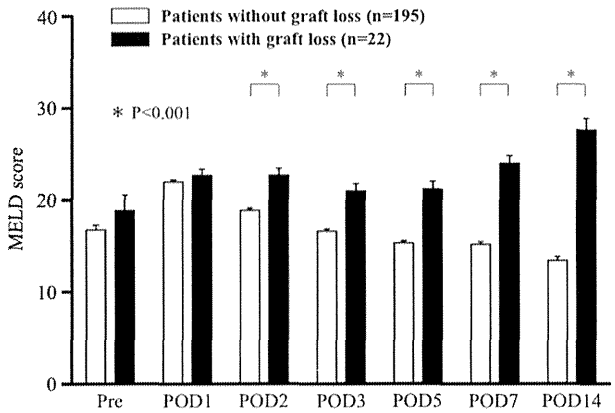


**Fig 1.** Chronological change in preoperative MELD score (MELD-Pre) and the MELD score on indicated postoperative days (MELD-POD) in patients without (n = 200) or with (n = 23) graft loss. The MELD-POD2 in patients with graft loss was significantly higher than that without graft loss (18.9 ± 0.3 vs 22.7 ± 0.8; $P < .05$). Later MELD scores were also significantly different.

than that in patients without graft loss (18.9 ± 0.3; vs primary graft dysfunction, $P = .025$; vs multiorgan failure, $P = .0003$; vs sepsis, $P = .002$), respectively, whereas the MELD-Pre scores in patients with graft loss by primary graft dysfunction (n = 6; 16.7 ± 2.9), multiorgan failure (n = 6; 22.2 ± 3.0), and sepsis (n = 5; 19.8 ± 3.2) were not significantly different from that in patients without graft loss (16.8 ± 0.5; vs primary graft dysfunction, $P = .969$; vs multiorgan failure, $P = .078$; vs sepsis, $P = .359$), respectively.

This indicates that MELD-POD2 is a better predictor of mortality following LDLT than MELD-Pre, which was not depend on the major cause of graft loss such as primary graft dysfunction, multiorgan failure, and sepsis.

### ROC Curves of MELD-Pre and MELD-POD as Predictors of Graft Loss

The AUROC curve of MELD-Pre and MELD-POD1, 2, 3, 5, 7, and 14, for the prediction of graft loss were 0.605, 0.553, 0.779, 0.775, 0.833, 0.933, and 0.938, respectively (Fig 2). The AUROC of MELD-POD values on POD2 and up to POD14 were significantly higher than that of MELD-Pre ($P < .05$) as predictors of graft loss. The sensitivity of the MELD-Pre score for predicting graft loss was poor at 44.6% with optimal cutoff of 20.0, whereas the sensitivity and specificity of the MELD-POD2 score were 68.2% and 79.5%, respectively, with optimal cutoff of 21.8 (Table 2). In addition, the AUROC c-statistic of the MELD-POD7 index was 0.933 with excellent sensitivity and specificity of 100% and 74.9, respectively.

### Multivariate Analysis of Risk Factors Related to Graft Loss

Multivariate analysis revealed that MELD-POD2 ≥19 (odds ratio [OR], 5.601; 95% confidence interval [CI], 1.395–4.508; $P = .0009$) and preoperative hospitalization

