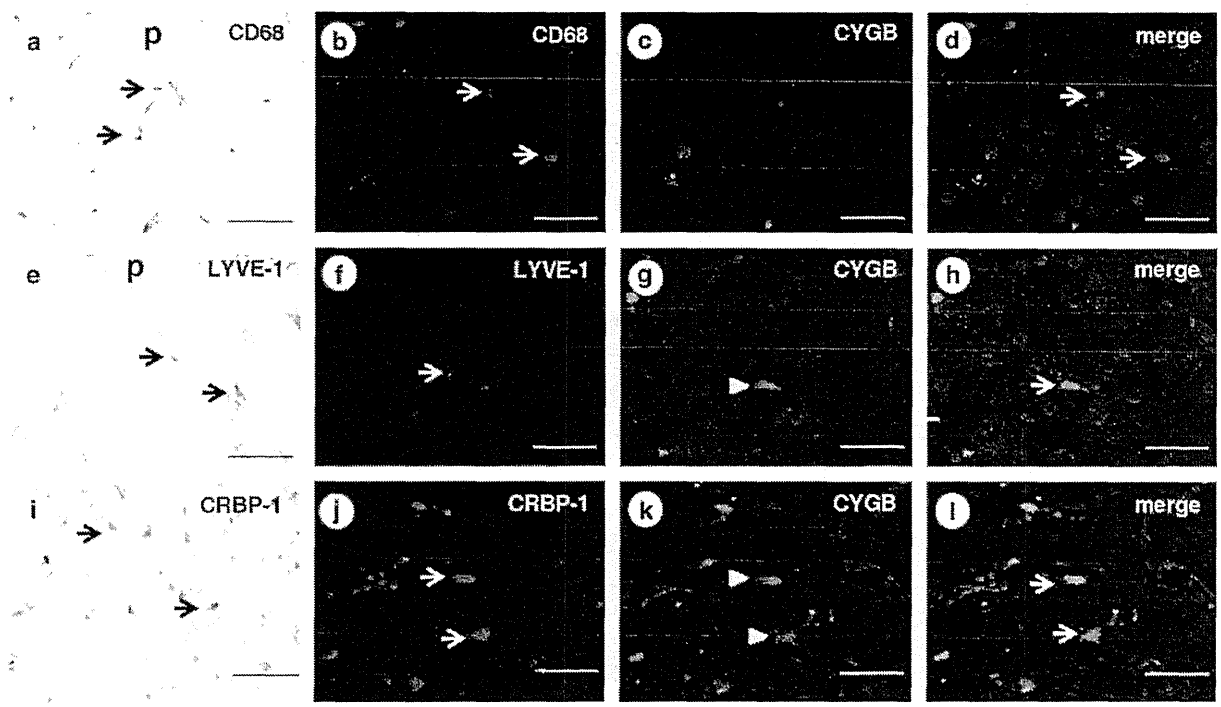


Figure 3 Immunohistochemistry for CD68, LYVE-1, and CRBP-1 in the intact human liver. (**a–d**) Immunohistochemistry for CD68 and CYGB. CD68-positive cells (arrows) were located inside the sinusoidal lumen (**a**). Double immunofluorescence showed that CYGB (**b**) and CD68 (c) did not colocalize as shown in panel **d**. p, portal vein. Bar, 100 μm. (**e–h**) Immunohistochemistry for LYVE-1 and CYGB. LYVE-1-positive cells (arrows) were localized along the hepatic sinusoids (**e**). Double immunofluorescence showed that LYVE-1 (**f**) and CYGB (**g**, arrowhead) did not colocalize, as shown in (**h**). Bar, 100 μm. (**i–l**) Immunohistochemistry for CRBP-1 and CYGB. CRBP-1-positive cells (arrows) were localized in the perisinusoidal space (**i**). Double immunofluorescence showed that CRBP-1 (**j**) and CYGB (**k**, arrowheads) were entirely colocalized, as shown in (**l**). Bar, 100 μm.

LYVE-1-positive cells are sinusoidal endothelial cells in the intact human liver (Figure 3e). Double immunofluorescence staining showed that LYVE-1 did not colocalize with CYGB (Figure 3f–h). Therefore, CYGB was not expressed in hepatocytes, Kupffer cells, or sinusoidal endothelial cells. Double immunostaining for CRBP-1 (Figure 2a and Figure 3i) and CYGB (Figure 3j–l) supported the notion that normal liver tissues contain CYGB- and CRBP-1-double-positive quiescent stellate cells.

CYGB Expression in Fibrotic and Cirrhotic Human Livers

Liver tissues were isolated from patients at different HCV-induced fibrosis stages (from F1 to F4) and subjected to histochemical and immunohistological examination (Figure 4). The extent of collagen deposition at each fibrosis stage was estimated by Sirius red staining, which is shown in Figure 4aA (for F1), E (F2), I (F3), and M (F4). In the liver parenchyma, cells that were positive for CYGB (Figure 4 B, F, J, and N) or CRBP-1 (Figure 4C, G, K, and O) were present along the hepatic sinusoids, indicating that they were stellate cells. α-SMA was expressed in cells around the periportal area, and its expression extended along the expansion of collagen deposition, as shown by Sirius red staining, and along the hepatic sinusoids (Figure 4 D, H, L, and P). Notably, CYGB- and CRBP-1-positive cells (stellate cells) were present inside the hepatic nodules; however, they were scarce in the fibrotic septum of cirrhotic (F4) livers (Figure 4N for CYGB and Figure 4O for CRBP-1), in contrast with the abundance of α-SMA-positive cells in the fibrotic septum (Figure 4P). We further studied the expression of CYGB, CRBP-1, and α-SMA in human NASH tissue (fibrosis stage F2). The results were similar to those obtained in HCV-induced fibrotic tissue: CYGB- and CRBP-1-positive cells were present along the hepatic sinusoids in the liver parenchyma, and α-SMA was expressed by cells around the portal area, with its expression extending along the deposited collagen (Figure 4Q, R, and S).

To quantify the immunohistochemistry results, we performed immunoblot analysis using rabbit polyclonal anti-human CYGB antibodies, which specifically react with human CYGB but not mouse CYGB, in human liver samples and fibrotic mouse livers. We detected a band at the position of purified recombinant human CYGB (21 kDa) in HCV-infected human fibrotic liver tissues at the F2 stage; however, this band was not detected in normal human liver samples. In addition, no CYGB band was observed in fibrotic liver tissues from mice treated with a choline-deficient amino acid-defined diet (for 32 weeks) or *N,N*-diethylnitrosamine

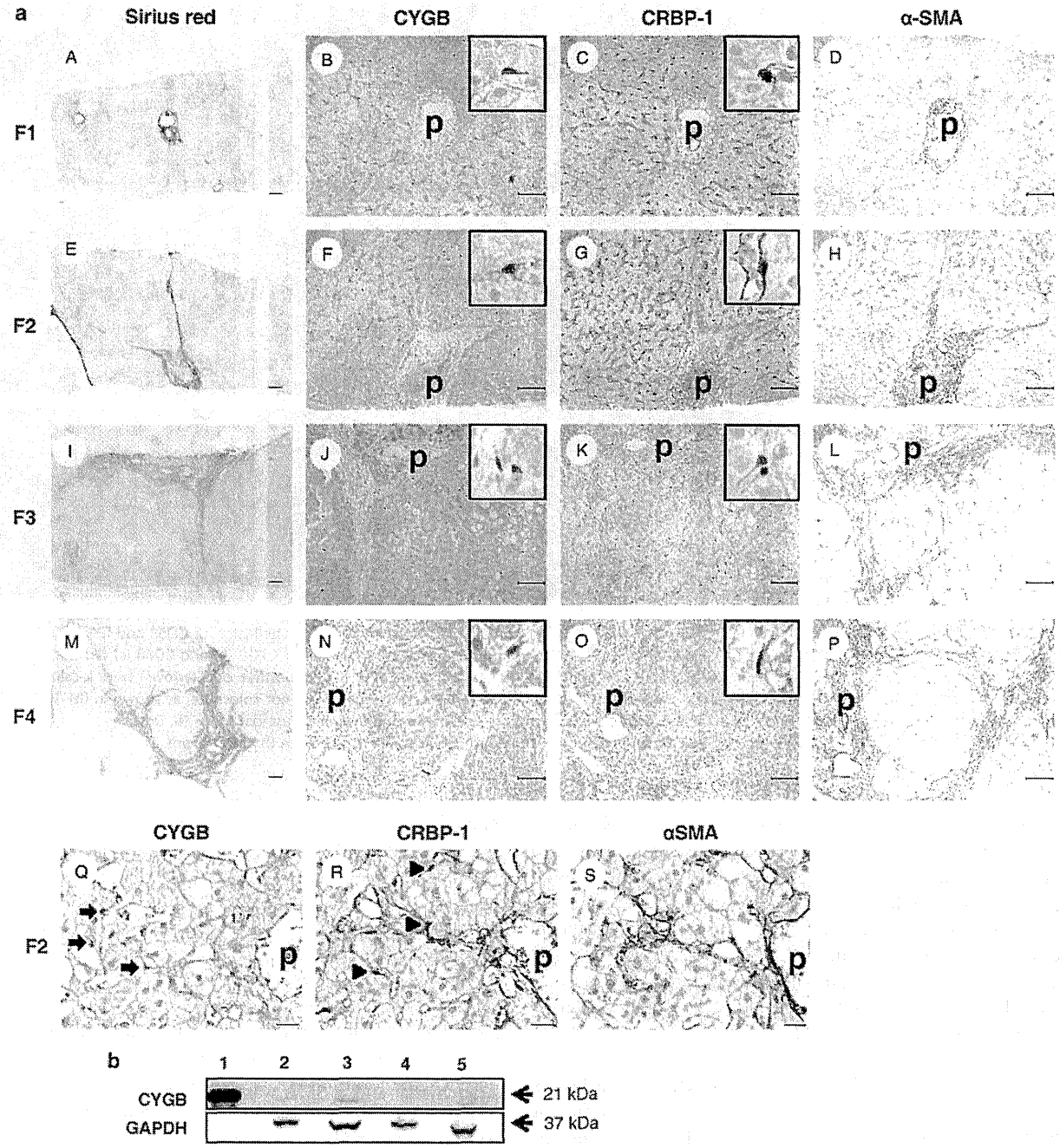


Figure 4 (**a**) Histological analyses of human liver fibrosis at different stages (F1 to F4) according to the new Inuyama classification. (A, E, I, M) Sirius red staining. Bar, 50 μm. (B, F, J, N, Q) Immunohistochemistry for CYGB. Bar, 50 μm. Inserts show high-magnification views of CYGB-positive cells in the liver parenchyma. (C, G, K, O, R) Immunohistochemistry for CRBP-1. Bar, 50 μm. Inserts show high-magnification views of CRBP-1-positive cells in the liver parenchyma. (D, H, L, P, S) Immunohistochemistry for α-SMA. Bar, 50 μm. p, portal vein. (**b**) Immunoblot analysis using rabbit polyclonal anti-human CYGB antibodies that specifically react with human CYGB but not mouse CYGB, with human liver samples and fibrotic mouse livers. (1) Recombinant human CYGB (10 μg); (2) normal human liver (25 μg); (3) hepatitis C virus-infected human liver (25 μg); (4) fibrotic liver from a mouse treated with a choline-deficient amino acid-defined diet (25 μg); and (5) fibrotic liver from a mouse treated with *N,N*-diethylnitrosamine (25 μg). Note that this analysis also revealed the induction of CYGB in fibrotic human livers.

