, sh-*ALDH1*-2 were 48.4 ± 4.6 and 47.7 ± 3.0, respectively. These results are representative of three independent experiments. (c) Fluorescence images of Huh1 and Huh7 cells. Aldehyde dehydrogenase 1 A1 (ALDH1) (red) and EpCAM or CD13 (green) expression was merged with nuclear 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) staining (blue). Scale bar = 100 μm.

sh-*ALDH3*-1 and sh-*ALDH3*-2 markedly suppressed *ALDH3* expression, and that sh-*ALDH3*-1 was more effective than sh-*ALDH3*-2 (Supplementary Figure S1d). Of note, *ALDH3* knockdown showed no remarkable changes in cell proliferation and sphere formation (Supplementary Figure S1e,f). Flow cytometric analyses revealed no significant differences in the proportion of EpCAM^{high} cells upon *ALDH3* knockdown as compared to the control cells (48.5 ± 2.7% vs 47.2 ± 2.5%) (Supplementary Figure S2a). Neither the proliferation nor the sphere-forming ability of purified EpCAM$^+$ Huh1 cells was significantly altered upon *ALDH3* knockdown (Supplementary Figure S2b,c). Furthermore, the expression of ALDH3 was not correlated to the expression of EpCAM or CD13 in Huh1 cells (Supplementary Figure S2d).

Expression of ALDH1 in HCC surgical samples

We examined ALDH1 expression in 49 primary HCC and the adjacent non-tumor tissues. All non-tumor tissues homogenously expressed ALDH1 at low levels. In contrast, HCC tumors contained cells with much higher levels of ALDH1 expression at varying frequencies (Fig. 4a,b). Immunoreactivity of ALDH1 was classified as score 0 (0% of cells), score 1 (1–9% of cells), score 2 (10–24% of cells) and score 3 (>25% of cells) according to the percentage of cells strongly expressing ALDH1. The numbers of HCC samples scored as 0, 1, 2 and 3 were 14 (28.6%), 12 (24.5%), 19 (38.8%) and four (8.2%), respectively. There were no HCC tumors in which ALDH1-overexpressing cells accounted for more than 50% of tumor cells. Based on the scoring system, HCC were divided into two groups, ALDH1-low (score 0 or 1) and ALDH1-high (score 2 or 3).

To determine whether ALDH1 and EpCAM or CD13 are co-expressed in tumor cells in primary HCC, we performed dual immunofluorescence staining on nine randomly selected HCC tissues scored as 2 or 3 (Fig. 4c). Of importance, no EpCAM expression was found in any of these HCC tissues. In contrast, CD13 expression was frequently observed in tumor cells of all cases. However, the expression level of ALDH1 was not obviously correlated with that of CD13 except for minor foci where ALDH1-negative cells showed a strong membranous expression of CD13 (Fig. 4c).

ALDH1 expression in severely injured liver

Next, we performed immunohistochemical analyses on 16 diseased livers from liver transplant recipients. Regardless of the cause of cirrhosis, the diseased livers showed diffuse and homogenous expression of ALDH1 as observed in non-tumor tissues. In contrast, ALDH1-overexpressing hepatocytes were observed in the regenerative nodules associated with subacute liver failure (Supplementary Figure S3a). EpCAM expression was observed in cells with low levels of ALDH1 rather than in ALDH1-overexpressing cells (Supplementary Figure S3b). Additionally, CD13 expression was widely observed in hepatocytes regardless of the levels of ALDH1 expression (Supplementary Figure S3c).

Evaluation of ALDH1 expression and clinicopathological features

We then analyzed the association between the ALDH1 expression and clinicopathological characteristics in HCC patients (Table 1). Of interest, ALDH1-high HCC were significantly related to well-differentiated pathology (Edmondson grade I or II) ($P = 0.03$) and low serum levels of α-fetoprotein (AFP) ($P < 0.01$), a hepatic stem/progenitor cell marker. We then performed prognostic analyses of 49 HCC patients by using a Kaplan–Meier analysis of recurrence and survival according to the ALDH1 expression score (Fig. 5a,b). During the follow-up period (28.6 ± 19.2 months), 25 patients developed recurrences of HCC and 10 patients died from HCC. The median RFS in patients with HCC scored as 0, 1, 2 and 3 based on ALDH1 expression was 22, 25, 27 and 30 months, respectively. Although ALDH1-high patients showed a comparatively more favorable prognosis for RFS than ALDH1-low patients, there were no statistically significant differences between the two groups (median RFS; 23 vs 29 months, $P = 0.08$). Even when we took into consideration Union for International Cancer Control (UICC) stages, liver fibrosis and Edmondson grades, the results showed a similar trend to the results for all cases (Fig. 5c–e).

Next, we examined the prognostic relevance of clinical variables (Table 2). The variables with $P < 0.20$ on univariate analysis (age, sex, chronic hepatitis, UICC stage, ALDH1 expression, Edmondson grade, serum AFP, serum total bilirubin and serum albumin, and platelets) were subjected to multivariate analysis. Multivariate Cox's regression analysis revealed that high ALDH1 expression and UICC stage I/II showed a statistically significant correlation with RFS (Table 3). These results indicate that high ALDH1 expression and early stage HCC are favorable prognostic factors after treatment.

DISCUSSION

ALDEHYDE DEHYDROGENASE 1 A1 and ALDH3 are members of the ALDH family, which catalyzes the oxidation of a wide range of aldehydes to carboxylic acids. ALDH1 and ALDH3 expression is closely associated with cancer cell growth and motility.[20] Recently, it has been reported that ALDH1 activity could be a general marker for normal and cancer stem cells. However, our knowledge on their implications in HCC remains limited.[21]

To gain insight into the role of ALDH1 in HCC cells, we performed loss-of-function assays in culture. Surprisingly, the knockdown of *ALDH1* showed no effect on cell proliferation and sphere-formation ability. Concordant with these findings, flow cytometric analyses demonstrated that there is little change in the proportion of