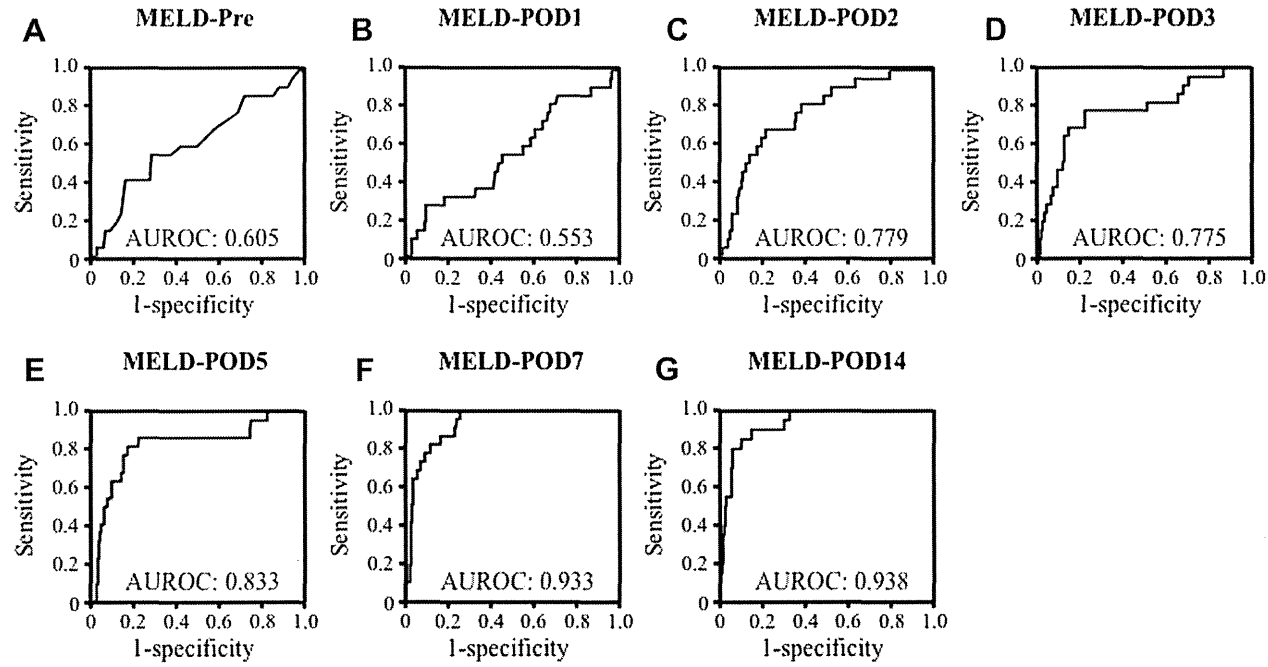


**Fig 2.** ROC curves of MELD-Pre and MELD-POD as predictors of graft loss. The areas under the ROC curve (AUROC) of MELD-Pre and MELD-POD1, 2, 3, 5, 7, and 14, for prediction of graft loss were 0.605, 0.553, 0.779, 0.775, 0.833, 0.933, and 0.938, respectively. Five MELD-POD values, namely, MELD-POD2, 3, 5, 7, and 14, were significantly higher than that of MELD-Pre ($P < .05$) in patients with graft loss.

**Table 2. Diagnostic Performance of MELD for Predicting Graft Loss After LDLT (n = 217)**

| Variables | Optimal Cutoff | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|---|
| MELD-Pre | 20.0 | 54.6 | 72.1 | 18.5 | 93.2 |
| MELD-POD1 | 26.0 | 27.3 | 80.8 | 25.0 | 91.7 |
| MELD-POD2 | 21.8 | 68.2 | 79.5 | 27.3 | 95.7 |
| MELD-POD3 | 19.3 | 77.3 | 77.9 | 34.0 | 96.8 |
| MELD-POD5 | 18.3 | 81.8 | 83.6 | 36.0 | 97.6 |
| MELD-POD7 | 17.0 | 100 | 74.9 | 31.0 | 100 |
| MELD-POD14 | 18.5 | 90.0 | 85.6 | 39.1 | 98.8 |

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.
Note: Optimal cutoff points gave the highest total sensitivity and specificity.

(OR, 3.330; 95% CI, 0.318–0.893; $P = .0151$) were significant and independent risk factors for graft loss (Table 3).

## DISCUSSION

The present study evaluated the value of the MELD scoring system on different PODs as a predictor of mortality following LDLT. The scores on POD2 and later were useful predictors, with the AUROC c-statistic approximately 0.75 or more. In addition, multivariate analyses of postoperative mortality revealed that a high MELD score on POD2 was an independent predictor of short-term graft loss following LDLT, in addition to preoperative hospitalization status. To our knowledge, this is the first report to demonstrate the feasibility of using postoperative clinical risk factors in the form of MELD-POD for early postoperative risk stratification following LDLT.

Because of a shortage of donor organs, transplantation units should aim for optimal organ allocation to lower the death rate of patients on the waiting list and to increase the post-transplantation survival rate. The objective evaluation of disease severity in patients awaiting a graft is the main argument for allocating livers based on the MELD score [3,5]. It was demonstrated that a longer waiting time was not associated with an increased risk of death while on the waiting list [24,25]. The model has proven to be predictive of death of patients on the waiting list, and ranks patients on disease severity rather than waiting time, but it does not have good predictive value for mortality following LDLT. Our study enforces the feasibility of the early postoperative MELD scoring system as a predictive index of mortality after LDLT rather than the usual MELD-Pre score. Therefore, it could enable early registration for a cadaveric LT or early consideration for a second LDLT before primary graft dysfunction occurred.

**Table 3. Multivariate Analysis of Risk Factors Related to Graft Loss After LDLT (n = 217)**

| Variables (n = 217) | OR | 95% CI | P |
|---|---|---|---|
| MELD score-POD2 ≥19 | 5.601 | 1.395–4.508 | .0009 |
| Preoperative status: hospitalized | 3.330 | 0.318–0.893 | .0151 |
| Donor age ≥ 35 y | 1.977 | 0.810–3.631 | .1537 |

Recently, Rahbari et al [16] first applied the MELD scoring system with postoperative parameters as a predictive index of postoperative outcome. They demonstrated that the MELD score on POD5 was associated with mortality (OR, 2.06; 95% CI, 1.41–3.02) after hepatectomy using multivariate analyses, and the AUROC value of the MELD score on POD5 was 0.862 for the prediction of mortality [16]. However, they did not state why the MELD score on POD5 only was applied, and the cases were limited to patients who underwent hepatectomy. We examined the values of the MELD score on various postoperative days, namely POD1, 2, 3, 5, 7, and 14, as predictors of mortality following LDLT. The scores on POD2 or later were found to be useful predictors whose AUROC c-statistics were almost 0.8 or more with good sensitivity and specificity. The sensitivity and specificity of the MELD-POD2 index for graft loss within 6 months were 68.2 and 79.5, respectively, whereas the AUROC c-statistic of the MELD-POD5 index was 0.833 with excellent sensitivity and specificity of 81.8 and 83.6, respectively. It clearly demonstrated that postoperative risk scores, MELD-POD2 onward, after LDLT were more powerful predictors of mortality than the usual preoperative MELD score. These data confirm the a priori hypothesis that early postoperative risk assessment allows for more accurate prediction of postoperative outcome compared with the commonly performed preoperative evaluations, although the latter may, however, be useful for evaluating the eligibility for LDLT.