(25 p.p.m. for 25 weeks). Thus, this analysis also revealed the induction of CYGB in fibrotic human livers (Figure 4b).

The scarcity of CYGB- and CRBP-1-positive cells in the fibrotic septum is convincingly shown in Figure 5. The distribution patterns of three myofibroblast biomarkers, α-SMA (Figure 5b, e, h, k, n), Thy-1 (Figure 5c, f, i, and l), and FBLN2 (Figure 5o), in the portal areas were nearly identical in fibrotic livers. The distribution of CYGB was mutually exclusive with the distribution of these three proteins (Figure 5a, d, g, j, and m).

The mutually exclusive localization patterns of these cell type-specific markers were further examined by double immunofluorescence staining (Figure 6). CYGB was expressed in cells close to the parenchymal area of F1 to F3 livers (Figure 6a, d, and g), and its expression did not overlap with that of α-SMA (Figure 6c, f, and i). However, in the F4 liver, cells near the extended fibrotic septum were double positive for CYGB and α-SMA, strongly suggesting that these cells were activated stellate cells. In the F3 liver, neither FBLN2 nor Thy-1 overlapped with CYGB (Figure 6m–r).

Taken together, these data reveal that CYGB and CRBP-1 are excellent markers of human stellate cells in both intact and fibrotic livers and that stellate cells become positive for α-SMA when activated. We hypothesize that cells positive for FBLN2 and Thy-1 are different from stellate cells and exhibit the phenotype of portal myofibroblasts that are α-SMA positive in intact human liver tissue.

Quantitative Analysis of the Contributions of Stellate Cells and Myofibroblasts to the Progression of Fibrosis

Of the 40 HCV-infected patients who underwent liver biopsy, the proportion of fibrotic area (as determined by Sirius red staining and immunostaining for α-SMA and Thy-1) was significantly correlated with the stage of liver fibrosis according to the new Inuyama classification. The Sirius red-positive area increased from 1.8% in F1 to 3.66%, 8.57%, and 16.8% in F2, F3, and F4, respectively (Figure 7a). Similarly, the α-SMA-positive area increased from 1.26% in F1 to 1.82%, 5.65%, and 8.31% in F2, F3, and F4, respectively (Figure 7b). The Thy-1-positive area increased from 1.13% in F1 to 2.13%, 5.43%, and 7.52% in F2, F3, and F4, respectively (Figure 7c). In contrast, the density of CYGB-positive cells (Figure 7d) and CRBP-1-positive cells (Figure 7e) was inversely correlated with the progression of liver fibrosis; the density of CYGB-positive cells was 17.9 ± 1.29, 19.7 ± 1.01, 16.2 ± 0.82, and 13.8 ± 1.06 cells/mm^2 in F1, F2, F3, and F4, respectively, and the density of CRBP-1-positive cells was 9.56 ± 1.24, 14.6 ± 0.77, 12.1 ± 0.83, and 9 ± 0.67 cells/mm^2 in F1, F2, F3, and F4, respectively.

CYGB Expression in Primary Mouse Stellate Cells

We determined above the *in vivo* expression profiles of cell type-specific biomarkers in stellate cells and myofibroblasts in intact and fibrotic human liver tissues. Next, we questioned whether these *in vivo* expression profiles could be reproduced in an *in vitro* system. We utilized primary cultures of mouse stellate cells rather than human stellate cells because human stellate cells in a normal quiescent stage are difficult to obtain for laboratory use. These cells were cultured for up to 7 days, during which changes in the expression levels of CYGB, α-SMA, and Thy-1 were immunohistochemically examined (Figure 8).

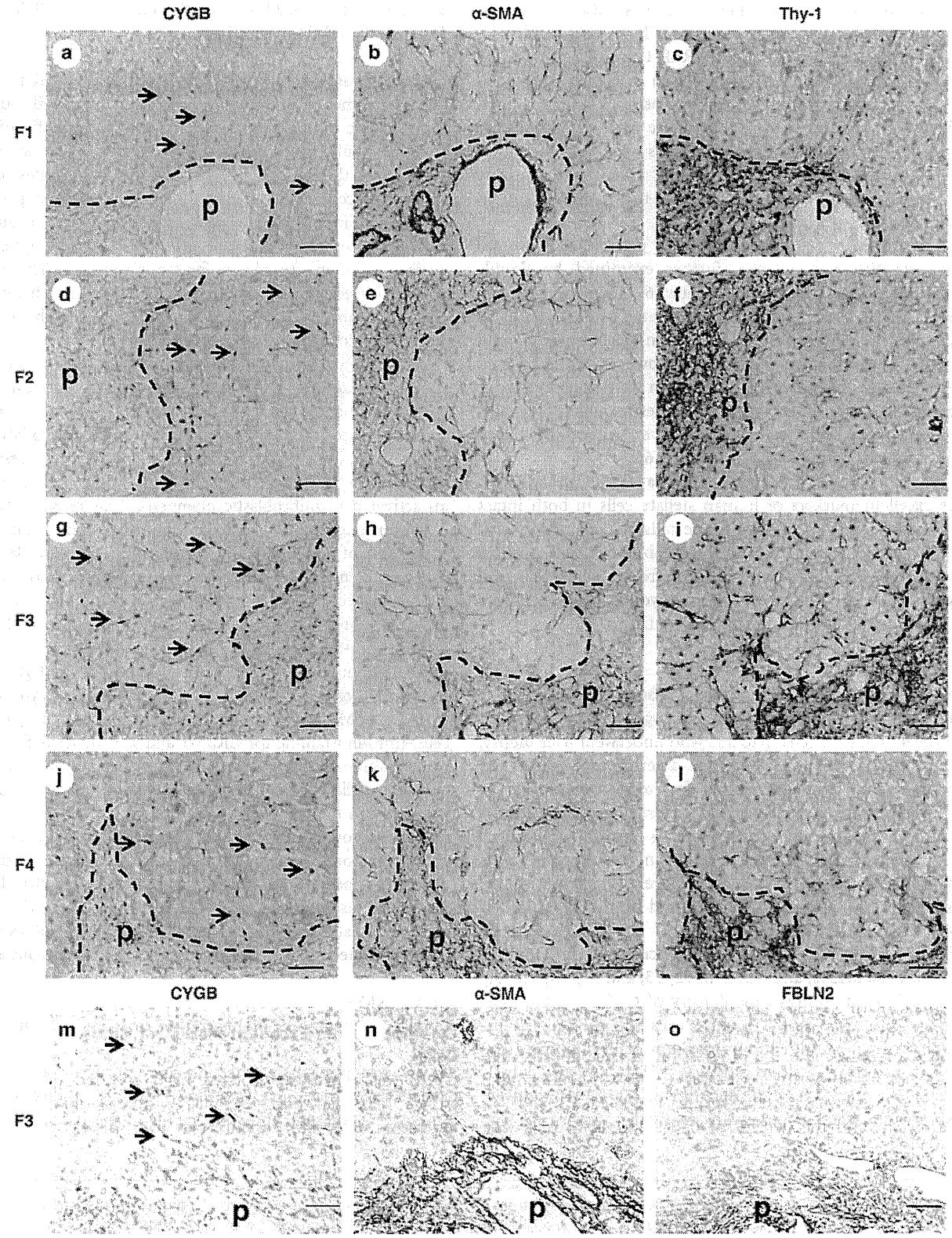
At 1 day of culture after isolation from the intact liver, mouse stellate cells adhered to plastic plates and exhibited round cell bodies with numerous lipid droplets similar to those observed in lipocytes (Figure 8aA). The cell bodies then began to gradually spread and flatten, and they successively increased in size and lost their lipid droplets, resulting in an activated myofibroblastic phenotype (Figure 8aB and C). Immunocytochemical analyses confirmed the consistent expression of CYGB until day 7 of culture (Figure 8aA–C). α-SMA was not observed at day 1 (Figure 8aD); however, it later appeared at days 4 and 7 (Figure 8aE and F). Double immunofluorescence analysis confirmed the presence of both CYGB and α-SMA in stellate cells at days 4 and 7 (Figure 8aH and J), although their intracellular localization patterns were different; Cygb was distributed diffusely in the cytoplasm, whereas α-SMA tended to accumulate at the periphery of the cells. Immunostaining for CRBP-1 and α-SMA yielded similar results, although CRBP-1 expression was decreased in 7-day-cultured stellate cells, presumably because of the loss of vitamin A in the cytoplasm (data not shown). Thy-1 was not observed throughout the culture period (Figure 8aM–O). The above-mentioned expression profiles of the marker proteins were confirmed by immunoblot analysis (Figure 8b). These results led us to hypothesize that the α-SMA-positive cells observed at later stages of culture were not myofibroblasts but rather activated stellate cells because they were Thy-1 negative.

DISCUSSION

Cytoglobin Is an Excellent Marker of Human Stellate Cells

CYGB was previously isolated from cultured rat hepatic stellate cells that have vitamin A storage ability in the quiescent state and function as liver-specific pericytes.[1]