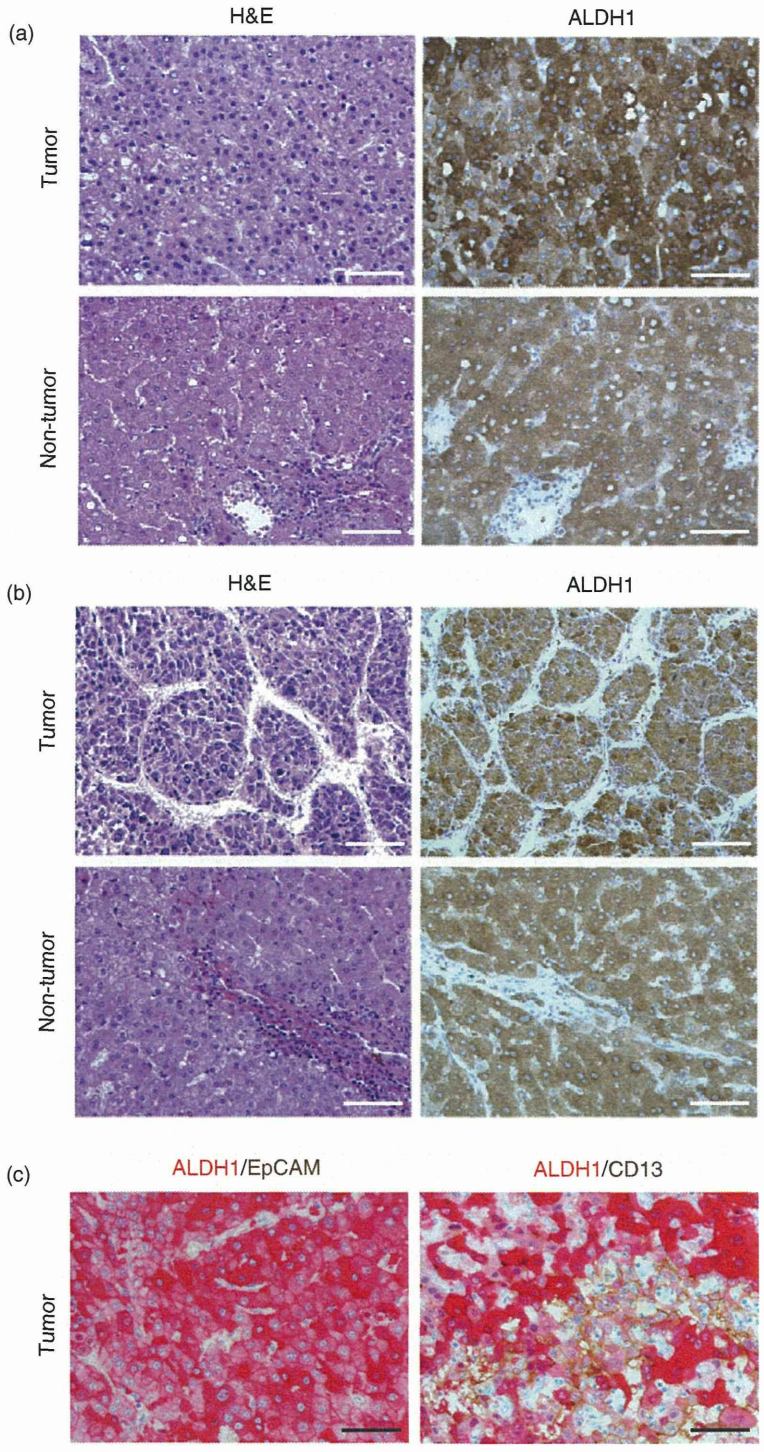

Figure 4 Aldehyde dehydrogenase 1 A1 (ALDH1) expression in hepatocellular carcinoma (HCC) clinical samples. (a) Representative hematoxylin–eosin (H&E) staining and immunohistochemical analysis of ALDH1 in well-differentiated HCC (Edmondson grade I) and the corresponding non-tumor tissue. Scale bar = 100 μm. (b) Representative H&E staining and immunohistochemical analysis of ALDH1 in moderately-differentiated HCC (Edmondson grade III) and the corresponding non-tumor tissue. Scale bar = 100 μm. (c) Representative dual immunohistochemical analysis of ALDH1 (red) and epithelial cell adhesion molecule (EpCAM) or CD13 (brown) in well-differentiated HCC (Edmondson grade I). Scale bar = 20 μm.

Table 1 Clinicopathological features of ALDH1-high and ALDH1-low HCC

Characteristics	ALDH1 expression		
	Low (score 0 or 1, $n = 26$)	High (score 2 or 3, $n = 23$)	*P*-value
Age (years)	65.7 ± 10.8	66.9 ± 8.3	0.23
Sex (male/female)	23/3	18/5	0.28
Etiology (HBV/HCV/other)	5/12/9	6/13/4	0.39
Liver cirrhosis (yes/no)	8/18	13/10	0.09
AFP (ng/mL)	10 381.1 ± 36 117.1	101.6 ± 191.6	<0.01†
DCP (mAU/mL)	4448.3 ± 11 567.5	4843.1 ± 12 759.0	0.76
Tumor diameter (mm)	47.2 ± 27.1	46.5 ± 38.4	0.27
Portal involvement (yes/no)	6/20	4/19	0.74
Edmondson‡ grade (I/II/III/IV)	1/2/17/6	4/4/15/0	0.03†
UICC§ stage (I/II/III/IV)	11/12/3/0	6/11/6/0	0.31

†Statistically significant.
‡Edmondson–Steiner.
§Union for International Cancer Control.
AFP, α-fetoprotein; ALDH1, aldehyde dehydrogenase 1 A1; DCP, des-γ-carboxy prothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus.

EpCAM$^+$ tumor-initiating HCC cells. Previously, we have reported that polycomb group gene products such as BMI1 and EZH2 are overexpressed in tumor-initiating HCC cells and are critical for the maintenance of these cells.[22,23] Given that knockdown of *BMI1* and *EZH2* impaired the sphere-forming ability and significantly reduced the number of EpCAM$^+$ cells, ALDH1 might be dispensable for the maintenance of stem cell features in HCC.

Table 2 Univariate analysis of the relative risks for recurrence-free survival of patients

Variables	Relative risk (95% CI)	*P*-value
Age >70 years	0.38 (0.13–1.07)	0.07
Sex (male)	0.40 (0.12–1.32)	0.13
Chronic hepatitis	1.17 (0.41–3.38)	0.77
UICC stage I/II	0.23 (0.09–0.62)	<0.01†
ALDH1 high expression	0.35 (0.11–1.13)	0.08
Edmondson grade I/II	0.34 (0.09–1.19)	0.09
Serum AFP (>100 ng/mL)	1.60 (0.68–3.76)	0.28
Total serum bilirubin (>1 mg/dL)	1.32 (0.48–3.58)	0.59
Serum albumin (<3.5 g/dL)	2.04 (0.72–5.75)	0.18
Platelets ($<15 \times 10^4/\mu L$)	0.98 (0.33–2.90)	0.97

†Statistically significant.
AFP, α-fetoprotein; ALDH1, aldehyde dehydrogenase 1 A1; CI, confidence interval; DCP, des-γ-carboxy prothrombin; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; UICC, Union for International Cancer Control.

Consistent with our results, it has been reported that ALDH1 is dispensable for the maintenance of HSC and NSC.[24] To examine whether ALDH3, but not ALDH1, could be a CSC marker as reported for breast cancer cells, we conducted loss-of-function assays with ALDH3. The basal expression of ALDH3 was extremely low in HCC cells and *ALDH3* knockdown did not affect the cell growth or tumorigenicity of purified EpCAM$^+$ Huh1 cells. Additionally, the level of ALDH3 expression was not correlated with that of EpCAM or CD13 in Huh1 cells. Although immunostaining analyses in primary HCC showed that ALDH3 was diffusely expressed in both tumor tissues and non-tumor tissues, the level of ALDH3 expression in tumor tissues was lower than that in non-tumor tissues (data not shown). Taken together, it is unlikely that ALDH3 is associated with the stem cell-like features of HCC cells. However, the possibility also exists that redundancy among other ALDH molecules weakens the phenotype of *ALDH1* knockdown HCC cells. Considering that the expression level of ALDH1 in HCC cells is higher than in other type of cancer cells, ALDH1 would be of importance in the biological aspect. Further analyses including the gain-of-function assay of ALDH1 would be necessary.

A high level of ALDH1 expression is closely associated with poor clinical outcomes in a variety of cancers such as breast cancer.[25] In contrast, it has been reported that ALDH1 expression was a favorable prognostic factor in ovarian carcinoma and pancreatic cancer.[26,27] In the