There are some concerns about Re-LT because of the unclear mortality after Re-LT, and the discrepancy between organ availability and the number of patients awaiting LT [26–28]. In spite of the steady improvement in survival of recipients after LT, a proportion of these recipients experience graft failure requiring Re-LT, and graft failure as a result of early or late complications and disease recurrence are more commonly encountered after Re-LT [29]. The only life-saving treatment for patients with liver allograft failure is Re-LT, and it requires extensive surgical expertise and experienced decision-making before, during, and after the surgical procedure [26–28]. The overall graft and patient survival is lower than in those undergoing a first LT, and the gap between liver graft supply and demand is widening globally [26–28]. Therefore, the use of either cadaveric or living donor liver grafts for Re-LT is controversial. The precise assessment of patient status after LT is indispensable to determine the need for Re-LT as soon as possible, or to register for cadaveric LT. Considering the limitation of established indices, such as the preoperative MELD score, our present index, the postoperative MELD score appears to provide an improved prediction of mortality following LT; therefore, it may have value for indicating the need for Re-LT with a precise evaluation of patient status following LDLT.

In conclusion, the present early postoperative MELD score is a feasible index for prediction of postoperative mortality following LDLT. It provides not only an accurate assessment of patient status after LDLT, but also an

indication of the need for Re-LT. Further prospective studies with a larger number of cases are warranted to confirm the value of the early postoperative MELD score.

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PLOS ONE

# 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* | $1.55 \times 10^{-7}$ | 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 $\alpha$ 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: $\text{logit}(p) = -3.905+0.083*\text{age}+(-0.929)*\text{sex}+(-0.684)*DPB1*02:01+1.814*c1+(-0.478)*c2+0.782*c3$. 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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LETTER TO THE EDITORS

# Fluctuations in the concentration/dose ratio of calcineurin inhibitors after simeprevir administration in patients with recurrent hepatitis C after liver transplantation

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Dear Sirs,

As the efficacy of dual therapy with peginterferon and ribavirin for recurrent hepatitis C after liver transplantation is limited, direct-acting antiviral agents (DAA) should be considered. First-generation NS3/4A inhibitors, such as telaprevir or boceprevir, for liver transplant recipients are problematic because of their inhibitory action on cytochrome P450 3A (CYP3A), an enzyme responsible for the metabolism of calcineurin inhibitors including tacrolimus and cyclosporine. In fact, administration of telaprevir resulted in elevation of blood concentrations and increase in the elimination half-life of calcineurin inhibitors (CNI) [1]. Therefore, when telaprevir is used for recurrent hepatitis C after liver transplantation, the dose of the CNI needs to be reduced to maintain proper blood concentrations, resulting in a significant increase in the concentration/dose (C/D) ratio of the CNI after the administration of telaprevir [2].

Since January 2014, we started using the second-generation NS3/4A inhibitor simeprevir along with peginterferon

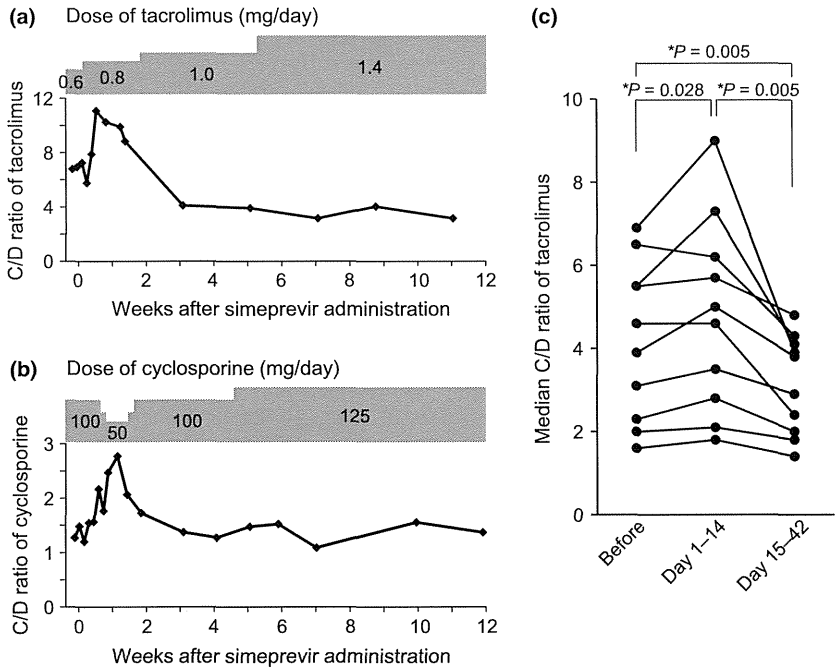


**Figure 1** (a,b) Time course of the concentration/dose (C/D) ratio of a calcineurin inhibitor for case 1 (a) and case 2 (b), both of which involved triple therapy with simeprevir, peginterferon, and ribavirin for recurrent hepatitis C after liver transplantation. The fine line represents the C/D ratio of tacrolimus (ng/ml per mg) in a or cyclosporine (ng/ml per mg) in b. The dose of calcineurin inhibitors is shown in gray boxes. (c) Median C/D ratio of tacrolimus in 10 patients with simeprevir-based triple therapy after liver transplantation. Significant differences between the 2 groups are indicated by * with *P* values analyzed by Wilcoxon's signed-rank test. Difference among the 3 groups is also significant by Friedman's test ($P < 0.001$).