Figure 5 Histological analyses of human liver fibrosis at different stages (F1 to F4) according to the new Inuyama classification. Immunohistochemistry for CYGB (**a**, **d**, **g**, **j**, **m**), α-SMA (**b**, **e**, **h**, **k**, **n**), Thy-1 (**c**, **f**, **i**, **l**), and FBLN2 (**o**). At each stage, CYGB (arrows) had limited expression in sinusoids, and the positive cells were deemed stellate cells. α-SMA was expressed in the vessel walls of the portal vein and artery, around bile ducts, and in the cells in Glisson's capsule. Some α-SMA-positive cells were also present in the parenchyma at stage F4 (**k**). Both Thy-1 and FBLN2 had limited expression in Glisson's capsule and the extended fibrotic septum in all stages of liver fibrosis. Bar, 50 μm. p, portal vein.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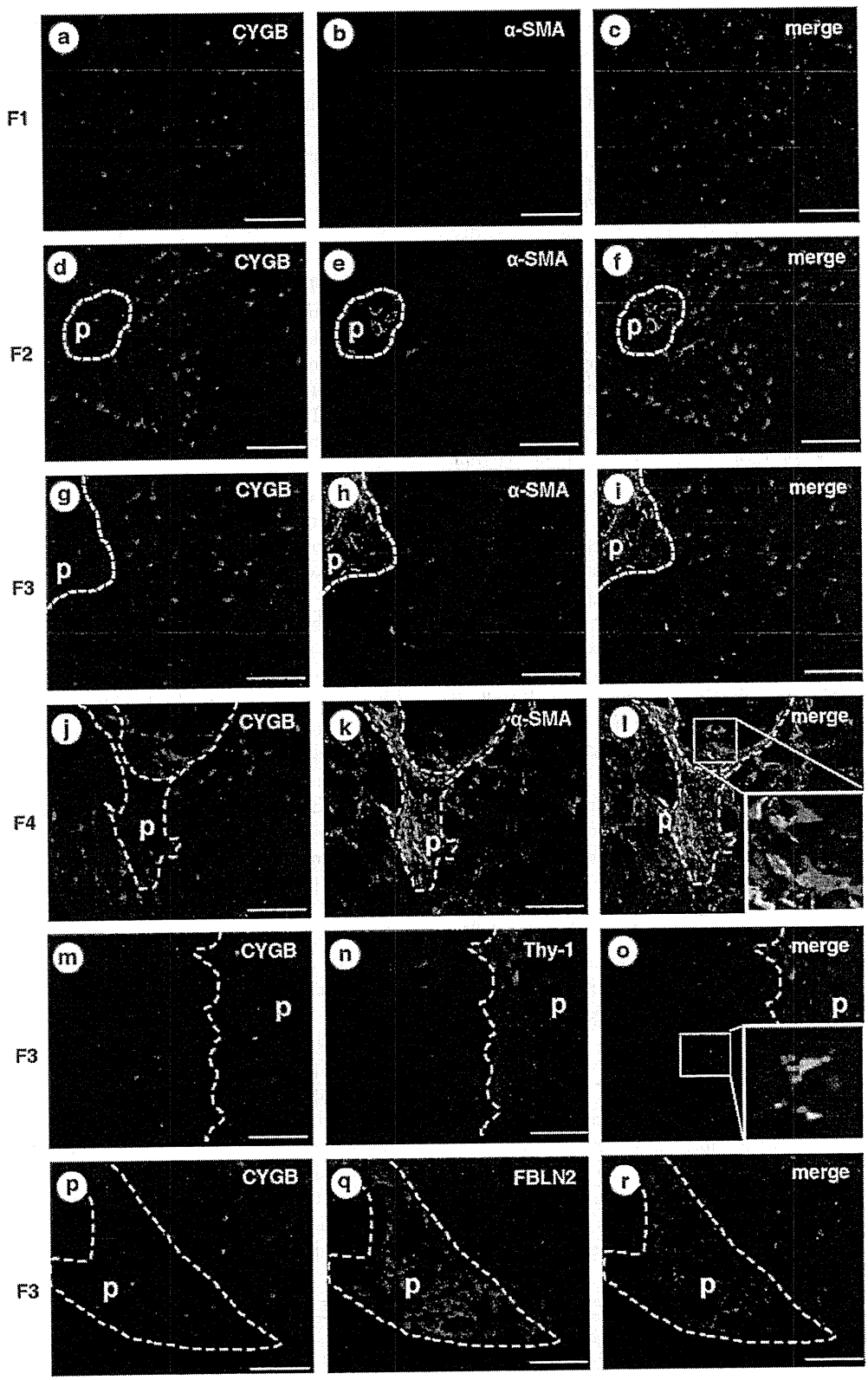
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Histoglobin, CYGB, and stellate cell activation-associated protein were classified as human, mouse, and rat homologs of a hexacoordinate globin that differs from the traditional pentacoordinate globins such as myoglobin and hemoglobin.[1–4] CYGB is induced during the activation of rat hepatic stellate cells, which become myofibroblast-like cells, and its expression is increased in fibrotic livers in rodent models.[1] However, it is unclear whether CYGB is expressed in both stellate cells and portal myofibroblasts. Previously, Ogawa *et al*[21] isolated vitamin A-free cells from the nonparenchymal cell fraction in rat livers using FACS analysis. Ogawa *et al*[21] then demonstrated that vitamin A-positive cells are desmin, CYGB, and α-SMA positive and also highly express oxidized low-density lipoprotein receptor 1, endothelin receptor B, and cardiac troponin T. In contrast, vitamin A-free cells are negative for desmin and CYGB but positive for α-SMA and FBLN2. These cell types express high levels of arginine vasopressin receptor V1a (Avpr1a), gremlin, osteopontin, collagen a3(V), and lumican. Thus, Ogawa *et al*[21] concluded that CYGB could be a promising molecular marker of rat hepatic stellate cells. Furthermore, Bosselut *et al*[29] performed a comparative proteomic study to identify markers and gain insight into the distinct functions of myofibroblasts derived from either hepatic stellate cells or portal mesenchymal cells in rats.[21] The two cell types were subjected to comparative analyses by 2-D MS/MS. CYGB was confirmed to have the highest level of overexpression in activated stellate cells, as confirmed by reverse-transcription quantitative real-time PCR, immunoblot, and immunocytochemical analyses. Thus, CYGB was identified as the best marker for distinguishing stellate cells from portal myofibroblasts. The results also suggested different functions for the two cell populations in the liver wound-healing response, with a prominent role for portal myofibroblasts in scar formation. It should be noted that these previous studies confirmed the expression of CYGB in rodent hepatic stellate cells and *in vivo* models, whereas the present study addressed the actual localization of CYGB in human stellate cells, but not in portal myofibroblasts that are positive for FBLN2 and Thy-1.

Definition of Hepatic Myofibroblasts

The term 'myofibroblast' was first proposed by Gabbiani *et al*[30] in 1972 to refer to fibroblastic cells located within granulation tissue that exhibit substantial cytoplasmic microfilaments composed of actin, myosin, and associated proteins.[29] In particular, the microfilaments of myofibroblasts contain α-SMA that is the actin isoform typical of smooth muscle cells located in the vessel wall[30] and has become the most reliable marker for myofibroblastic cells.[31,32] Myofibroblasts are additionally positive for Thy-1.[12,13]

In the liver, hepatic stellate cells and portal fibroblasts are able to acquire a myofibroblastic phenotype,[8,33] although it has remained difficult to distinguish myofibroblastic (activated) stellate cells from portal myofibroblasts in human liver tissue. In the current study, we showed that cells that are positive for both CYGB and CRBP-1 represent the quiescent phenotype of human stellate cells that are uniquely localized in the perisinusoidal space, and cells that are additionally positive for α-SMA are myofibroblastic (activated) human stellate cells that are predominantly present near the fibrotic septum of advanced fibrotic liver tissues. Furthermore, we observed that cells positive for Thy-1 and FBLN2 in normal liver tissues (ie, portal myofibroblasts) were present but scarce around the portal vein area.

Dudas *et al*[12] first reported that Thy-1 is an *in vivo* and *in vitro* marker of rat hepatic myofibroblasts, and later confirmed that Thy-1 is not present in normal or capillarized sinusoids or in isolated rat stellate cells, and that it is neither inducible in isolated stellate cells nor upregulated in myofibroblasts.[14] In accordance with this report, we detected Thy-1 positivity to a limited extent around the portal vein area in the intact human liver and in the extended fibrotic septum of the fibrotic human liver, where portal myofibroblasts are located. Culture experiments using mouse stellate cells confirmed that these cells express CYGB throughout the culture period until day 7 and α-SMA at days 4 and 7, whereas Thy-1 is not expressed throughout this period. Thus, our data also support the hypothesis that Thy-1 is not a marker of hepatic stellate cells in humans or mice, although it is expressed in myofibroblasts around the portal vein area. The reason for the minimal expression of Thy-1 in portal myofibroblasts is not known, although Thy-1 regulates fibroblast focal adhesions, cytoskeletal organization, and cell migration.[34]

FBLN2 is an extracellular matrix protein of the fibulin family that binds various extracellular ligands and calcium. FBLN2 is present in the basement membrane and stroma of several tissues and may play a role in organ development, particularly during the differentiation of heart, skeletal, and neuronal structures. Knittel *et al*[15] reported that FBLN2-positive MFs are detectable in the portal field, vessel walls, and hepatic parenchyma of the normal liver, and their number is increased in the septal regions during liver fibrogenesis in rat models. These findings are similar to

Figure 6 Double immunofluorescence staining. CYGB (red in panels **a**, **d**, **g**, and **j**; green in panels **m** and **p**), α-SMA (green in panels **b**, **e**, **h**, and **k**), DAPI (blue), Thy-1 (red in panel **n**), and FBLN2 (red in panel **q**) are shown. Merged photographs of CYGB and α-SMA, FBLN2, or Thy-1 are also presented. CYGB was expressed in the parenchyma and inside hepatocytic nodules. Cells constituting the fibrotic septum in advanced fibrosis (F3) were positive for α-SMA but negative for CYGB. CYGB and α-SMA double-positive cells were occasionally present around the fibrotic septum of F4 liver (**i**). CYGB-positive cells did not overlap with cells that were positive for Thy-1 or FBLN2 (**o** and **r**). Bar, 100 μm.

our present observations in diseased human livers. Thus, FBLN2 and Thy-1 are reliable cellular markers of portal myofibroblasts that differ from stellate cells that are positive for CYGB and CRBP-1 in intact and fibrotic human livers. In the portal area, MFs that are negative for CYGB are the main cells that induce fibrotic septum formation. Thus, targeting of these cells in addition to stellate cells has therapeutic potential for controlling fibrotic septum development.

Cellular Markers of Hepatic Stellate Cells

Based on our present results, we emphasize the superiority of CYGB as a marker of human stellate cells in both intact and fibrotic livers. Several markers of stellate cells have been reported in rodents and humans. Stellate cells store vitamin A-containing lipid droplets, suggesting that vitamin A may be a useful marker of stellate cells.[35] However, a specific staining method to identify vitamin A or related compounds,