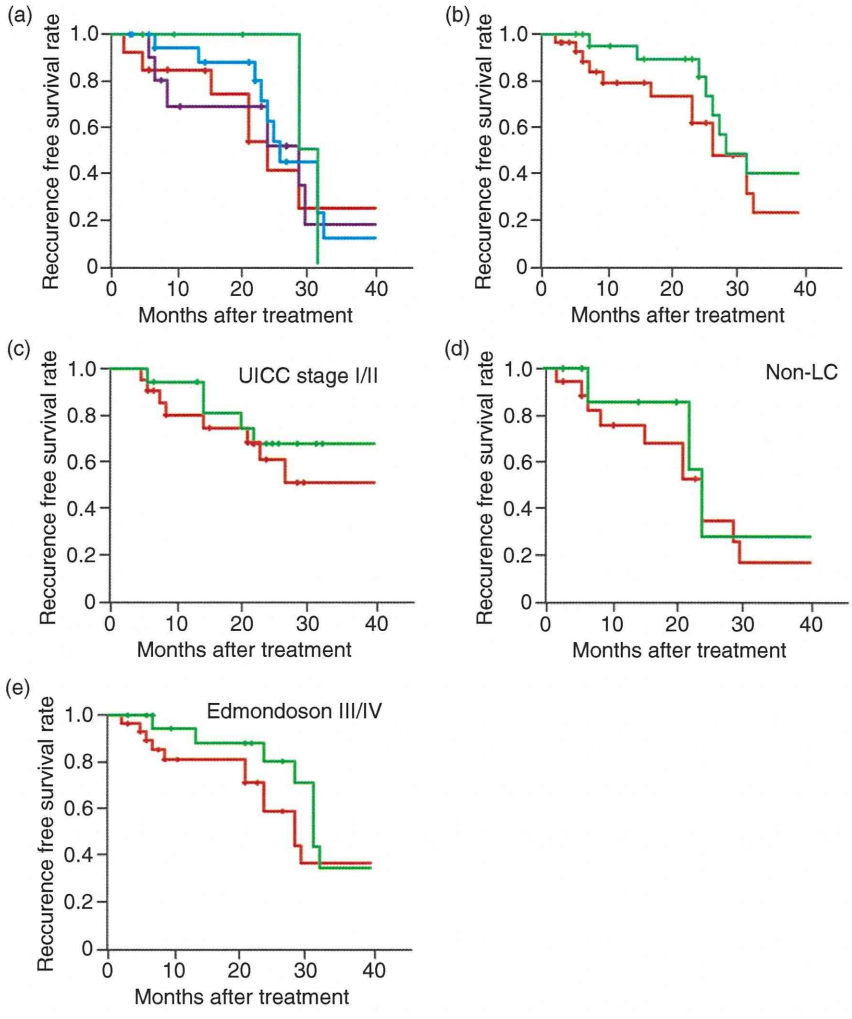

Figure 5 Prognostic analyses based on aldehyde dehydrogenase 1 A1 (ALDH1) expression. (a, b) Cumulative recurrence-free survival (RFS) rate with regard to ALDH1 expression. (c–e) RFS rate of HCC patients with Union for International Cancer Control (UICC) stage I/II (c), non- cirrhosis (d) and Edmondson III/IV (e). (a) ▬, score 0 ($n = 14$); ▬, score 1 ($n = 12$); ▬, score 2 ($n = 19$); ▬, score 3 ($n = 4$). (b) ▬, ALDH1-high ($n = 23$); ▬, ALDH1-low ($n = 26$). (c) ▬, ALDH1-high ($n = 17$); ▬, ALDH1-low ($n = 23$). (d) ▬, ALDH1-high ($n = 10$); ▬, ALDH1-low ($n = 18$). (e) ▬, ALDH1-high ($n = 15$); ▬, ALDH1-low ($n = 23$).

Table 3 Multivariate analysis of the relative risks for recurrence-free survival of patients

Variables	Relative risk (95% CI)	*P*-value
Age >70 years	0.45 (0.19–1.06)	0.07
Sex (male)	0.44 (0.15–1.33)	0.15
UICC stage I/II	0.12 (0.04–0.35)	<0.01†
ALDH1 high expression	0.32 (0.12–0.86)	0.02†
Edmondson grade I/II	0.57 (0.25–1.31)	0.19
Serum albumin (<3.5 g/dL)	1.80 (0.68–4.78)	0.24

†Statistically significant.
ALDH1, aldehyde dehydrogenase 1 A1; CI, confidence interval; UICC, Union for International Cancer Control.

present study, we performed immunohistochemical analyses of primary HCC samples and examined whether ALDH1 expression serves as a predictor of clinical prognosis. ALDH1-high HCC was significantly associated with low serum AFP levels ($P < 0.01$) and well-differentiated pathology ($P = 0.03$). Although the correlation between des-γ-carboxy prothrombin (DCP) level and histological grade has been reported in HCC,[28] a difference in DCP levels was not observed between ALDH1-low and ALDH1-high HCC in the present analysis. Further examinations with a large number of HCC cases are necessary to address this issue.

In agreement with these findings, high ALDH1 expression serves as a favorable prognostic factor for RFS. Taking into consideration that HCC with a similar gene signature to hepatic stem/progenitor cells shows a poor prognosis,[29,30] ALDH1 appears to function as a

differentiation marker rather than a stem cell marker in HCC. Considering that ALDH1 and ALDH2 are highly expressed in liver and are involved in alcohol metabolism, the biological importance of ALDH1 in the liver could differ from that in other organs. This might account in part for the differences in malignant phenotypes and clinical outcomes between HCC and other types of cancers.

A recent report documented that ALDH1$^+$ cells function as murine hepatic progenitor cells.[31] Of interest, our immunohistochemical analyses showed that regenerative nodules associated with subacute liver failure contained a large number of cells that expressed a high level of ALDH1. Because EpCAM and CD13 has also been reported as hepatic stem/progenitor markers,[17,18] we examined the co-expression of ALDH1 and EpCAM or CD13 in these samples. Unexpectedly, but importantly, the level of ALDH1 expression was not associated with that of EpCAM or CD13. Further analyses to determine the role of these cells in human liver regeneration would be of importance.

In summary, *ALDH1* knockdown showed no impact on the cell growth ability or the maintenance of stem cell-like features in HCC cells. Additionally, high expression levels of ALDH1 in HCC tissues were correlated with a favorable clinical outcome. Therefore, ALDH1 might have different functions in different types of cancer.

ACKNOWLEDGMENTS

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Original Article

Hepatitis A, B, C and E virus markers in Chinese residing in Tokyo, Japan

Jun Yan,[1,2] Tatsuo Kanda,[1] Shuang Wu,[1] Fumio Imazeki[1] and Osamu Yokosuka[1]

[1]*Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, and* [2]*Kyowa Clinic, Tokyo, Japan*

Aim: Recently, the number of foreigners living in Japan has been increasing, with the majority originating from China. It is important for us to know the prevalence of hepatitis virus markers among them, as proper medical practices and vaccinations should be prepared when seeing them and their offspring.

Methods: We examined the relationship between the prevalence of hepatitis virus markers: hepatitis B surface antigen (HBsAg), anti-HBs, anti-hepatitis C virus (HCV), anti-hepatitis A virus (HAV) and anti-hepatitis E virus immunoglobulin (Ig)G, and background such as age, birthplace and length of stay in Japan, of 568 Chinese residing in Tokyo, and also of 55 indigenous Japanese.

Results: The prevalence of HBV and HAV markers in Chinese staying in Tokyo is higher than in indigenous Japanese (HBsAg, 10% vs 1.8%; anti-HBs, 45% vs 9.0%; anti-HAV, 90% vs 14%). There were no differences in anti-HCV and anti-HEV IgG between the two groups.

Conclusion: Indigenous Japanese subjects have less immunity against HAV and HBV. The HBV carrier rate is higher in Chinese subjects, and attention should be paid to this issue in clinical practice. It might be important to control hepatitis viruses in Chinese subjects when doctors see them in Japan.

Key words: Chinese, HAV, HBV, HCV, HEV, Tokyo

INTRODUCTION

HEPATITIS A, B, C and E virus (HAV, HBV, HCV and HEV, respectively) cause acute hepatitis, and occasionally fulminant hepatitis, and HBV and HCV also lead to chronic hepatitis, cirrhosis and hepatocellular carcinoma in Japan as well as throughout the world.[1–6] In general, the prevalence of hepatitis viruses follows a wide range of diverse patterns, being dependent on different areas and countries.[7–10]

In Japan, hepatitis B surface antigen (HBsAg) and antibody to HCV (anti-HCV), respectively, were detected in 0.63% and 0.49% in sera from first-time blood donors aged 16–64 years.[5] It was also reported that only fewer than 50% of people have immunity against HAV, estimated from anti-HAV prevalence.[11] Of qualified blood donors, 3.4% were regarded as positive for anti-HEV immunoglobulin (Ig)G.[12] On the other hand, as an example, in China, the prevalence of HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG was reported to be 5.84%, 41.3%, 0.58%, 72.8% and 17.66%, respectively, although this prevalence pattern is well known to differ among different areas in China.[13]

By the end of 2009, 2 186 121 foreigners were living in Japan, and the largest proportion, 31.6%, was born in China, Taiwan and Hong Kong.[14] With increasing numbers of foreigners living in Japan, we will have more opportunities to see them as patients in clinical practice. It is important for us to know, among other things, their prevalence of hepatitis virus markers, as vaccinations and appropriate medical practices should be provided when seeing them and their offspring.

Therefore, in the present study, we examined the relationship between the prevalence of hepatitis virus markers and background such as age, birthplace and their duration of domicile in Japan among Chinese living in Tokyo, Japan.