 

and ribavirin for patients with recurrent hepatitis C after liver transplantation. The dose of the CNI was adjusted using therapeutic drug monitoring (TDM) of either tacrolimus or cyclosporine. In 11 cases, we identified fluctuations in the C/D ratio during the simeprevir-based triple therapy. Six of the 11 patients were men, and the median age was 64 years (range, 46–73 years). Before the treatment, fibrosis scores F1 and F2 based on the METAVIR score was found in five and six patients, respectively. Tacrolimus-based immunosuppression with ($n$ = 4) or without ($n$ = 6) mycophenolate mofetil was administered to 10 patients, and cyclosporine with mycophenolate mofetil was administered to one patient. Median serum alanine aminotransferase (ALT) level before treatment was 51 IU/l (range, 21–115), and ALT of all patients decreased to the normal range in the first 2 weeks of treatment.

For the first 2 cases, the time course of the C/D ratio of tacrolimus in case 1 and cyclosporine in case 2 is shown in Fig. 1a and b. Blood concentrations of tacrolimus and cyclosporine were adjusted to trough levels 6–8 and 150–200 ng/ml, respectively, using TDM after simeprevir administration (100 mg/day). The C/D ratio of calcineurin inhibitors were elevated in the first 2 weeks in both cases, but decreased thereafter, necessitating an increase in the dose of the calcineurin inhibitor. The median C/D ratio of tacrolimus before, the first 2 weeks after, and 3–6 weeks after simeprevir administration in the 10 consecutive cases of patients receiving tacrolimus and simeprevir-based triple therapy in our hospital is shown in Fig. 1c. The median C/D ratio significantly increased from 4.25 ng/ml/mg before simeprevir administration to 4.8 ng/ml per mg in the first 2 weeks, but significantly decreased to 3.35 ng/ml per mg after 3 weeks of simeprevir administration.

These findings revealed the importance of TDM of CNI in transplant recipients undergoing simeprevir-based triple therapy. During the first 2 weeks, elevation of the C/D ratio would be caused by the interaction of simeprevir with CNI, because simeprevir is metabolized by the enzyme CYP3A, which is responsible for the metabolism of CNI. Notably, the C/D ratio was significantly decreased after 2 weeks of simeprevir-based triple therapy, despite continuous simeprevir administration at the same dose. The mechanism for the decrease in concentration of CNI with effective antiviral therapy has been proposed to be due to an increased metabolism of CNI by improvement in liver function [3]. Changes of CNI concentrations would be more dynamic using DAA, because of the drug–drug interaction and strong anti-HCV effect. Therefore, we should be cautious of the fluctuations in the CNI concentrations especially during DAA-based therapy and thus recommend TDM during the entire period of antiviral therapy.

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## Conflict of interest

Y Ueda and S Uemoto have received research grants from Astellas Pharma Inc.

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# Impact of *cytochrome P450 3A5* polymorphism in graft livers on the frequency of acute cellular rejection in living-donor liver transplantation

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***Objective*** We investigated whether the *cytochrome P450 3A5**3 (*CYP3A5**3) genotype affects tacrolimus pharmacokinetics and the risk of acute cellular rejection in living-donor liver transplant patients in Japan.

***Materials and methods*** Between July 2004 and June 2011, we enrolled 410 living-donor liver transplant patients receiving tacrolimus. Biopsy specimens of intestinal mucosa and graft liver at surgery were obtained to examine the mRNA expression of CYP3A subfamilies as well as the genotyping of *CYP3A5**3 polymorphism.

***Results*** The *CYP3A5* genotype in the native intestine had no significant effect on the occurrence of acute cellular rejection between postoperative days 14 and 23 in cases with identical or compatible ABO blood types (11.5% for the *CYP3A5**1 allele vs. 7.4% for *CYP3A5**3/*3; $P = 0.2643$), although the concentration/dose ratio of tacrolimus was significantly higher in patients with the intestinal *CYP3A5**3/*3 genotype than in those with the *CYP3A5**1 allele for 5 post-transplant weeks. However, patients who received a graft liver with the *CYP3A5**1 allele showed a higher rate of acute cellular rejection than those who received a graft liver with the *CYP3A5**3/*3 genotype (14.5 vs. 5.7%; $P = 0.0134$). The relative risk for acute cellular rejection associated with the *CYP3A5**1 liver allele was 2.629 ($P = 0.018$, Cox regression model). Consequently, graft liver *CYP3A5**1 genotype might increase the risk for acute cellular rejection after living-donor liver transplantation, possibly by associating with the local hepatic tacrolimus concentration.