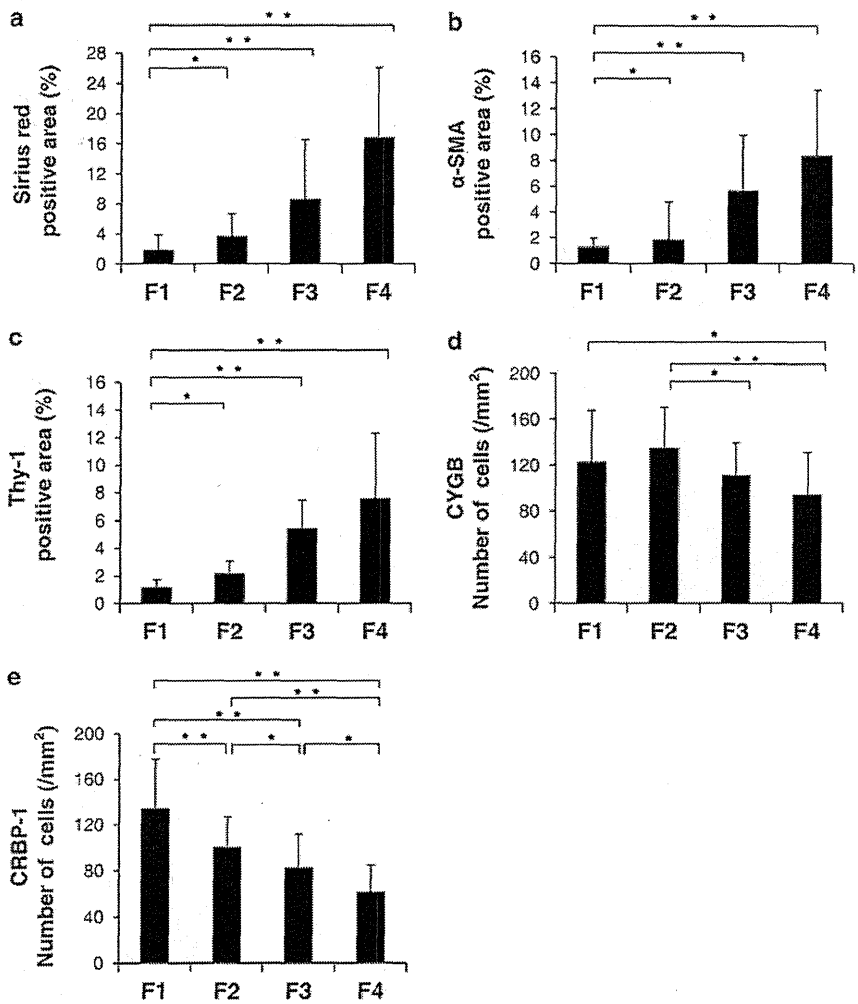
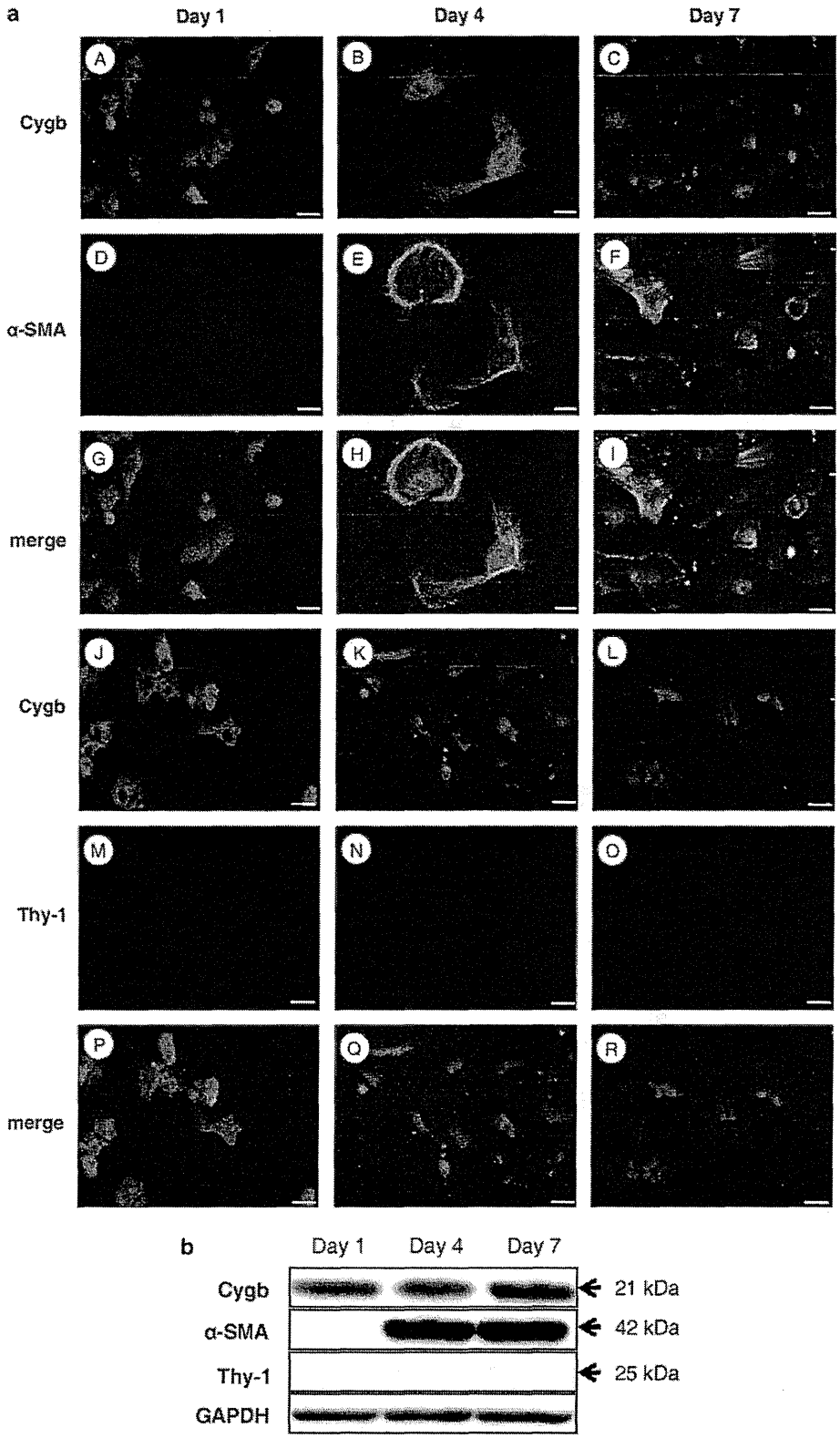


Figure 7 Morphometric analysis. (**a–c**) Correlation between hepatic fibrosis stage (according to the new Inuyama classification) and the ratio of the Sirius red-positive area (**a**), α-SMA-positive area, (**b**) or Thy-1-positive area (**c**). Positive areas for Sirius red, α-SMA, or Thy-1 immunohistochemistry were determined using Lumina Vision 2.4 bio-imaging software (Mitani Corporation, Tokyo, Japan). Note that the Sirius red-positive area, α-SMA-positive area, and Thy-1-positive area increased with the progression of liver fibrosis. (**d**, **e**) CYGB or CRBP-1-positive cells were counted in a 1.4 mm^2 area under a × 100 objective. Note that the CYGB- and CRBP-1-positive cell numbers decreased as liver fibrosis progressed. $^*P<0.05$, $^{**}P<0.01$.

Figure 8 CYGB expression in primary cultured mouse stellate cells. After 1 day of culture following isolation, mouse HSCs adhered to plastic plates and exhibited round cell bodies with numerous lipid droplets similar to those observed in lipocytes. Cell bodies then began to gradually spread and flatten, increasing in size and losing lipid droplets, resulting in the activated myofibroblastic phenotype. (**a**) Immunocytochemical analyses confirmed the expression of CYGB throughout the experimental period (A–C), and α-SMA was detected at days 4 and 7 (D–F). Double immunofluorescence showed that activated mouse stellate cells were positive for both Cygb and α-SMA (G, H, I). Under identical culture conditions, Thy-1 was not observed in mouse stellate cells (M, N, O) that were positive for Cygb (J–L, P–R). Bar, 20 μm. (**b**) Immunoblot analyses confirmed the presence of CYGB at days 1, 4, and 7 and α-SMA at days 4 and 7. Thy-1 was not detected throughout the culture period.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Cygb
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Day 1
Day 4
Day 7
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α-SMA
Thy-1
GAPDH
21 kDa
42 kDa
25 kDa

such as retinol and retinoic acid, has not been developed, and detection of these compounds via fluorescence microscopy is inconvenient for fixed human liver tissues obtained via clinical procedures. In this context, the use of CRBP-1, a carrier protein of intracellular retinol, is reasonable.[11] CRBP-1 was observed to be downregulated in human livers with advanced fibrosis (Figure 7), presumably because of the loss of vitamin A in stellate cells upon cell activation.

As discussed above, α-SMA is frequently used as a marker of activated and myofibroblastic stellate cells.[29–33] However, this cytoskeletal protein is also expressed in portal myofibroblasts and vascular smooth muscle cells in the arteries, portal vein, and central veins, indicating that α-SMA is not specific for stellate cells. Vinculin, a membrane-cytoskeletal protein in focal adhesion plaques, and synemin, an intermediate filament, show localization patterns similar to that of α-SMA.[36,37]

Desmin, a 52 kD protein that is a subunit of intermediate filaments in skeletal muscle, smooth muscle, and cardiac muscle, was originally identified as a stellate cell marker by Yokoi *et al*[10] in 1984. Desmin is clearly detectable in mouse and rat stellate cells in tissue and in primary culture but not expressed by human stellate cells. Furthermore, desmin expression in rodent hepatic stellate cells has been reported to be both heterogeneous and location dependent.[38] Thus, desmin is no longer considered to be a specific marker of stellate cells. Although neural cell adhesion molecule (also known as CD56) and the intermediate proteins glial fibrillary acidic protein and vimentin have frequently been used as markers of stellate cells, these proteins are also expressed by myofibroblasts.[39,40] In addition, although neurotrophin-3 is specific for stellate cells, it disappears in activated stellate cells in human tissue.[41]

CONCLUSIONS

Taken together, our findings reveal that CYGB is an excellent marker for quiescent and activated stellate cells in both intact and fibrotic human liver. Because the identity of the cell types that participate in collagen production and the fibrotic process in the diseased human liver (caused by hepatitis B or C virus infection, alcohol abuse, obesity, or autoimmune disease) is controversial and because myofibroblasts can be derived from stellate cells, portal myofibroblasts, mesothelial cells,[42] and the epithelial–mesenchymal transition,[43] a molecular marker that is able to uniquely trace stellate cells inhuman liver tissues will be valuable for studying the pathogenesis and fibrotic process of human liver disease.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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New Susceptibility and Resistance HLA-DP Alleles to HBV-Related Diseases Identified by a Trans-Ethnic Association Study in Asia

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Abstract

Previous studies have revealed the association between SNPs located on human leukocyte antigen (*HLA*) class II genes, including *HLA-DP* and *HLA-DQ*, and chronic hepatitis B virus (HBV) infection, mainly in Asian populations. *HLA-DP* alleles or haplotypes associated with chronic HBV infection or disease progression have not been fully identified in Asian populations. We performed trans-ethnic association analyses of *HLA-DPA1*, *HLA-DPB1* alleles and haplotypes with hepatitis B virus infection and disease progression among Asian populations comprising Japanese, Korean, Hong Kong, and Thai subjects. To assess the association between *HLA-DP* and chronic HBV infection and disease progression, we conducted high-resolution (4-digit) *HLA-DPA1* and *HLA-DPB1* genotyping in a total of 3,167 samples, including HBV patients, HBV-resolved individuals and healthy controls. Trans-ethnic association analyses among Asian populations identified a new risk allele *HLA-DPB1*09:01* ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59) and a new protective allele *DPB1*02:01* ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) to chronic HBV infection, in addition to the previously reported alleles. Moreover, *DPB1*02:01* was also associated with a decreased risk of disease progression in chronic HBV patients among Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65). Trans-ethnic association analyses identified Asian-specific associations of *HLA-DP* alleles and haplotypes with HBV infection or disease progression. The present findings will serve as a base for future functional studies of HLA-DP molecules in order to understand the pathogenesis of HBV infection and the development of hepatocellular carcinoma.