Correspondence: Associate Professor Tatsuo Kanda, Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. Email: kandat-cib@umin.ac.jp

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METHODS

Study subjects and serum collection

THE SUBJECTS IN this study were 623 consecutive outpatients attending the Kyowa Clinic in Tokyo. Of these patients, 568 (80%) were Chinese who were staying in Japan. The others were 55 indigenous Japanese, and all patients were seen between August 2010 and January 2011 (Table 1). The duration of the Chinese subjects' stay in Japan was 103 ± 76 days. There were no differences in age, sex or alanine aminotransferase (ALT) levels between the two groups, but the platelet counts of the Chinese were lower than those of the Japanese subjects (Table 1). Chinese patients were divided into eight groups according to their birthplace in China, as follows: 32, nine, one, 180, 331, 10, zero and five were from North China (Beijing, Tianjing, Hebei, Shanxi and Inner Mongolia), Central China (Henan, Hunan and Hubei), South China (Guangdong, Guangxi and Hainan), East China (Shanghai, Jiangsu, Zhejiang, Fujian, Shandong, Jiangxi and Anhui), North-East China (Heilongjiang, Liaoning and Jilin), South-West China (Sichuan, Chongqing, Yunnan, Guizhou and Tibet), North-West China (Xinjiang, Shanxi, Gansu, Ningxia and Qinghai) and Hong Kong, Macao and Taiwan, respectively. All patients were adults and the most common symptoms were other than liver diseases. Family history of liver diseases, history of surgeries, blood transfusion, drug abuse and tattoo were investigated from patients' interviews and medical records.

Serological diagnosis

All patients were screened by serological tools for hepatitis A, B, C and E virus infections. HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG were tested in each sample by magnetizing particle aggregation (MAT; Shino-Test Tokyo, Japan), particle agglutination (PA; Fujirebio, Tokyo, Japan), chemiluminescent enzyme immunoassay (CLEIA; Fujirebio), chemiluminescent immunoassay (CLIA; Abbott Laboratories, North Chicago, IL, USA) and enzyme immune assay (EIA; Institute of Immunology, Tokyo, Japan), respectively. A positive reaction was indicated when the cut-off index (COI) exceeded 1.0 in anti-HCV, anti-HAV and anti-HEV IgG. The lower detection limit for HBsAg tested by MAT was 8 IU/mL, corresponding to approximately 10 COI measured by CLEIA method. The lower detection limit for anti-HBs examined by PA corresponded to 30 mIU/mL measured by CLEIA.

Hepatitis B virus genotype of patient sera was determined by ELISA (Institute of Immunology) based on the methodology described by Usuda *et al.*[7,15] Informed consent was obtained at the time of blood sampling from each patient included in the study. This study was approved by the ethics committee of Chiba University, Japan, and that of Kyowa Clinic, and conformed to the Declaration of Helsinki. Sera were collected as part of clinical practice and stored at −20°C until laboratory testing was performed.

Table 1 Background of study patients and hepatitis virus markers

	Total subjects	Chinese staying in Japan	Indigenous Japanese	*P*-value*
No. of patients	623	568	55	
Age, years	47 ± 14	47 ± 14	45 ± 15	NS
Sex (M/F)	292/331	264/304	28/27	NS
ALT (IU/L)	26 ± 44	26 ± 46	25 ± 19	NS
Platelets ($\times 10^4/mm^3$)	22 ± 5.4	22 ± 5.8	24 ± 5.1	0.013
HBsAg (+/−)	63/556	62/502	1/54	0.031
Anti-HBs (+/−)	258/362	259/305	5/50	<0.0001
Anti-HCV (+/−)	11/607	10/553	1/54	NS
Anti-HAV (+/−)	518/100	510/53	8/47	<0.0001
Anti-HEV IgG (+/−)	128/493	120/446	8/47	NS

**P*-value between Chinese subjects staying in Japan and indigenous Japanese subjects.
+, Positive; −, negative; ALT, alanine aminotransferase; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HEV, hepatitis E virus; IgG, immunoglobulin G; NS, not significant.

Data analysis

Data were expressed as mean ± standard deviation. Differences were evaluated by Student *t*-test or χ^2-test. $P < 0.05$ was considered statistically significant. For all tests, two-sided *P*-values were calculated and the results were considered statistically significant at $P < 0.05$. Statistical analysis was performed using the Excel statistics program for Windows ver. 7 (SSRI, Tokyo, Japan) and DA Stats software (O. Nagata, Nifty Serve: PAF01644).

RESULTS

Chinese subjects staying in Tokyo have more immunity against HAV and HBV

AMONG 623 STUDY subjects, 549 (88%) had normal ALT levels (ALT ≤40 IU/L). HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG were determined in 619 (99%), 620 (99%), 618 (99%), 618 (99%) and 621 (99%), respectively. The overall prevalence of HBsAg, anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG in the present study was 10%, 45%, 1.7%, 90% and 21%, respectively (Table 1). The prevalence of HBV and HAV markers of Chinese staying in Japan was higher than that of indigenous Japanese (HBsAg, 10% vs 1.8%; anti-HBs, 45% vs 9.0%; anti-HAV, 90% vs 14%), but there were no differences in anti-HCV and anti-HEV IgG between the two groups (Table 1). These results suggest that Chinese have more immunity against HAV and HBV than indigenous Japanese. A greater proportion of Chinese subjects was HBsAg positive compared to indigenous Japanese subjects.

Sex differences in hepatitis virus markers

Next, we examined the sex differences in the two groups (Table 2). There were no sex differences concerning HBsAg, anti-HBs, anti-HCV and anti-HAV in each of the two groups. Among Chinese subjects staying in Japan, men with anti-HEV IgG were predominant, but this predominance was not seen in the Japanese group (Table 2).

Age differences in relation to prevalence of hepatitis virus markers

Among Chinese subjects staying in Japan, the HBsAg positive rate under 30 years was higher than in those in their 30s ($P = 0.0018$), 40s ($P < 0.0001$) and over 50 years ($P < 0.0001$), and the HBsAg positive rate of those in their 30s was also higher than those over 50 years ($P < 0.053$) (Fig. 1a). Only one HBsAg positive Japanese subject was a 53-year-old man. There were no differences in each age group between Chinese and Japanese subjects (Fig. 1a).

Positive rates of anti-HBs in those under 30 years, in their 30s, 40s and over 50 years in Chinese subjects staying in Japan were higher than those in indigenous Japanese ($P = 0.0037$, 0.0020, 0.0065 and 0.0034,

Table 2 Background of study patients and hepatitis virus markers according to sex differences

	Chinese staying in Japan			Indigenous Japanese		
	Male ($n = 264$)	*P*	Female ($n = 304$)	Male ($n = 28$)	*P*	Female ($n = 27$)
Age, years	47 ± 14	NS	47 ± 13	46 ± 13	NS	43 ± 18
ALT (IU/L)	29 ± 43	NS	23 ± 49	31 ± 19	0.0083	18 ± 16
Platelets ($\times 10^4$/mm^3)	21 ± 5.7	<0.0001	23 ± 5.9	24 ± 5.4	NS	24 ± 4.9
Length of stay (days)	103 ± 78	NS	104 ± 75			
Family of liver diseases (+/−)	13/248	NS	24/273	2/26	NS	0/27
Transfusion (+/−)	5/259	NS	6/297	1/27	NS	1/26
Surgery (+/−)	24/240	0.017	49/254	4/24		4/23
Drug abuse (+/−)	0/264	NA	0/303	0/28	NA	0/27
Tattoo (+/−)	0/264	NS	1/302	0/28	NA	0/27
HBsAg (+/−)	30/232	NS	32/270	1/27	NS	0/27
Anti-HBs (+/−)	119/144	NS	140/162	2/26	NS	3/24
Anti-HCV (+/−)	3/259	NS	7/292	1/27	NS	0/27
Anti-HAV (+/−)	235/28	NS	275/25	3/25	NS	5/22
Anti-HEV IgG (+/−)	68/195	0.015	52/251	2/26	NS	6/21

+, Positive; −, negative; ALT, alanine aminotransferase; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HEV, hepatitis E virus; IgG, immunoglobulin G; NS, not significant.