***Conclusions*** The target level of tacrolimus may be affected by the *CYP3A5**3 genotype of the liver, rather than by that of the small intestine, after postoperative day 14.
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Keywords: acute cellular rejection, *CYP3A5**3, liver transplantation, tacrolimus

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## Introduction

Liver transplantation is a life-saving treatment for end-stage liver disease caused by cirrhosis, autoimmune hepatitis, and biliary atresia. Tacrolimus, a calcineurin inhibitor, is used widely after liver transplantation as a primary immunosuppressive agent. Therapeutic drug monitoring is essential to maintain the appropriate concentration of tacrolimus in the blood as it has a narrow therapeutic range and pharmacokinetics that vary widely within and between individuals [1,2]. Single nucleotide polymorphisms (SNPs) in the genes encoding drug-metabolizing enzymes are reported to be among the factors that affect the pharmacokinetics of tacrolimus. Cytochrome P450 3As (CYP3As) are involved in the metabolism of many drugs, including tacrolimus. Previously, we showed that in living-donor liver transplant patients, lower tacrolimus concentration/dose (C/D) ratios were associated with graft livers (donor genotype) and native small intestines (recipient genotype) that had the *CYP3A5**1 allele than were associated with those that were *CYP3A5**3 (rs776746)/*3 homozygotes [3–6]. The *CYP3A5**3 genotype causes an abnormal mRNA splicing and results in a lack of functional CYP3A5 protein. Insufficient or excessive immunosuppression with tacrolimus in transplant patients causes acute cellular rejection and severe adverse effects, including infectious complications, hypertension, and nephrotoxicity [7]. The association between the *CYP3A5**3 genotype and increased risk for acute cellular rejection in renal transplantation has been controversial [8–12]. However, there has been only a preliminary report describing the relationship between *CYP3A5**3 SNP and infectious complications in liver transplant patients [13]. We investigated the effect of the *CYP3A5**3 genotype on the

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pharmacokinetics of tacrolimus and on the risk of acute cellular rejection in living-donor liver transplant patients.

## Materials and methods

### Patients, clinical samples, and criteria for acute cellular rejection

Between July 2004 and June 2011, we enrolled 410 Japanese living-donor liver transplant patients who were treated with tacrolimus as a primary immunosuppressant at Kyoto University Hospital and 412 donors in this study. All participants provided written informed consent. Two recipients underwent retransplantation during this period. Patients with ABO blood type incompatibility were excluded from retrospective observational analyses of acute cellular rejection because of their different immunosuppressive regimens that included additional rituximab and hepatic artery infusion of steroid and/or cyclophosphamide and/or prostaglandin E1. In addition, patients with retransplantations were excluded from analyses of acute cellular rejection because it was assumed that the state of immunoreactivity in the retransplanted patients, who received oral immunosuppressants until just before their retransplantation, differed from that of the de-novo transplantees. This study was carried out in accordance with the Declaration of Helsinki and its amendments and was approved by the Kyoto University Graduate School and Faculty of Medicine, Ethics Committee.

Intestinal samples were obtained from the upper jejunum between July 2004 and May 2010; part of the Roux-en-Y limb was used for biliary reconstruction or the biopsy specimen at the upper jejunum was obtained during the placement of a biliary drainage tube. Liver samples were obtained between July 2004 and June 2011 from biopsy specimens that were obtained for pathological testing of the graft at surgery (zero biopsy) [14].

Acute cellular rejection was diagnosed on the basis of an increase in transaminase and/or the histological evaluation of liver biopsy specimens between postoperative days 11 and 26. Most patients who experienced acute cellular rejection were treated with intravenous high-dose steroid (10 mg/kg/day).

To examine the influence of genotype matching, we classified genotypes into two groups and patients into four groups. The genotype groups were the 3A5*1 group (*CYP3A5**1/*1 and *CYP3A5**1/*3), which included CYP3A5 expressers, and the 3A5*3 group (*CYP3A5**3/*3), which had defective CYP3A5. The patient groups were based on the genotypes of the donor and the recipient. The four groups of patients were the liver 3A5*1/intestine 3A5*1 group, in which both the liver (donor) and native intestine (recipient) carried a *CYP3A5**1 allele; the liver 3A5*1/intestine 3A5*3 group, in which the donor carried a *CYP3A5**1 allele and the recipient had the *CYP3A5**3/*3 genotype; the liver 3A5*3/intestine 3A5*1 group, in which the donor had the *CYP3A5**3/*3 genotype and the recipient carried the *CYP3A5**1 allele; and the liver 3A5*3/intestine 3A5*3 group, in which both the donor and the recipient had the *CYP3A5**3/*3 genotype (Table 2).

### Measurement of tacrolimus concentrations

Before patients received their morning dose of tacrolimus, whole-blood samples were obtained to determine the tacrolimus trough levels. The blood concentration of tacrolimus was measured using a microparticle enzyme-linked immunoassay (IMx; Abbott, Tokyo, Japan) between July 2004 and March 2009, and a chemiluminescent enzyme immunoassay (ARCHITECT; Abbott) after April 2009. The equivalence of the data obtained using these two methods was validated (data not shown). The daily oral dose of tacrolimus was adjusted to achieve target trough blood concentrations of 10–15 ng/ml during the first 2 weeks following surgery, ~ 10 ng/ml during the next 2 weeks, and 5–7 ng/ml thereafter.

### Genotyping of the *CYP3A5**3 polymorphism

Genomic DNA was extracted from homogenates of liver biopsy specimens and the intestinal mucosa using MagNAPure LC DNA Isolation kit I (Roche, Mannheim, Germany) or the AllPrep DNA/RNA Mini kit (Qiagen, Hilden, Germany). Genomic DNA was extracted from peripheral blood using either MagNAPure LC DNA Isolation kit I (Roche) or EZ1 DNA Blood kit (Qiagen). All DNA extraction was performed according to the manufacturers' instructions. The *CYP3A5**3 polymorphism was examined using the previously described PCR-restriction fragment length polymorphism method [6].