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Introduction

Hepatitis B virus (HBV) infection is a major global health problem, resulting in 0.5–1.0 million deaths per year [1]. The prevalence of chronic HBV infection varies. About 75% of the chronic carriers in the world live in Southeast Asia and East Pacific [2]. Due to the introduction of vaccination programs, the prevalence of HBV infection in many countries has gradually been decreasing with consequent decreases in HBV-related hepatocellular carcinoma (HCC) [3]. Although some HBV carriers spontaneously eliminate the virus, about 10–15% of carriers develop liver cirrhosis (LC), liver failure and HCC [4]. Moreover, the progression of liver disease was revealed to be associated with the presence of several distinct mutations in HBV infections [5]. Genetic variations in *STAT4* and *HLA-DQ* genes were recently identified as host genetic factors in a large-scale genome-wide association study (GWAS) for HBV-related HCC in China [6].

With regard to the genes associated with susceptibility to chronic HBV infection, *HLA-DP* and *HLA-DQ* genes were identified by GWAS in Japanese and Thai populations in 2009 [7] and 2011 [8], respectively. In addition, our previous GWAS confirmed and identified the association of SNP markers located on *HLA-DPA1* (rs3077) and *HLA-DPB1* (rs9277535) genes with susceptibility to chronic hepatitis B (CHB) and HBV clearance in Japanese and Korean subjects[9]. The significant associations of *HLA-DP* with CHB and HBV clearance have mainly been detected in Asian populations, such as Japanese [8,9], Thai [7], Chinese [10–12], and Korean [9]. In 2012, the association between *HLA-DPA1* gene SNPs and persistent HBV infection was replicated in a Germany non-Asian population for the first time; however, this showed no association with HBV infection [13]. These results seem to be explained by the fact that allele frequencies of both rs3077 (0.155, 0.587 and 0.743 for C allele, on HapMap CEU, JPT, and YRI) and rs9277535 (0.261, 0.558 and 0.103 for G allele, on HapMap CEU, JPT, and YRI) are markedly different between populations. Moreover, the previous study showed that HBsAg seropositivity rates were higher in Thailand and China (5–12%) than in North America and Europe (0.2–0.5%) [2]. These results suggest that comparative analyses of *HLA-DP* alleles and haplotypes in Asian populations would clarify key host factors of the susceptible and protective *HLA-DP* alleles and haplotypes for CHB and HBV clearance. Here, we performed trans-ethnic analyses of *HLA-DP* alleles and haplotypes in Asian populations comprising Japanese, Korean, Hong Kong and Thai individuals. The findings from this study will serve as a base for future functional studies of HLA-DP molecules.

Results

Characteristics of studied subjects

The characteristics of a total of 3,167 samples, including Japanese, Korean, Hong Kong and Thai subjects, are shown in Table 1. Each population included three groups of HBV patients, resolved individuals and healthy controls. The clinical definitions of HBV patients and resolved individuals are summarized in Materials and Methods. Some of the Japanese and all of the Korean samples overlapped with the subjects in our previous study [9,14].

We performed genotyping for *HLA-DPA1* and *HLA-DPB1* in all 3,167 samples, and a total of 2,895 samples were successfully genotyped. The characteristics of successfully genotyped samples are shown in Table S1.

Association of *HLA-DPA1* and *HLA-DPB1* alleles in Asian populations

As for a general Asian population, including 464 Japanese, 140 Korean, 156 Hong Kong, and 122 Thai subjects, five *HLA-DPA1* alleles and twenty-four *HLA-DPB1* alleles were observed (Table S2). The frequencies of *HLA-DPA1* and *HLA-DPB1* alleles were similar between Japanese and Korean subjects. On the other hand, the number of alleles with frequencies of 1–2% was larger in Hong Kong and Thai populations, despite the small sample size. Although the frequencies of *HLA-DP* alleles varied in Asian populations, *HLA-DPB1*05:01* was the most prevalent with over 30% in all populations.

The associations of *HLA-DPA1* and *HLA-DPB1* alleles with chronic HBV infection (i.e., comparison between HBV patients and healthy controls) are shown in Table S2. To avoid false positives caused by multiple testing, the significance levels were corrected based on the numbers of *HLA-DPA1* and *HLA-DPB1*

Table 1. Number of individuals in this study.

Population	Japanese	Korean	Hong Kong	Thai
Total number of samples	1,291	586	661	629
HBV patients	489	340	281	390
IC	114	-	-	-
CH	147	175	187	198
AE	21	-	-	-
LC	38	-	-	-
HCC	169	165	94	192
Mean age (y)	57.1	44.7	57.9	52.0
(min-max)	(20–84)	(18–74)	(32–86)	(21–84)
Gender (M/F)	338/151	265/75	239/42	289/101
Resolved individuals*	335	106	190	113
HCV (−)	249	106	190	113
HCV (+)	86	-	-	-
Mean age (y)	59.7	43.1	40.0	48.2
(min-max)	(18–87)	(12–66)	(18–60)	(39–66)
Gender (M/F)	173/162	61/45	113/77	83/30
Healthy controls	467	140	190	126
Mean age (y)	39.0**	33.7	26.2	46.6
(min-max)	(23–64)	(1–59)	(16–60)	(38–79)
Gender (M/F)	370/97	67/73	87/103	73/53

Abbreviation: IC, Inactive Carrier; CH, Chronic Hepatitis; AE, Acute Exacerbation; LC, Liver Cirrhosis; HCC, Hepatocellular Carcinoma.

* Resolved individuals were HBsAg negative and HBcAb positive.

** 419 of 467 healthy controls were de-identified, without information on age.

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alleles in the focal population. Briefly, the significance level was set at 0.05/(# of observed alleles at each locus) in each population (see Materials and Methods). With regard to high-risk alleles of *HLA-DPA1*, the most prevalent allele *HLA-DPA1*02:02* was significantly associated with susceptibility to HBV infection in Japanese ($P = 3.45 \times 10^{-4}$; OR = 1.39; 95% CI, 1.16–1.68) and Korean subjects ($P = 2.66 \times 10^{-5}$; OR = 1.89; 95% CI, 1.39–2.58), whereas this association was not observed in Hong Kong or Thai subjects. The association of *HLA-DPA1*02:01* with susceptibility to HBV infection was significant only in Japanese ($P = 2.61 \times 10^{-7}$; OR = 1.88; 95% CI, 1.46–2.41). The significant association of *HLA-DPA1*01:03* with protection against HBV infection was commonly observed among four Asian populations (Table S2). The pooled OR and 95% CI were 0.51 and 0.41–0.63, respectively in a meta-analysis ($P = 3.15 \times 10^{-10}$) (Fig. S1A).

As shown in Table S2, *HLA-DPB1* shows higher degree of polymorphism than *HLA-DPA1*. The most common allele in Asian populations, *HLA-DPB1*05:01*, was significantly associated with HBV susceptibility in both Japanese and Korean subjects. Although *HLA-DPB1*05:01* showed no significant association in the Hong Kong and Thai populations, the same direction of association (i.e., HBV susceptibility) was observed. Meta-analysis of the four populations revealed a significant association between *HLA-DPB1*05:01* and susceptibility to HBV infection ($P = 1.51 \times 10^{-4}$; OR = 1.45; 95% CI, 1.19–1.75) (Fig. S1B). The frequency of *HLA-DPB1*09:01* was significantly elevated in Japanese HBV patients (15.7%) as compared with healthy controls (8.7%) ($P = 3.70 \times 10^{-6}$; OR = 1.94; 95% CI, 1.45–2.62), and this association was most significant (i.e., the smallest P value) in the Japanese population. Because of lower allele frequencies of *HLA-DPB1*09:01* or lack of statistical power in the other populations, no significant associations were observed. A common allele in Thai subjects, *HLA-DPB1*13:01*, was significantly associated with susceptibility to HBV infection ($P = 2.49 \times 10^{-4}$; OR = 2.17; 95% CI, 1.40–3.47) with the same direction of associations in Japanese and Hong Kong (OR = 1.52 and 1.40, respectively).

*HLA-DPB1*04:02* was identified as the most protective allele for HBV infection in Japanese ($P = 1.59 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55) and Korean subjects ($P = 1.27 \times 10^{-7}$; OR = 0.19; 95% CI, 0.10–0.38). Both *HLA-DPB1*02:01* and *HLA-DPB1*04:01* were also significantly associated with protection in the Japanese population, and the former was significantly associated with protection in Hong Kong subjects ($P = 9.17 \times 10^{-4}$; OR = 0.49; 95% CI, 0.32–0.76). This common allele among four Asian populations, *HLA-DPB1*02:01*, showed a significant association with protection against HBV infection ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) in a meta-analysis (Fig. S1B).

The frequencies of associated *HLA-DP* alleles in a comparison of HBV patients with healthy controls (Table S2) or with HBV-resolved individuals (Table S3) were similar in all four Asian populations. In the Japanese population, the associations of susceptible and protective *HLA-DPB1* alleles to chronic HBV infection seem weaker in the comparison of HBV patients with HBV-resolved individuals than in the comparison of HBV patients with healthy controls. Moreover, the results of association analyses showed no difference in the comparison of HBV patients with HBV-resolved individuals, including or excluding HCV positive individuals (Table S3). In contrast, the association became stronger in the comparison of HBV patients with HBV-resolved individuals among the Korean subjects. The protective allele *HLA-DPB1*04:01* was also identified to have a strong association with HBV clearance in Hong Kong subjects (Table S3). Moreover, in Hong Kong subjects, the *HLA-DPB1*05:01* associated with the risk for HBV infection showed lower frequency in HBV-resolved individuals (42.9%) than in the healthy controls (48.1%), which accounts for a strong association in the comparison of HBV patients with HBV-resolved individuals ($P = 6.24 \times 10^{-3}$; OR = 1.64; 95% CI, 1.14–2.36). Although the number of samples was insufficient, *HLA-DP*100:01* showed a significant association with protection against HBV infection in the Hong Kong population ($P = 3.05 \times 10^{-6}$; OR = 0.03; 95% CI, 0.0007–0.20).

Table 2. Association of number of *DPB1*02:01* alleles (i.e., 0, 1 or 2) with disease progression in CHB patients assessed by multivariate logistic regression analysis adjusted for age and sex.

Population	P value	OR (95% CI)
Japanese	0.000177	0.47 (0.32–0.70)
Korean	0.025358	0.55 (0.33–0.93)
Hong Kong	0.040842	0.46 (0.22–0.97)
Thai	0.087782	0.58 (0.31–1.08)
All*	1.55×10^{-7}	0.50 (0.39–0.65)

*Population was adjusted using dummy variables.

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As for disease progression in CHB patients among Asian populations, a protective effect of *HLA-DPB1*02:01* on disease progression was observed in the Japanese ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67) and Korean populations ($P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75) (Table S4). Multivariate logistic regression analysis adjusted for age and sex revealed that the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) was significantly associated with disease progression in CHB patients in Japanese ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70) (Table 2). Moreover, protective effects of *DPB1*02:01* on disease progression in Asian populations ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) were detected in a multivariate logistic regression analysis adjusted for age, gender, and population (Table 2).