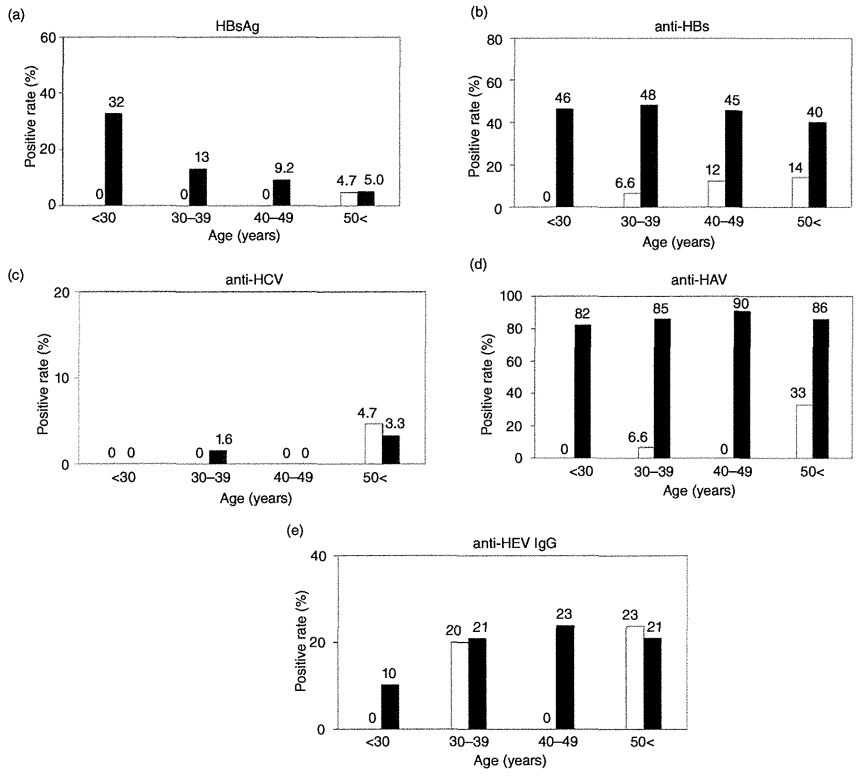

Figure 1 Hepatitis virus markers in study subjects according to age. (a) Hepatitis B surface antigen (HBsAg); (b) anti-HBs antibody; (c) anti-hepatitis C virus (HCV) antibody; (d) anti-hepatitis A virus (HAV) antibody; and (e) anti-hepatitis E virus (HEV) immunoglobulin (Ig)G antibody. White bar, indigenous Japanese; black bar, Chinese staying in Japan. Positive rates (%) are indicated.

respectively). There were no differences between each age group of Chinese subjects and also no differences between each age group of indigenous Japanese subjects in the present study (Fig. 1b).

There were no significant differences in anti-HCV positive rates in each age group of Chinese subjects or in each age group of Japanese indigenous subjects. There were also no significant differences in anti-HCV positive rates of each age group between Chinese and Japanese groups (Fig. 1c).

The positive rate of anti-HAV in subjects under 30 years was lower than in those over 50 years in the indigenous Japanese group ($P = 0.030$) (Fig. 1d). There were no differences among the respective age groups in Chinese subjects. Among the same age groups, the positive rates of anti-HAV in Chinese subjects were higher than those in indigenous Japanese subjects ($P < 0.0001$, each) (Fig. 1d).

There were no significant differences of anti-HEV IgG positive rates in each age group of Japanese indigenous subjects. As for Chinese subjects, there was a difference in anti-HEV IgG positive rate between the groups under 30 years and those in their 40s ($P = 0.029$). There were no significant differences in anti-HEV positive rates of each age group between the Chinese and Japanese groups (Fig. 1e).

Prevalence of hepatitis virus markers in Chinese subjects according to birthplace

Next, we examined the prevalence of hepatitis virus markers in Chinese subjects according to birthplace (Tables 3,4). Although the number of study subjects was limited, the prevalence of anti-HBs, anti-HCV, anti-HAV and anti-HEV IgG was quite similar in Chinese subjects independent of their place of birth. Interestingly, the HBsAg carrier rate was higher in the patients from East China than in those from North-East China (Table 4, $P < 0.0001$). In the background between these two areas (Table 3), young age, male dominance and longer term stays from East China were more than those from North-East China ($P < 0.0001$, $P = 0.029$ and $P < 0.0001$, respectively). As for risk factors of hepatitis virus infection, a history of surgery was seen more frequently in those from North China ($P = 0.023$, Table 3). We determined HBV genotypes in 57 of 63 HBsAg positive subjects and revealed that HBV genotype B was more common in those from East China than in those from North-East China ($P = 0.013$, Table 4). We also

Table 3 Background and risk factors of hepatitis virus infection in Chinese subjects staying in Japan: comparison with indigenous Japanese subjects

Birthplace	Chinese staying in Japan							Japanese
	North China	Central China	South China	East China	North-East China	South-West China	Hong Kong and Taiwan	Indigenous
No. of patients	32	9	1	180	331	10	5	55
Age, years	53 ± 13	40 ± 14	39	41 ± 12	50 ± 13	42 ± 11	60 ± 13	45 ± 15
Sex (M/F)	14/18	8/1	0/1	95/85	140/191	4/6	3/2	28/27
ALT (IU/L)	29 ± 53	22 ± 12	10	26 ± 46	26 ± 47	20 ± 9.8	20 ± 9.8	25 ± 19
Platelets ($\times 10^4/mm^3$)	22 ± 5.2	22 ± 7.3	27	22 ± 5.5	22 ± 6	21 ± 6.5	21 ± 6.5	24 ± 5
Length of their stay (days)	137 ± 90	66 ± 71	46	80 ± 68	113 ± 75	94 ± 76	192 ± 103	
Family history of liver diseases (+/−)	2/30	0/9	0/1	14/162	21/304	0/10	0/5	2/53
History (+/−)								
Transfusion	0/32	1/8	0/1	3/177	6/324	1/9	0/5	2/53
Surgery	7/25	1/8	0/1	14/166	50/280	1/9	0/5	8/43
Drug abuse	0/32	0/9	0/1	0/180	0/330	0/10	0/5	0/54
Tattoo	0/32	0/9	0/1	0/18	1/329	0/10	0/5	0/5

+, Positive; −, negative; ALT, alanine aminotransferase.

Table 4 Hepatitis virus markers in study subjects according to birthplace in Chinese subjects staying in Japan: comparison with indigenous Japanese subjects

Birthplace	Chinese staying in Japan							Japanese
	North China	Central China	South China	East China	North-East China	South-West China	Hong Kong and Taiwan	Indigenous
No. of patients	32	9	1	180	331	10	5	55
Hepatitis virus markers: +/−								
HBsAg	5/27	1/8	0/1	37/142	17/311	1/9	1/4	1/54
HBV genotype (B/C)	1/4	0/1	0/0	12/20	0/16	1/0	0/1	0/1
Anti-HBs	10/22	4/5	0/1	83/95	158/172	5/5	0/5	5/50
Anti-HCV	0/32	0/9	0/1	3/177	7/320	0/10	0/5	1/54
Anti-HAV	25/7	8/1	1/0	147/29	315/15	9/1	5/0	8/47
Anti-HEV IgG	10/22	2/7	0/1	41/138	60/270	5/5	2/3	8/47

+, Positive; −, negative; ALT, alanine aminotransferase; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HEV, hepatitis E virus; IgG, immunoglobulin G; NS, not significant.

determined HCV genotype by direct sequencing HCV core region in two cases from North-East China and one indigenous Japanese, with all three showing genotype 2a (data not shown).

DISCUSSION

THE PRESENT STUDY revealed that Chinese subjects staying in Tokyo have more immunity against HAV and HBV than indigenous Japanese subjects and that approximately 10% of Chinese subjects staying in Tokyo are HBsAg carriers. The HBsAg carrier rate seems to be higher in patients from East China than those from North-East China (Table 4). This might be useful to see the Chinese patients from these areas in clinical practices. There have been several reports about the HBsAg carrier rates of East China[16,17] and North-East China.[13,18] Hepatitis B vaccine was first recommended for routine vaccination of infants in China in 1992.[19] Because of high vaccine prices and user fees charged to parents by local health departments for vaccine purchase and administration, until 2002, infant hepatitis vaccination occurred primarily in large cities of wealthier eastern provinces.[19] In the 2004 survey, estimated vaccine coverage was higher in East China than in North-East China.[19] It is a possible reason why the difference in HBsAg prevalence between these areas was observed in the present study. We do not know the exact reason for this difference, and we consider that further studies will be needed.