### Evaluation of hepatic and intestinal mRNA expression of CYP3As

Total RNA was extracted from homogenates of graft liver and intestinal mucosa biopsy specimens using MagNAPure LC RNA Isolation kit II (Roche) or the AllPrep DNA/RNA Mini kit (Qiagen). The mRNA expression levels of CYP3A4, CYP3A5, CYP3A7, and CYP3A43 in the graft liver and native intestine were quantified by real-time PCR using an absolute calibration method with a standard curve generated using known amounts of standard plasmid DNA [6] and an ABI prism 7700 sequence detector (Applied Biosystems, Foster City, California, USA). The lower limit of quantification was set to a cycle threshold ($C_t$) value of 40. The primer/probe sets used for these CYP3As were those reported by Koch *et al.* [15]. GAPDH was used as an internal control as described previously [16]. The level of mRNA expression in each sample was corrected by the amount of GAPDH.

### Statistical analysis

Differences between mRNA expression levels in the *CYP3A5**3 genotype groups were analyzed using the Mann–Whitney *U*-test. The Kruskal–Wallis test, followed by Dunn's post-hoc test was used to perform comparisons between three or more groups. The probability of acute

cellular rejection was estimated using the Kaplan–Meier method and probabilities were compared using log-rank analysis. *P* values less than 0.05 were considered to be statistically significant. Statistical analyses were carried out using Prism version 5.0 software (GraphPad Software Inc., San Diego, California, USA). A Cox proportional hazards regression model was used for multivariate time to event analysis using SPSS version 19.0 (IBM Corp., Armonk, New York, USA).

## Results

### Relationship between the *CYP3A5**3 polymorphism and the level of mRNA expression of CYP3A subfamilies in the liver and intestine

The demographics of recipients and donors in this study, including age, sex, graft-to-recipient body weight ratio, and primary diseases, are shown in Table 1. Of the ABO identical or compatible recipients, four individuals died within 14 days after transplant because of a viral infection (two cases), thrombotic microangiopathy (one case), or pulmonary hemorrhage (one case). These four patients were excluded from the analyses of acute cellular rejection and *CYP3A5* genotype, but not from the analyses of mRNA expression and tacrolimus pharmacokinetics. We quantified the levels of CYP3A4, CYP3A5, CYP3A7, and CYP3A43 mRNA expression in the graft liver and native intestine using real-time PCR. Because they were below the lower limitation of quantification for mRNA expression of CYP3A subfamilies, 11 CYP3A7 and six CYP3A43 liver samples, and four CYP3A4, one CYP3A5, 144 CYP3A7, and 194 CYP3A43 small intestine samples were assigned a value of half of the minimum detectable level. Figure 1 shows the mRNA expression levels of CYP3A4 (Fig. 1a and e), CYP3A5 (Fig. 1b and f), CYP3A7 (Fig. 1c and g), and CYP3A43 (Fig. 1d and h) in graft liver (Fig. 1a–d) and native intestine (Fig. 1e–h). The expression levels of CYP3A5 (Fig. 1b) and CYP3A7 (Fig. 1c) were significantly higher in graft livers that carried the *CYP3A5**1 allele than in those that had the *CYP3A5**3/*3 genotype (CYP3A5; $P<0.0001$, CYP3A7; $P=0.0004$, Mann–Whitney *U*-test), whereas CYP3A4 (Fig. 1a) and CYP3A43 (Fig. 1d) were expressed at the same level irrespective of the *CYP3A5**3 polymorphism. In the small intestine, only the level of CYP3A5 mRNA expression was significantly higher in those carrying the *CYP3A5**1 allele than in those who had the *CYP3A5**3/*3 genotype (Fig. 1f; $P<0.0001$, Mann–Whitney *U*-test). Next, we examined the relationship between the mRNA expression levels of CYP3As in the liver and the age of the donor and between those in the intestine and the age of the recipient in living-donor liver transplantation (see Supplemental digital content 1, *http://links.lww.com/FPC/A734*, showing the correlation between CYP3A mRNA expression levels and age in living-donor liver transplantation) because few previous studies had examined this relationship. Although we had no data on mRNA expression levels in neonates, neither intestinal nor hepatic mRNA expression of CYP3As showed any relationship with age. Drug-metabolizing enzymes such as CYP3A subfamilies were physiologically essential for defense mechanism irrespective of age, but it is unknown whether the mRNA expression levels are related to the in-vivo enzymatic activity both in children and in adults. Because the CYP3A mRNA expression levels showed a weak correlation with age, we did not complete an analysis of covariance (i.e. did not adjust for age). In addition, we examined the influence of ABO blood group match and *CYP3A5* genotype on mRNA expression of CYP3A subfamilies in the liver and intestine, respectively (see Supplemental digital content 2, *http://links.lww.com/FPC/A735*, showing the relationship between mRNA levels in graft liver or intestine, combined with the *CYP3A5* genotype and ABO blood group compatibilities). In this