Associations of *DPA1-DPB1* haplotypes in Asian populations

The estimated frequencies of *HLA DPA1-DPB1* haplotypes are shown in Table S5. The most frequent haplotype among the four Asian populations was *DPA1*02:02-DPB1*05:01*. The number of haplotypes with low frequencies of 1–2% was 10 in both Japanese and Korean subjects, whereas more haplotypes appeared with frequencies of 1–2% in Hong Kong and Thai subjects. The associations of *DPA1-DPB1* haplotypes with HBV infection are shown in Table S5. In the Japanese population, *DPA1*02:01-DPB1*09:01* showed the most significant association with susceptibility to HBV infection ($P = 3.38 \times 10^{-6}$; OR = 1.95; 95% CI, 1.46–2.64). The most common haplotype in the four Asian populations, *DPA1*02:02-DPB1*05:01*, was found to be significantly associated with susceptibility to HBV infection in the Japanese and Korean subjects ($P = 7.40 \times 10^{-4}$; OR = 1.37; 95% CI, 1.14–1.66 for Japanese, and $P = 4.50 \times 10^{-6}$; OR = 2.02; 95% CI, 1.48–2.78 for Korean). In the Thai subjects, *HLA-DPB1*13:01* was the most significant risk allele for HBV infection (Table S2); however, no significant associations were found for the three different haplotypes bearing *HLA-DPB1*13:01*: *DPA1*02:01-DPB1*13:01*, *DPA1*02:02-DPB1*13:01*, and *DPA1*04:01-DPB1*13:01*,indicating that the association of *HLA-DPB1*13:01* with susceptibility to HBV infection did not result from a specific *DPA1-DPB1* haplotype or combination with a specific *DPA1* allele.

In the Japanese population, both haplotypes *DPA1*01:03-DPB1*04:01* and *DPA1*01:03-DPB1*04:02* showed significant associations with protection against HBV infection ($P = 1.17 \times 10^{-5}$; OR = 0.32; 95% CI, 0.18–0.56 for *DPA1*01:03-DPB1*04:01* and $P = 1.95 \times 10^{-7}$; OR = 0.37; 95% CI, 0.24–0.55 for *DPA1*01:03-DPB1*04:02*). In the Korean subjects, a significant association of *DPA1*01:03-DPB1*04:02* was also demonstrated; however, no association was observed for *DPA1*01:03-DPB1*04:01*. Because the observed number of each haplotype was small, none of the other haplotypes showed a significant association with protection against HBV infection.

In order to identify trans-ethnic DPA1-DPB1 haplotypes associated with HBV infection, a meta-analysis was performed. A meta-analysis further revealed that the *DPA1*01:03-DPB1*02:01* haplotype was significantly associated with protection against HBV infection ($P = 1.45 \times 10^{-5}$; OR = 0.69; 95% CI, 0.58–0.82) (Fig. S1C).

Discussion

Among 2.2 billion individuals worldwide who are infected with HBV, 15% of these are chronic carriers. Of chronic carriers, 10–15% develops LC, liver failure and HCC, and the remaining individuals eventually achieve a state of nonreplicative infection, resulting in HBsAg negative and anti-HBc positive, i.e. HBV-resolved individuals. To identify host genetic factors associated with HBV-related disease progression may lead HBV patients to discriminate individuals who need treatment.

The *HLA-DPA1* and *HLA-DPB1* genes were identified as host genetic factors significantly associated with CHB infection, mainly in Asian populations [7–12], and not in European populations [13]. In the previous association analyses of *HLA-DPB1* alleles with HBV infection, one risk allele *HLA-DPB1*05:01* (OR = 1.52; 95% CI, 1.31–1.76), and two protective alleles, *HLA-DPB1*04:01* (OR = 0.53; 95% CI, 0.34–0.80) and *HLA-DPB1*04:02* (OR = 0.47; 95% CI, 0.34–.64), were identified in the Japanese population [7]. In this study, we further identified a new risk allele *HLA-DPB1*09:01* (OR = 1.94; 95% CI, 1.45–2.62) for HBV infection and a new protective allele *HLA-DPB1*02:01* (OR = 0.71; 95% CI, 0.56–0.89) in the Japanese population, in addition to the previously reported alleles (Table S2) [7]. The discrepancy in the association of *HLA-DPB1*09:01* allele with risk for HBV infection in a previous study [7] results from the elevated frequency of *HLA-DPB1*09:01* in the controls (12.2%), which is higher than our controls (8.7%). In this study, healthy subjects were recruited as controls. In contrast, individuals that were registered in BioBank Japan as subjects with diseases other than CHB were recruited as controls in the previous study [7], which may have included patients with diseases with which *HLA-DPB1*09:01* is associated. Although no significant association of *HLA-DPB1*09:01* with risk for HBV infection was observed in the Korean subjects, *HLA-DPB1*09:01* appears to have a susceptible effect on HBV infection, as it showed the same direction of association. When the association analyses in Japanese and Korean subjects were combined in meta-analysis, the association was statistically significant ($P = 1.36 \times 10^{-6}$; OR = 1.97; 95% CI, 1.50–2.59). Thus, *HLA-DPB1*09:01* may be a Northeast Asian-specific allele associated with risk for HBV infection.

Moreover, a significant association of *HLA-DPB1*13:01* with risk of HBV infection (OR = 2.17; 95% CI, 1.40–3.47) was identified in the Thai subjects. However, the frequency of *HLA-DPB1*13:01* in Thai healthy controls (11.5% in the present study) reportedly varies, ranging from 15.4% to 29.5%, due to the population diversity [15–17]. Therefore, a replication analysis is required to confirm the association of *HLA-DPB1*13:01* with HBV infection in the Thai subjects. There were four other marginally associated *HLA-DPB1* alleles with low allele frequencies below 5% in HBV patients and healthy controls, including *HLA-DPB1*28:01*, *-DPB1*31:01*, *-DPB1*100:01*, and *-DPB1*105:01*, in the Hong Kong and Thai subjects. Because these infrequent alleles may have resulted from false positive associations, the association needs to be validated in a large number of subjects.

*HLA-DPB1*02:01* showed a significant association with protection against HBV infection in both Japanese and Hong Kong populations (Table S2); however, the *HLA-DPB1*02:01* allele was not associated with HBV infection in the previous study [7]. Although *HLA-DPB1*02:01* showed no association in either Korean or Thai populations, a significant association of *HLA-DPB1*02:01* with protection against HBV infection among four Asian populations was detected in meta-analysis ($P = 5.22 \times 10^{-6}$; OR = 0.68; 95% CI, 0.58–0.81) (Fig. S1B). We therefore conclude that the present finding is not a false positive.

A recent report showed that *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, **05:01*, **09:01*, and **14:01* were significantly associated with response to booster HB vaccination in Taiwan neonatally vaccinated adolescents [18]. The *HLA-DPB1*02:01:02*, **02:02*, **03:01:01*, **04:01:01*, and **14:01* were significantly more frequent in recipients whose post-booster titers of antibodies against HBV surface antigen (anti-HBs) were detectable, on the other hand, *HLA-DPB1*05:01* and **09:01* were significantly more frequent in recipients who were undetectable. Moreover, the *HLA-DPB1*05:01* and **09:01* significantly increase the likelihoods of undetectable pre-booster anti-HBs titers. These results seem consistent with our findings, in which *HLA-DPB1*05:01* and **09:01* are associated with susceptibility to chronic hepatitis B infection.

We also identified a protective effect of *HLA-DPB1*02:01* allele on disease progression in Asian populations. Previous studies identified the association of HLA class II genes including *HLA-DQ* and *HLA-DR* with development of HBV related hepatocellular carcinoma in the Chinese population [6,19,20]. In this study using Japanese and Korean samples, we identified significant associations between *HLA-DPB1*02:01* and disease progression in CHB patients ($P = 4.26 \times 10^{-5}$; OR = 0.45; 95% CI, 0.30–0.67, for Japanese and $P = 8.74 \times 10^{-4}$; OR = 0.47; 95% CI, 0.29–0.75 for Korean) (Table S4). Although the association of *HLA-DPB1*02:01* with disease progression was weaker after adjustment for age and gender in Korean subjects ($P = 2.54 \times 10^{-2}$; OR = 0.55; 95% CI, 0.33–0.93), the same direction of association was observed (i.e. protective effect on disease progression) (Table 2). The protective effects of *HLA-DPB1*02:01* on disease progression showed a significant association after adjustment for age and gender in the Japanese population ($P = 1.77 \times 10^{-4}$; OR = 0.47; 95% CI, 0.32–0.70); moreover, a significant association between *HLA-DPB1*02:01* was observed among four Asian populations, under which population was adjusted by using dummy variables in a multivariate logistic regression analysis ($P = 1.55 \times 10^{-7}$; OR = 0.50; 95% CI, 0.39–0.65) (Table 2).

The *HLA-DPA1* and *HLA-DPB1* belong to the HLA class II alpha and beta chain paralogues, which make a heterodimer consisting of an alpha and a beta chain on the surface of antigen presenting cells. This HLA class II molecule plays a central role in the immune system by presenting peptides derived from extracellular proteins. We identified two susceptible haplotypes (*DPA1*02:02-DPB1*05:01* and *DPA1*02:01-DPB1*09:01*) and three protective haplotypes (*DPA1*01:03-DPB1*04:01*, *DPA1*01:03-DPB1*04:02*, and *HLA-DPA1*01:03-DPB1*02:01*) to chronic hepatitis B infection, which may result in different binding

affinities between HLA-DP subtypes and extracellular antigens. Although functional analyses of HLA-DP subtypes to identify HBV-related peptides are not fully completed, identification of susceptible and protective haplotypes as host genetic factors would lead us to understand the pathogenesis of HBV infection including viral factors.

In summary, we identified a new risk allele *HLA-DPB1*09:01*, which was specifically observed in Northeast Asian populations, Japanese and Korean. Moreover, a new protective allele *HLA-DPB1*02:01* was identified among four Asian populations: Japanese, Korean, Hong Kong and Thai. The protective allele *HLA-DPB1*02:01* was associated with both chronic HBV infection and disease progression in chronic HBV patients. Identification of a total of five alleles, including two risk alleles (*DPB1*09:01* and *DPB1*05:01*) and three protective alleles (*DPB1*04:01*, *DPB1*04:02* and *DPB1*02:01*), would enable HBV-infected individuals to be classified into groups according to the treatment requirements. Moreover, the risk and protective alleles for HBV infection and disease progression, identified in this study by means of trans-ethnic association analyses, would be key host factors to recognize HBV-derived antigen peptides. The present results may lead to subsequent functional studies into HLA-DP molecules and viral factors in order to understand the pathogenesis of HBV infection and development of hepatocellular carcinoma.