Several medical institutes at which mostly Chinese gather have existed in Japan. Kyowa Clinic, one such facility, is located in Okachimachi, Tokyo, an important juncture of traffic networks. Because Japanese newspapers advertise this clinic, and the doctor sees the patients using both Chinese and Japanese languages, this outpatient-only clinic is known to Chinese subjects' staying in Japan. The patients of this clinic consist of 90% Chinese and 10% Japanese. Most of the less than 65-year-old male Chinese patients are cooks in Chinese restaurants, interior decorators and students, most of the less than 65-year-old female Chinese patients are housewives and students, and most of the Chinese patients 65 years or older are unemployed. Most of the Japanese patients are employees of small businesses and residents near this clinic. The present study has an authentic potential in terms of the clinical practice being different from previous studies, such as those concerning blood donors, in spite of the population selection of the present study seeming unnatural. Although selection biasness of patients with Japanese and Chinese background might exist, we included these Japanese patients, who come to the same clinic as controls to compare with Chinese in the present study. Although the number of hepatitis cases is decreasing, hepatitis is still a major health problem in Japan.[5,8,10,20] In China as well, hepatitis is a major public health burden.[13] As more foreigners take up residence in Japan, we are likely to see more Chinese patients in clinical practice, as approximately one-third of such foreigners come from China.[14] The present study might provide us with important information.

The number of cases of adult hepatitis A has been decreasing in Japan in accordance with socioeconomic and sanitation improvements.[9,10] In 1986, a national prevention program was launched in Japan with selective vaccination of babies born to carrier mothers with hepatitis B e antigen (HBeAg).[21] In 1995, this was extended to babies born to HBeAg negative carrier mothers. As a result, the prevalence of HBsAg among younger people born since 1986 has decreased dramatically.[21,22] Because there are no universal vaccination programs against HAV or HBV in Japan, HAV and HBV infections are still seen as important issues.[10]

Hepatitis A virus is a single-stranded RNA virus and usually spreads via the fecal–oral route, similarly to HEV. Of interest is the fact that the distribution of anti-HEV IgG among Chinese subjects staying in Tokyo is similar to that of indigenous Japanese subjects, although the prevalence of anti-HAV in Chinese staying in Tokyo is higher than that of indigenous Japanese (Fig. 1d,e). This may be related to differences in infectious routes of transmission of these two viruses or in differences of HAV vaccination between the two countries, as a certain number of HAV-vaccinated young Chinese adults seemed to be included in the present study.[23,24] In any event, a large proportion of Chinese adults seem to be protected by latent infection or immunization against HAV.[13,25]

The positive rate of anti-HEV IgG in the Kanto metropolitan area of Japan was previously reported as 8.6% in qualified blood donors[12] and 6.5% in health checkups.[26] In general, the positive rate of anti-HEV IgG in China has been recognized to be higher than that in Japan,[27] and the same report described a positive rate of anti-HEV IgG of more than 20% in indigenous Japanese aged 70 years or older. In the present study, the mean age of indigenous Japanese was 45 years (Table 1), and anti-HEV IgG positive indigenous Japanese numbered three in their 30s, one in their 50s, three in their 60s and one in their 70s, with the anti-HEV IgG positive rate being higher than in previous reports.[12,25,27] In most areas of

Japan, the positive rate of anti-HEV IgG in males was higher than that in females. We do not know the exact reasons why our anti-HEV IgG patients were not male-dominant (Table 2). The population selection of the present study may not be unbiased. However, as it seems that Japanese females have in recent years developed a taste for broiled pig innards on skewers compared to before, the potential of HEV infection is likely to grow, and greater attention should also be paid to Japanese females.

As HCV is a blood-borne RNA virus, and blood screening for HCV is a standard procedure in Japan, the distribution of anti-HCV of indigenous Japanese subjects is similar to that of Chinese subjects staying in Tokyo. HBV is an incomplete double-stranded DNA virus that infects through blood products and sexual contact as well as mother-to-baby transmission. The differences in the distribution of anti-HBs may be dependent on a different HBV vaccination status or different past HBV infection.

In conclusion, indigenous Japanese subjects have less immunity against HAV and HBV. As the HBV carrier rate is higher in Chinese subjects, this should receive some attention in clinical practice, and it might be important to control hepatitis viruses in Chinese subjects when they are seen by doctors in Japan.

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HBVコア関連抗原を用いた肝内cccDNAレベル評価：抗ウイルス治療評価の新たな指標

髭　　修平*

索引用語：HBVコア関連抗原，閉環2本鎖DNA，抗ウイルス治療

1 はじめに

B型肝炎ウイルス(HBV)は，通常は不完全2本鎖構造を示すDNAウイルスとして存在しているが，ウイルスが肝細胞内に侵入した後に完全二本鎖となり，covalently closed circular DNA（cccDNA)と呼ばれる閉環・らせん状構造をとる．HBV粒子の構造や複製・増殖の機構の詳細が明らかにされ，HBVの増幅開始段階で転写や増幅の鋳型となる上記のcccDNAが，肝組織内のHBVレベルを規定すると考えられている．

肝組織内のHBVレベルやcccDNAを，血液などの臨床検体で評価することは容易ではないが，最近，HBVマーカーの測定系にも進展がみられる．そのひとつとして，HBVのcore，precore領域と関連したウイルス蛋白量を測定するHBVコア関連抗原(以下，HBcr抗原)がわが国で開発され，2008年から保険適応下に測定可能となった．

近年のB型肝炎治療の進歩により，HBVマーカーの評価や意義も変わってきており，本稿では，HBcr抗原と肝内cccDNA量との関連や，抗HBV治療の効果判定におけるHBcr抗原測定の意義などについて示す．

2 HBVの構造と肝内cccDNA（図1）

HBVは，血液中ウイルス粒子内では不完全2本鎖DNAとして存在しているが，肝細胞内に取り込まれた後は，核内で不完全2重鎖のDNAから完全2本鎖となり，さらに閉環2本鎖DNA（covalently closed circular DNA：cccDNA)として存在する．このDNAの(－)鎖DNAを鋳型とし，宿主のRNAポリメラーゼを利用して4種類のmRNA（3.5 kb，2.4 kb，2.1 kb，0.7 kb)が作られる[1]．このcccDNAを鋳型として転写されたpregenomic RNAからは，逆転写反応により(－)鎖DNAが合成され，さらに(＋)鎖が合成されて，不完全2本鎖DNAができる(複製)．

Syuhei HIGE : Evaluation of intrahepatic cccDNA levels by HBV core-related antigen: a new marker for the evaluation of antiviral treatment
*札幌厚生病院第3消化器科［〒060–0033 北海道札幌市中央区北3条東8丁目5番地］