**Table 1 Demographics of living-donor liver transplant recipients and donors**

| Recipients | *n*=410 | Donors | *n*=412 |
|---|---|---|---|
| Age (years)[a] | 46 (0.1–69) | Age (years)[a] | 39 (20–66) |
| Sex (male/female) | 185/225 | Sex (male/female) | 213/199 |
| Body weight (kg)[a] | 52 (3.1–106) | | |
| GRWR (%)[a] | 1.1 (0.5–5.3) | | |
| ABO blood group match | | | |
| Identical/compatible/incompatible | 231/78/103 | | |
| Primary disease | | | |
| Cirrhosis | 226 | | |
| Hepatitis C virus infection[b] | 106 | | |
| Hepatitis B virus infection[b] | 58 | | |
| Primary biliary cirrhosis | 35 | | |
| Alcoholic cirrhosis | 11 | | |
| Other cirrhosis | 16 | | |
| Biliary atresia | 100 | | |
| Primary sclerosing cholangitis | 8 | | |
| Fulminant hepatic failure | 5 | | |
| Hepatoblastoma | 7 | | |
| After liver transplantation | 24 | | |
| Others[c] | 42 | | |

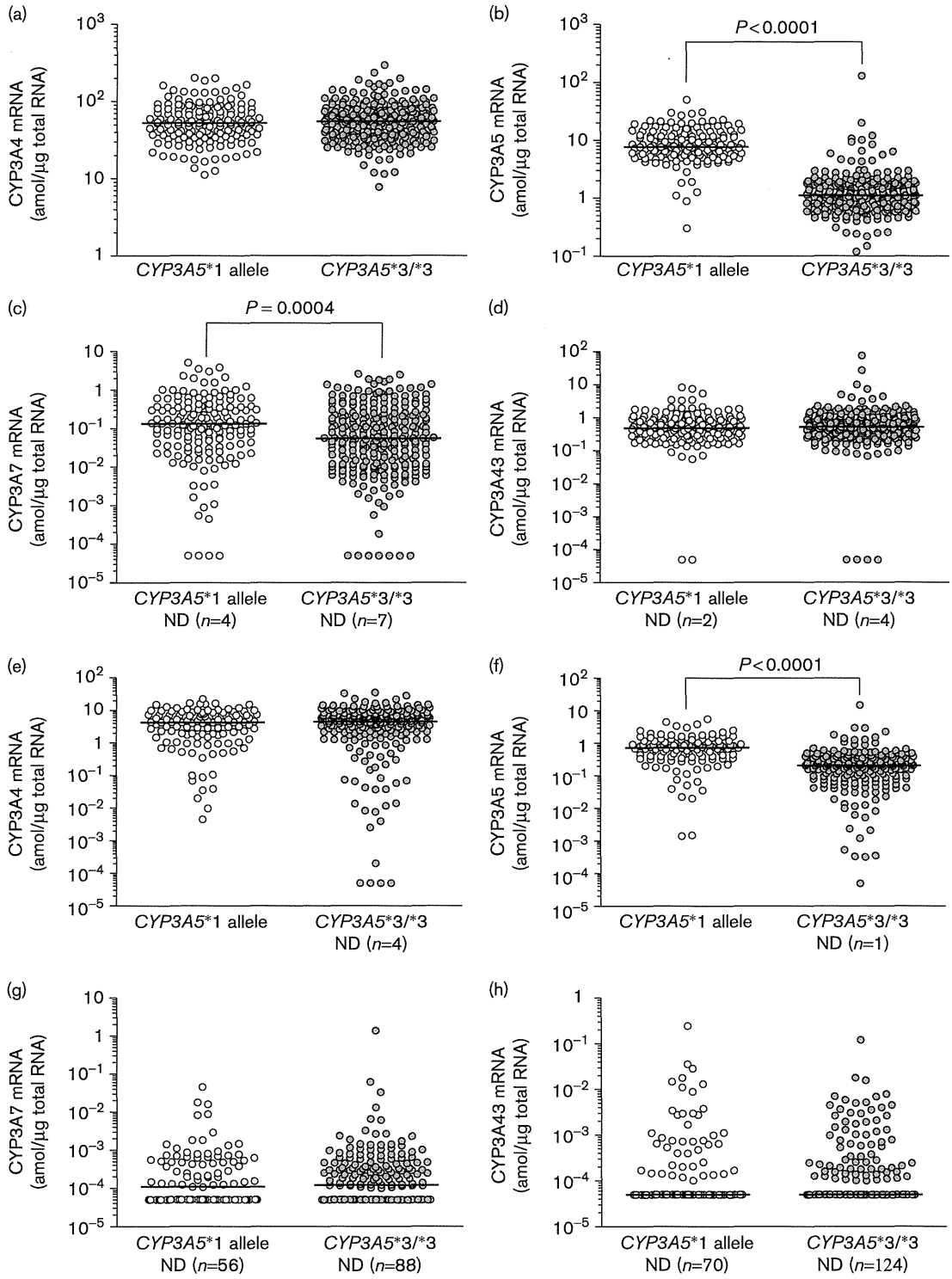
GRWR, graft-to-recipient body weight ratio.
[a]Data are expressed as medians with ranges in parentheses.
[b]Two patients with hepatitis C also had complication of hepatitis B virus and two also had complication of alcoholic cirrhosis. One patient with hepatitis B had complication of alcoholic cirrhosis.
[c]The primary diseases were autoimmune hepatitis (4), Byler disease (2), Budd–Chiari syndrome (4), Caroli disease (1), or Wilson disease (3), nonalcoholic steatohepatitis (4), biliary dilation (2), hyperoxaluria (2), hypertyrosinemia (1), polycystic liver disease (2), Alagille syndrome (3), ornithine carbamoyltransferase deficiency disease (1), glycogenosis (2), Jeune's syndrome (1), congenital extrahepatic portosystemic shunt (1), carbamyl phosphate synthetase deficiency (1), somatostatinoma (1), argininosuccinate lyase deficiency (1), amyloidosis (1), hemangioendothelioma (1), and portal vein deficiency (4). The number of patients is indicated in parentheses.