Materials and Methods

Ethics Statement

All study protocols conform to the relevant ethical guidelines, as reflected in the *a priori* approval by the ethics committee of National Center for Global Health and Medicine, and by the ethics committees of all participating universities and hospitals, including The University of Tokyo, Japanese Red Cross Kanto-Koshinetsu Block Blood Center, The University of Hong Kong, Chulalongkorn University, Yonsei University College of Medicine, Nagoya City University Graduate School of Medical Sciences, Musashino Red Cross Hospital, Tokyo Medical and Dental University, Teine Keijinkai Hospital, Hokkaido University Graduate School of Medicine, Kurume University School of Medicine, Okayama University Graduate School of Medicine, Yamaguchi University Graduate School of Medicine, Tottori University, Kyoto Prefectural University of Medicine, Osaka City University Graduate School of Medicine, Nagoya Daini Red Cross Hospital, Ehime University Graduate School of Medicine, Kanazawa University Graduate School of Medicine, National Hospital Organization Osaka National Hospital, Iwate Medical University, Kawasaki Medical College, Shinshu University School of Medicine, Saitama Medical University, Kitasato University School of Medicine, Saga Medical School, and University of Tsukuba.

Written informed consent was obtained from each patient who participated in this study and all samples were anonymized. For Japanese healthy controls, 419 individuals were de-identified with information about gender, and all were recruited after obtaining verbal informed consent in Tokyo prior to 1990. For the 419 Japanese healthy individuals, written informed consent was not obtained because the blood sampling was conducted before the "Ethical Guidelines for Human Genome and Genetic Sequencing Research" were established in Japan. Under the condition that DNA sample is permanently de-linked from the individual, this study was approved by the Research Ethics Committee of National Center for Global Health and Medicine.

Characteristics of studied subjects

All of the 3,167 genomic DNA samples were collected from individuals with HBV, HBV-resolved individuals (HBsAg-negative and anti-HBc-positive) and healthy controls at 26 multi-center hospitals throughout Japan, Korea, Hong Kong, and Thailand (Table 1). In a total of 1,291 Japanese and 586 Korean samples, 1,191 Japanese individuals and all 586 Korean individuals were included in our previous study [9]. With regard to additional Japanese individuals, we collected samples from 48 healthy controls at Kohnodai Hospital, and 52 HBV patients at Okayama University Hospital and Ehime University Hospital, including 26 individuals with LC and 26 individuals with HCC. A total of 661 Hong Kong samples and 629 Thai samples were collected at Queen Mary Hospital and Chulalongkorn University, respectively.

HBV status was measured based on serological results for HBsAg and anti-HBc with a fully automated chemiluminescent enzyme immunoassay system (Abbott ARCHITECT; Abbott Japan, Tokyo, Japan, or LUMIPULSE f or G1200; Fujirebio, Inc., Tokyo, Japan). For clinical staging, inactive carrier (IC) state was defined by the presence of HBsAg with normal ALT levels over 1 year (examined at least four times at 3-month intervals) and without evidence of liver cirrhosis. Chronic hepatitis (CH) was defined by elevated ALT levels (>1.5 times the upper limit of normal [35 IU/L]) persisting over 6 months (by at least 3 bimonthly tests). Acute exacerbation (AE) of chronic hepatitis B was defined as an elevation of ALT to more than 10 times the upper limit of normal (ULN, 58 IU/L) and bilirubin to at least three times ULN (15 μmol/L). LC was diagnosed principally by ultrasonography (coarse liver architecture, nodular liver surface, blunt liver edges and hypersplenism), platelet counts<100,000/cm^3, or a combination thereof. Histological confirmation by fine-needle biopsy of the liver was performed as required. HCC was diagnosed by ultrasonography, computerized tomography, magnetic resonance imaging, angiography, tumor biopsy or a combination thereof.

The Japanese control samples from HBV-resolved subjects (HBsAg-negative and anti-HBc-positive) at Nagoya City University-affiliated healthcare center were used by comprehensive agreement (anonymization in a de-identified manner) in this study. Some of the unrelated and anonymized Japanese healthy controls were purchased from the Japan Health Science Research Resources Bank (Osaka, Japan). One microgram of purified genomic DNA was dissolved in 100 μl of TE buffer (pH 8.0) (Wako, Osaka, Japan), followed by storage at −20°C until use.

Genotyping of *HLA-DPA1* and *HLA-DPB1* alleles

High resolution (4-digit) genotyping of *HLA-DPA1* and *-DPB1* alleles was performed for HBV patients, resolved individuals, and healthy controls in Japan, Korea, Hong Kong, and Thailand. LABType SSO HLA DPA1/DPB1 kit (One Lambda, CA) and a Luminex Multi-Analyte Profiling system (xMAP; Luminex, Austin, TX) were used for genotyping, in according with the manufacturer's protocol. Because of the small quantity of genomic DNA in some Korean samples, we performed whole genome amplification for a total of 486 samples using GenomiPhi v2 DNA Amplification kit (GE Healthcare Life Sciences, UK), in accordance with the manufacturer's instruction.

A total of 2,895 samples were successfully genotyped and characteristics of these samples are summarized in Table S1.

Statistical analysis

Fisher's exact test in two-by-two cross tables was used to examine the associations between *HLA-DP* allele and chronic HBV infection or disease progression in chronic HBV patients,

using statistical software R2.9. To avoid false-positive results due to multiple testing, significance levels were adjusted based on the number of observed alleles at each locus in each population. For *HLA-DPA1* alleles, the number of observed alleles was 3 in Japanese, 4 in Korean, 5 in Hong Kong, and 5 in Thai subjects. Therefore, the significant levels for α were set at $\alpha = 0.05/3$ in Japanese, $\alpha = 0.05/4$ in Korean, $\alpha = 0.05/5$ in Hong Kong, and $\alpha = 0.05/5$ in Thai subjects. In the same way, significant levels for *HLA-DPB1* alleles were $\alpha = 0.05/10$, $0.05/11$, $0.05/12$, and $0.05/16$, respectively. Multivariate logistic regression analysis adjusted for age and sex (used as independent variables) was applied to assess associations between the number of *DPB1*02:01* alleles (i.e., 0, 1, or 2) and disease progression in CHB patients. To examine the effect of *DPB1*02:01* allele on disease progression in all populations, population was further adjusted by using three dummy variables (i.e., (c1, c2, c3) = (0, 0, 0) for Japanese, (1, 0, 0) for Korean, (0, 1, 0) for Hong Kong, and (0, 0, 1) for Thai) in a multivariate logistic regression analysis. We obtained the following regression equation: $\text{logit}(p) = -3.905 + 0.083 * \text{age} + (-0.929) * \text{sex} + (-0.684) * \textit{DPB1*02:01} + 1.814 * c1 + (-0.478) * c2 + 0.782 * c3$. Significance levels in the analysis of disease progression in CHB patients were set as $\alpha = 0.05/10$ in Japanese, $\alpha = 0.05/11$ in Korean, $\alpha = 0.05/15$ in Hong Kong, and $\alpha = 0.05/15$ in Thai subjects. The phase of each individual (i.e., a combination of two *DPA1-DPB1* haplotypes) was estimated using PHASE software [21], assuming samples are selected randomly from a general population. In comparison of the estimated *DPA1-DPB1* haplotype frequencies, significant levels were set as $\alpha = 0.05/14$ in Japanese, $\alpha = 0.05/17$ in Korean, $\alpha = 0.05/17$ in Hong Kong, and $\alpha = 0.05/18$ in Thai subjects. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) in order to calculate pooled OR and its 95% confidence interval (95% CI). We applied meta-analysis for alleles with frequency>1% in all four Asian populations. The significance levels in meta-analysis were adjusted by the total number of statistical tests; $\alpha = 0.05/20$ for *DPA1* alleles, $\alpha = 0.05/57$ for *DPB1* alleles, and $\alpha = 0.05/74$ for *DPA1-DPB1* haplotypes.

Supporting Information

Figure S1 Comparison of odds ratios in association analyses for *HLA-DP* with chronic HBV infection among four Asian populations: (A) *HLA-DPA1* alleles; (B) *HLA-DPB1* alleles; and (C) *HLA DPA1-DPB1* haplotypes. Meta-analysis was performed using the DerSimonian-Laird method (random-effects model) to calculate pooled OR and its 95% confidence interval (95% CI). Bold depicts a statistically significant association after correction of significance level.
(DOCX)

Table S1 Individuals with successfully genotyped for *HLA-DPA1* and *HLA-DPB1*.
(DOCX)

Table S2 Frequencies of HLA-DP alleles in HBV patients and healthy controls among Asian populations.
(XLSX)

Table S3 Frequencies of HLA-DP alleles in HBV patients and resolved individuals among Asian populations.
(XLSX)

Table S4 Associations of HLA-DPB1 alleles with disease progression in CHB patients among Asian populations.
(XLSX)

Table S5 Estimated frequencies of HLA DPA1-DPB1 haplotypes in HBV patients and healthy controls among Asian populations.
(XLSX)

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Author Contributions

Conceived and designed the experiments: NN HS MS KT M. Mizokami. Performed the experiments: NN HS KK Y. Mawatari M. Kawashima M. Minami. Analyzed the data: NN HS M. Kawashima JO. Contributed reagents/materials/analysis tools: W-KS M-FY NP YP SHA K-HH K. Matsuura YT M. Kurosaki YA NI J-HK SH TI KY IS Y. Murawaki YI AT EO YH MH SK EM KS KH ET SM MW YE NM K. Murata M. Korenaga KT M. Mizokami. Wrote the paper: NN HS JO KT M. Mizokami.

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EDITORIAL

HLA class II associated with outcomes of hepatitis B and C infections

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Abstract

Several factors influence the clinical course of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection. The human leukocyte antigen (HLA) system, the major histocompatibility complex (MHC) in humans, has been considered one of the most important host factors with respect to outcomes. To date, conventional genotyping studies have shown that *HLA* class II loci are mainly associated with spontaneous clearance of HBV and HCV. However, the specific HLA locus associated with the outcomes of hepatitis virus infection remains unclear. A recent genome-wide association study (GWAS) using a comprehensive approach for human genotyping demonstrated single nucleotide polymorphisms (SNPs) associated with the outcomes of hepatitis virus infection. Examination of large numbers of cohorts revealed that several SNPs in both *HLA-DPA1* and *HLA-DPB1* loci are associated with persistent HBV infection in Asian populations. To date, however, few studies have focused on *HLA-DP* because polymorphisms of *HLA-DP* haplotype do not vary greatly as compared with other loci of *HLA*. There are not enough studies to reveal the function of *HLA-DP*. GWAS additionally detected candidate SNPs within HLA loci associated with chronic HBV or HCV hepatitis, hepatic fibrosis, and the development of hepatocellular carcinoma. The results of one cohort were not always consistent with those of other cohorts. To solve several controversial issues, it is necessary to validate reported SNPs on *HLA* loci in global populations and to elucidate the *HLA*-allele-regulated molecular response to hepatitis virus infection.