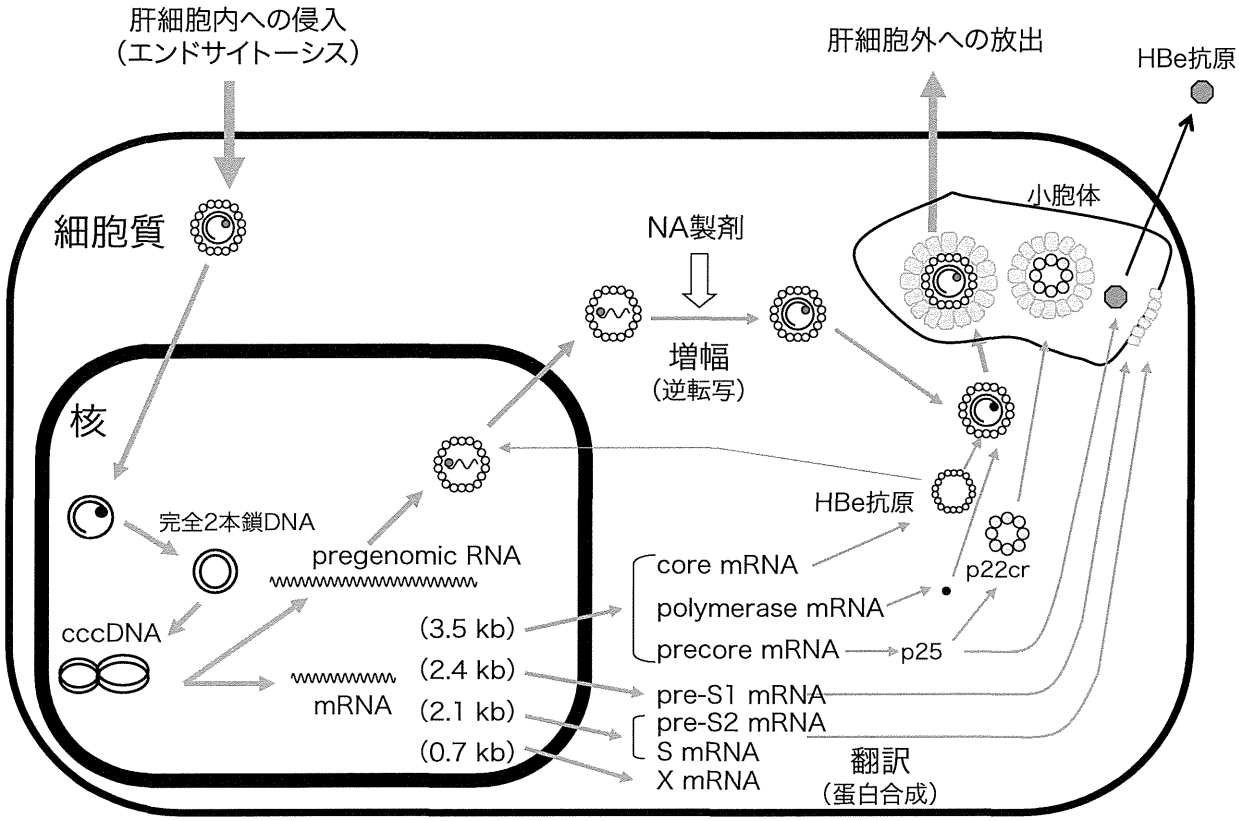

図1　B型肝炎ウイルスの複製と増殖
白抜き矢印(⇩)は，核酸アナログ(NA)製剤の作用部位で，逆転写酵を阻害する．

一方，ウイルス粒子に必要な蛋白や酵素の合成(翻訳)もcccDNAを鋳型として作られる[2]．これらのHBV DNA合成はウイルスのコア内で行われ，コア粒子が小胞体を通過する際にエンヴェロープに包まれてウイルス粒子(Dane粒子)となり血中に放出される．

このように，肝細胞に感染したHBVが増殖するためには，HBV自身の複製とウイルス蛋白の翻訳の2つの過程が必要であり，最も基本的な構造がHBV cccDNAとなる．

B型肝炎に関連したさまざまな病態，例えば，自然経過における肝炎活動性，病期の進行程度，発癌リスクや，治療の反応性，あるいは，治療効果などを評価する際に，正確なHBVレベルの評価が非常に重要であるが，HBV cccDNAは上記の病態把握に最も根本的な情報を示すものと考えられる．

しかし，HBV cccDNAの測定には肝組織検体が必要であり，Addisonら[3]，Heら[4]により定量測定方法が報告されているものの，現実的には頻回の検査は困難である．したがって，臨床的には，肝組織内のcccDNA量を反映する血液中のHBVマーカー測定系が望まれる．

3　血液中で測定可能なHBVマーカー検査の進歩

血液中のHBVに関連する測定系にも，進展がみられる．従来からある検査法では，HBs抗原やHBV DNA量の測定法の改良が，さらに，近年わが国で開発されたマーカーとしてHBcr抗原があげられる．

1. HBs抗原

HBVの抗原測定の基本はHBs抗原である．

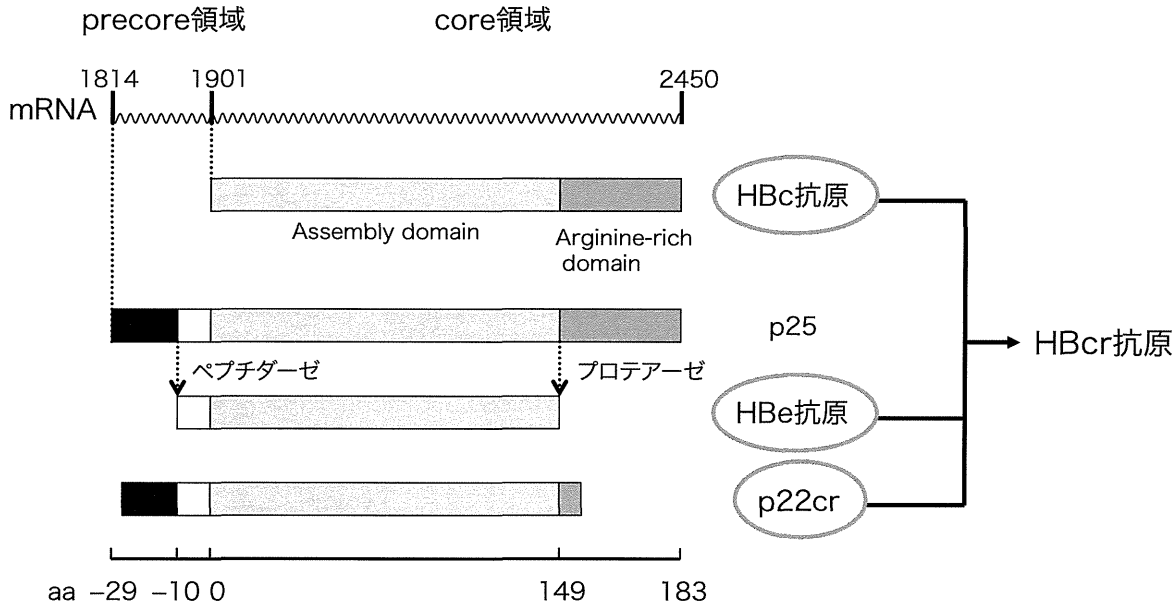

図2　HBcr抗原と関連蛋白
HBcr抗原は，HBV precoreおよびcore mRNAからの翻訳蛋白からなる．

HBs抗原は，感染性を有するDane粒子以外に，小型球形粒子，棹状粒子として存在し，large，middle，smallの3種類のHBs蛋白で構成されている．commercial assayでは，これらのタイプのHBs蛋白をあわせて検出している[5]．最近は，化学発光免疫測定(CLIA)法，あるいは化学発光酵素免疫測定(CLEIA)法により，感度や定量測定性の向上がみられる[6]．

2. HBV DNA量

HBV DNA量の定量測定については，real time PCR法の導入により感度が上昇している．

3. HBcr抗原(図2)

HBc抗原は，粒子形成に作用する領域(assembly domain)と，RNAの内部取り込みに作用する領域(arginine-rich domain)から成り立っている．HBe抗原は，precore mRNAから作られた蛋白であるp25の両端が分解酵素で切断され，細胞外に分泌される．Kimuraら[7]は，precore mRNAから前半部分がpeptidaseで切断されず，後半部分のarginine-rich domainが不十分な蛋白(p22cr)が合成され，これが，HBV-DNAを内包しないDane粒子類似のウイルス粒子となることを報告している．

HBcr抗原は，HBc抗原／HBe抗原／p22crの3種類の蛋白量を同時に測定するもので，HBVのcore，precore遺伝子から翻訳される蛋白の定量法として，新しく開発された[8]．HBcr抗原の測定は，2ステップサンドイッチ法に基づいたCLEIA法によるキット(ルミパルス®HBcrAg)を用いて実施される．血液中のウイルス蛋白を変性し，立体構造を破壊した後で，共通のエピトープを認識するように設定したモノクローナル抗体を用いて測定する．

測定結果は対数で表示され，通常の測定範囲は3.0～7.0 (LogU/ml)である[9]．

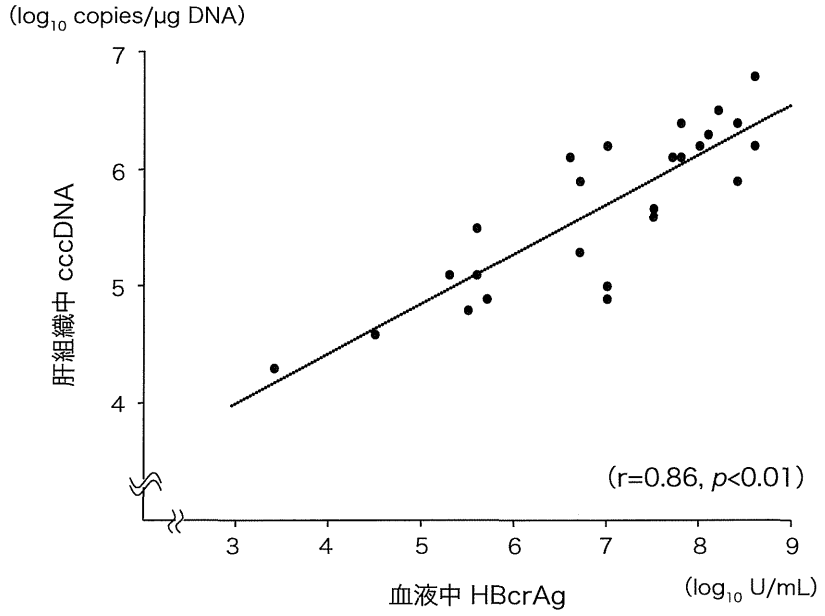

図3　肝組織中cccDNA量と血液中HBcr抗原量の相関

4 血液中HBcr抗原と肝内cccDNAの相関性

B型肝疾患患者の検体を用いて，肝生検組織中HBV cccDNA量と血液中HBcr抗原量を測定した(図3)．その結果，両者には良好な相関性があることが示された(相関係数0.85, p<0.01)．すなわち，血液検体を用いたHBcr抗原量測定にて肝組織中のcccDNA量を推測することが可能であることが示された．両者の相関性については，Suzuki[10]らによっても同様の成績が報告されている．

また，上記の肝組織中のcccDNA量は，血液中のHBs抗原量やHBV DNA量とも相関することを確認している．(相関係数，p値は，それぞれ，0.52，p<0.01，0.78，p<0.01)

5 抗ウイルス治療とHBVマーカーの変化

現在のB型肝炎に対する抗ウイルス治療は，作用機序により，核酸アナログ製剤とインターフェロン(IFN)製剤の使用に大別される．

核酸アナログ製剤は，わが国では，ラミブジン，アデホビル，エンテカビルの3種類が使用可能である．主な作用機序は，逆転写酵素阻害によるウイルス増幅の抑制である．

IFN製剤は，従来型IFNの他に，徐放型のペグIFNの使用も可能となった．IFNは，核酸アナログ製剤と同様のHBV増幅抑制作用のほかに，免疫賦活など複数の薬理作用を有しており，HBV感染肝細胞自体の排除にも直接作用を有すると考えられる[11]．

図1に示されるようなHBVの増殖サイクルと抗ウイルス薬の作用機序を総合すると，薬剤の作用機序の違いが，治療後のHBVマーカーの変化に影響することが推測される．

6 HBcr抗原による抗HBV治療の評価

1. 抗HBV治療開始後のウイルスレベル低下における，薬剤とHBVマーカーの関連

B型慢性肝炎症例に対してエンテカビル単独投与を行った11例と，IFNとエンテカビルを治療開始から16週間併用後，IFNのみ

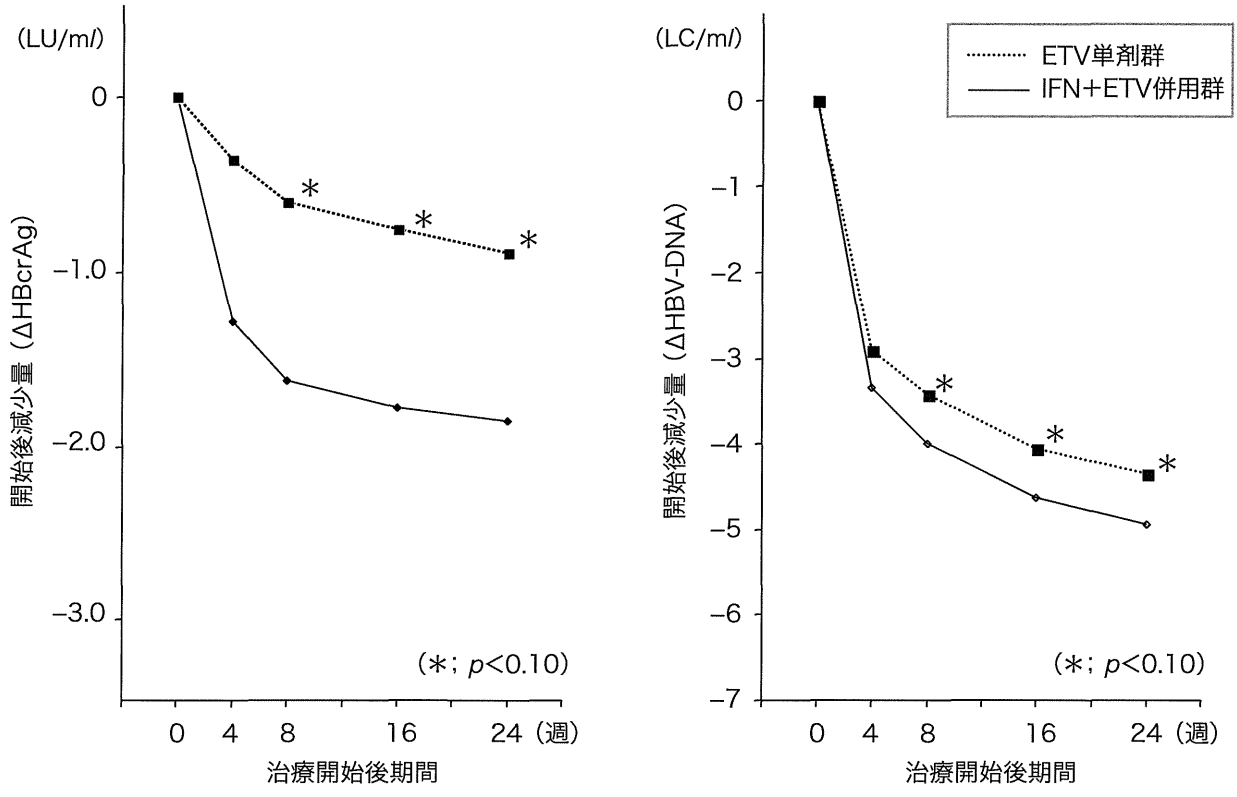

図4　抗HBV治療開始後のHBcr抗原量およびHBV DNA量の減少
ETV：エンテカビル，IFN：インターフェロン

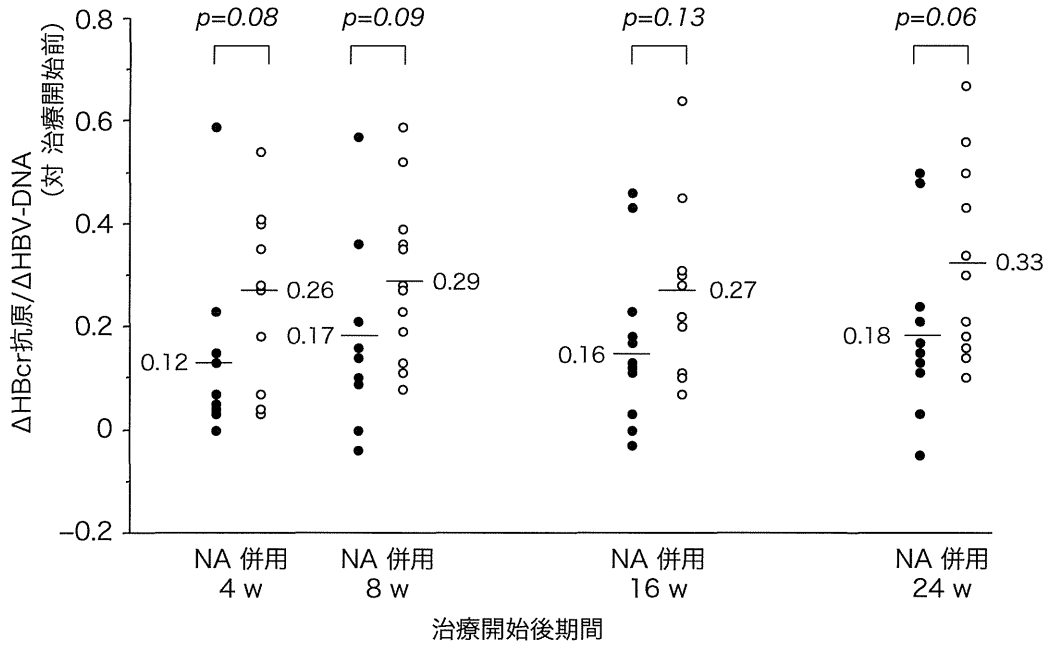

図5　治療開始後のHBcr抗原減少量/HBV-DNA減少量比
治療開始前から各時点までのHBcr抗原量とHBV DNA量の変化量比率を示す．
NA：核酸アナログ投与例，併用：IFNとNA製剤の併用例