Key words: Hepatitis B virus; Hepatitis C virus; Hepatocarcinogenesis; Human leukocyte antigen; Genome-wide association studies; Genotyping; Persistent infection

Core tip: Conventional genotyping studies have shown that human leukocyte antigen (*HLA*) typing was one of the most important host factors with respect to outcomes of hepatitis B and C virus infections. However, the specific HLA locus associated with the outcomes remains unclear. Recently a genome-wide association study for human genotyping demonstrated single nucleotide polymorphisms associated with the outcomes of hepatitis virus infection. Now it has been confirmed that several single nucleotide polymorphisms in both *HLA-DP* loci were associated with persistent hepatitis B virus infection in Asian populations.

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INTRODUCTION

The human leukocyte antigen (HLA) system, the major histocompatibility complex (MHC) in humans, has long been considered the most important region in the human genome with respect to infection, inflammation, autoimmunity, and transplantation medicine[1,2]. In humans, *HLA* complex consists of more than 200 genes located

close together on chromosome 6. Genes in this complex are categorized into three basic groups: class I (*HLA-A, -B*, and *-C*), class II (*HLA-DR, -DQ*, and *-DP*), and class III (some genes involved in inflammation and other immune-system activities). Interactions among HLA-restricted T lymphocytes, B lymphocytes, natural killer (NK) cells, and cytokines influence immune response to viral infection. *HLA* class I and II molecules are expressed as cell surface antigens that bind to peptide epitopes on $CD8^+$ T cells and $CD4^+$ T cells, respectively. Effective presentation of viral antigens by the HLA system induces good immune response.

It is well known that some patients infected with hepatitis B virus (HBV) or hepatitis C virus (HCV) spontaneously recover and can escape from persistent infection[3-5]. Progression of liver diseases by chronic viral infection also differs among patients. In addition, the response to HBV vaccination is different in each person. To identify immune systems against invaders in individual patients, *HLA* haplotypes related to persistent viral infection or providing protection against such infection have been examined. Singh *et al*[6] reported a detailed review about associations of *HLA* types with HBV and HCV infections among global populations. They speculated that there was a limited chance of detecting globally common *HLA* types related to outcomes or disease progression associated with hepatitis viral infection because *HLA* loci are diverse owing to racial admixture, environmental and selection pressure, and inherent polymorphic nature, leading to allelic variations among different ethnic groups.

Recent genome-wide association studies (GWAS) have demonstrated single nucleotide polymorphisms (SNPs) associated with the outcomes of hepatitis virus infection[7-14]. Imputation-based association analysis showed that some of the SNPs are located near *HLA* loci in chromosome 6p21[7,8,15]. Conventional genotyping and GWAS are different approaches for analysis. Conventional genotyping examines selected targeted genes, while GWAS can comprehensively examine hundreds of thousands of SNPs[16]. Although both approaches have suggested that *HLA* loci play important roles in the outcomes of viral hepatitis, the precise regions of *HLA* loci detected by each approach differed. In the present review, we summarize and compare the latest data obtained by GWAS with previous data obtained by conventional *HLA* typing.

ASSOCIATION BETWEEN HEPATITIS VIRAL INFECTION AND *HLA* ALLELES IDENTIFIED BY GENOTYPING

Singh *et al*[6] suggested that an association of *HLA DR*13* alleles in *HLA* Class II was protective in both HBV and HCV infections in several populations. *HLA DRB1*11* and *HLA DQB1*0301* were protective in HCV infection, but were associated with persistent HBV infection.

A recent meta-analysis showed that *HLA-DR*03* and *HLA-DR*07* were associated with an increased risk of persistent HBV infection in 19 individual case-control studies including 9 Han Chinese cohorts, 3 Korean cohorts, 2 Iranian cohorts, and 1 cohort each of Caucasian, Gambian, Taiwanese, Thai, and Turkish subjects[17]. In contrast, *HLA-DR*04* and *HLA-DR*13* were associated with clearance of HBV infection. In Chinese Han populations, *HLA-DR*01* was associated with clearance of HBV infection, while in other ethnic groups there was no association between *HLA-DR*01* and HBV infection.

As for HCV infection, a study performed in patients from the United Kingdom and the United States reported that the inhibitory NK cell receptor KIR2DL3 and *HLA-C1* ligand, *HLA* class I interact directly to promote spontaneous viral clearance[18]. In global populations, *HLA* class II, especially several alleles in *HLA-DRB1*, has been linked to persistent HCV infection[19,20]. Interestingly, Spanish and American groups reported an association between *MICA* genotypes in *HLA* class III and clearance of HCV[21,22].

ASSOCIATION BETWEEN HEPATITIS VIRAL INFECTION AND SNPS IN *HLA* LOCUS IDENTIFIED BY GWAS

A recent GWAS discovered many SNP candidates associated with common diseases[16]. In research on viral hepatitis, several SNPs associated with outcomes, including the *HLA* coding region of chromosome 6p21.3, were detected by GWAS.

HBV infection

Kamatani *et al*[7] reported the results of a case-control association study of HBV infection in 2009. They showed that rs3077 SNP near *HLA-DPA1* gene and rs9277535 SNP near *HLA-DPB1* were associated with persistent HBV infection in Japanese cohorts. In addition, *HLA* haplotype analysis showed that *HLA-DPA1*0202-DPB1*0501* and *HLA-DPA1*0202-DPB1*0301* were risk types for persistent HBV infection, and *HLA-DPA1*0103-DPB1*0402* and *HLA-DPA1*0103-DPB1*0401* were protective types for HBV infection. The same group performed a second GWAS analysis involving a larger number of cohorts[8]. The study validated that rs3077 SNP near *HLA-DPA1* gene and rs9277535 SNP near *HLA-DPB1* were strongly associated with persistent HBV infection. Other SNPs, rs2856718 and rs7453920 within the *HLA-DQ* locus, were also associated with persistent HBV infection. Moreover, HLA haplotype analysis indicated that *HLA-DQA1*0102-DQB1*0303* and *HLA-DQA1*0301-DQB1*0601* were risk types for persistent HBV infection, while *HLA-DQA1*0102-DQB1*0604* and *HLA-DQA1*0101-DQB1*0501* were protective types for HBV infection. GWAS of Han Chinese populations also showed that the *HLA-DPA1* and *HLA-DPB1* genes were related to persistent HBV infection. The first study from China indicated that 4 SNPs related to *HLA-*

Table 1 Single nucleotide polymorphisms within human leukocyte antigen loci associated with outcomes of hepatitis B virus infection

Ethnic group	Outcome	HLA locus	SNP	Odds	95%CI	HLA haplotype	Odds	Ref.
Japanese	Chronic infection	HLA-DPA1	rs3077	0.56	0.51-0.61			[7]
		HLA-DPB1	rs9277535	0.57	0.52-0.62			
						DPA1*0202-DPB1*0501	1.45	
						DPA1*0202-DPB1*0301	2.31	
						DPA1*0103-DPB1*0402	0.52	
						DPA1*0103-DPB1*0401	0.57	
Japanese	Chronic infection	HLA-DQ	rs2856718	1.43	1.33-1.54			[8]
			rs7453920	1.66	1.49-1.85			
						DQA1*0102-DQB1*0303	19.3	
						DQA1*0301-DQB1*0601	5.02	
						DQA1*0102-DQB1*0604	0.16	
						DQA1*0101-DQB1*0501	0.39	
Chinese	Chronic infection	HLA-DPA1	rs2395309	0.71	0.59-0.86			[9]
		HLA-DPA1	rs3077	0.64	0.53-0.78			
		HLA-DPA1	rs2301220	0.67	0.56-0.81			
		HLA-DPA1	rs9277341	1.77	1.39-2.25			
		HLA-DPB1	rs3135021	0.78	0.64-0.94			
		HLA-DPB1	rs9277535	0.56	0.47-0.68			
		HLA-DPB1	rs10484569	1.60	1.33-1.93			
		HLA-DPB1	rs3128917	1.91	1.59-2.30			
		HLA-DPB1	rs2281388	1.66	1.38-2.01			
		HLA-DPB1	rs3117222	0.51	0.42-0.61			
		HLA-DPB1	rs9380343	0.61	0.50-0.73			
Indonesian	Vaccine response	HLA-DR	rs3135363	1.59	1.45-1.73			[10]
		HLA-DPB1	rs9277535	0.82	0.71-0.96			
		HLA-III	rs9267665	2.13	1.82-2.49			
Chinese	HCC	HLA-DQA1/DRB1	rs9272105	1.28	1.22-1.35			[11]
		GRIK1*	rs455804	0.84	0.80-0.89			
Japanese, Korean	Chronic infection	HLA-DPA1	rs3077	0.46	0.39-0.54			[12]
		HLA-DPB1	rs9277542	0.50	0.43-0.60			
Chinese	Chronic infection	HLA-DPB1	rs9277535	0.60	0.51-0.70			[13]
		HLA-DPA1	rs3077	0.81	0.75-0.95			
		HLA-DQ	rs7453920	0.60	0.49-0.73			
		HLA-DQ	rs2856718	0.75	0.64-0.89			
	HCC	HLA-DQ	rs2856718	0.70	0.59-0.83			
		HLA-DPA1	rs3077	0.78	0.67-0.92			
Chinese	HCC	HLA-DQ	rs9275319	1.51	1.38-1.66			[14]
		STAT4*	rs7574865					

HLA: Human leukocyte antigen; SNP: Single nucleotide polymorphism; HCC: Hepatocellular carcinoma.

DPA1 gene, including rs3077, and 7 SNPs related to *HLA-DPB1*, including rs9277535, were associated with chronic HBV infection[9]. Another study showed that rs7453920 and rs2856718 SNPs near *HLA-DQ* were associated with persistent HBV infection in addition to the rs3077 and rs9277535 SNPs[10] (Table 1).

A recent report from another Japanese group showed that rs3077 SNP near *HLA-DPA1* gene and rs9277542 SNP near *HLA-DPB1* gene were associated with persistent HBV infection[12]. Studies using genotyping methods validated that the rs3077 and rs2395309 SNPs near *HLA-DPA1* gene and the rs9277542 SNP near *HLA-DPB1* were associated with HBV infection in Han Chinese populations[23-25].

GWAS revealed three independent variants within the *HLA* complex that were related to a poor response to HB vaccine in the Indonesian population. Specifically, rs3135363 SNP near *HLA-DR*, rs9277542 SNP near *HLA-DPB1*, and rs9267665 in *HLA* class III were associated with antibody titers after HB vaccination[10].

A comparison between cohorts with and without hepatocellular carcinoma (HCC) showed that rs9272105 SNP near *HLA-DQA1/DRB1* and rs455804 SNP near *GRIK1* were significantly associated with HCC development in Chinese patients with HBV[11]. There was a partial association of the genotype of rs9272105 to *HLA-DRB1*0405* and **0901*. Another study showed that rs2856718 SNP at *HLA-DQ* and rs3077 SNP at *HLA-DPA1* had a protective effect against HCC progression as compared with the dominant SNP of rs2856718 in Han Chinese populations[13]. In 2013, it was reported that rs9275319 at *HLA-DQ* and rs7574865 at *STAT4* were