**Table 2 Categorization of patients according to the combination of the *CYP3A5**3 genotype of the donor and the recipient**

| Category | Graft liver (donor) *CYP3A5**3 genotype | Native intestine (recipient) *CYP3A5**3 genotype |
|---|---|---|
| L*1/S*1 | *1/*1, *1/*3 | *1/*1, *1/*3 |
| L*1/S*3 | *1/*1, *1/*3 | *3/*3 |
| L*3/S*1 | *3/*3 | *1/*1, *1/*3 |
| L*3/S*3 | *3/*3 | *3/*3 |

L, graft liver from donor; S, small intestine of recipients.

**Fig. 1**



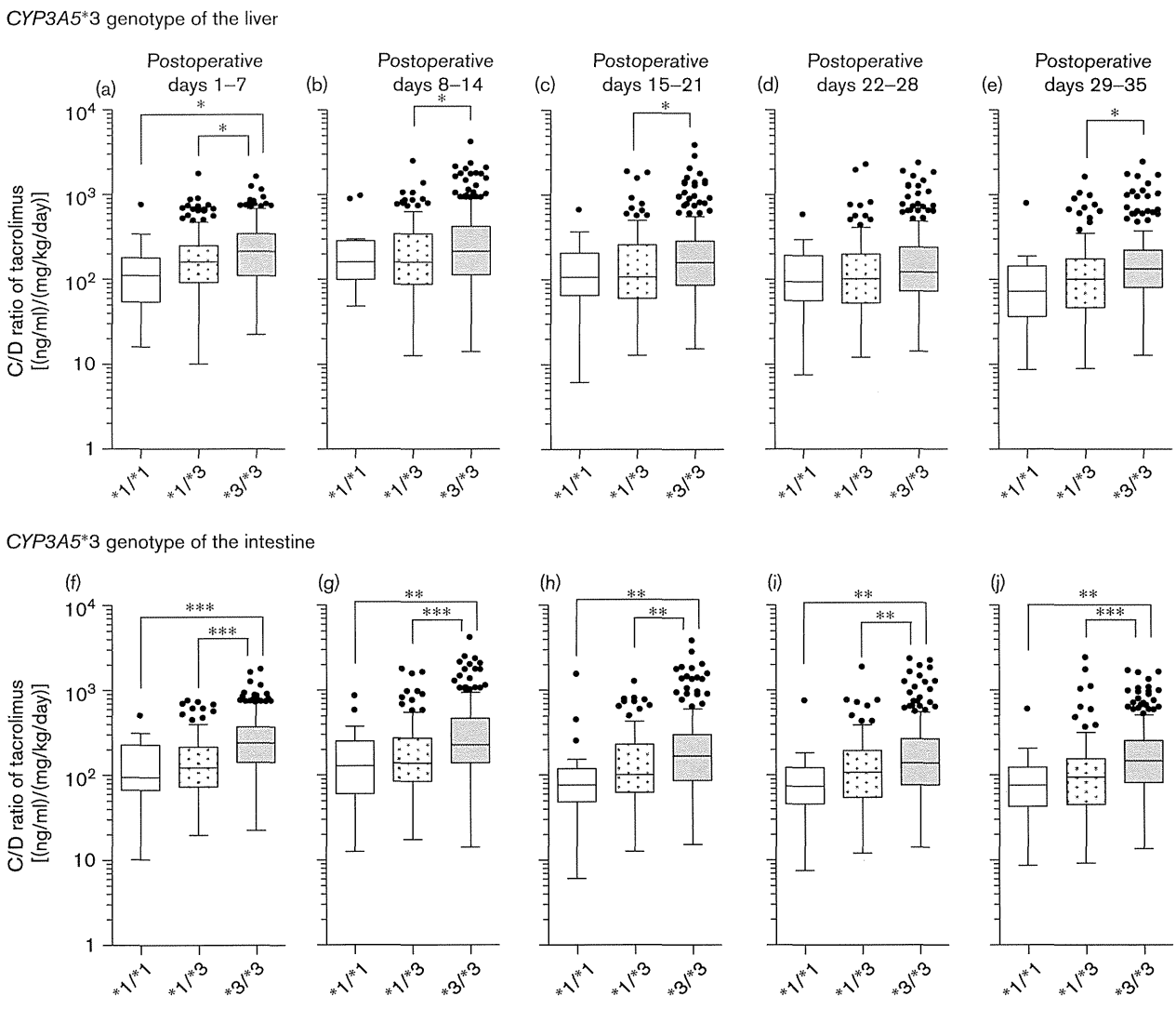

mRNA expression levels of cytochrome P450 (CYP) 3A4 (a, e), CYP3A5 (b, f), CYP3A7 (c, g), and CYP3A43 (d, h) in graft liver (a–d) and native intestine (e–h), and their relationship with the *CYP3A5*1* allele in graft liver or small intestine of living-donor liver transplant patients. The patients were divided into two groups on the basis of the *CYP3A5* genotypes of each tissue as follows: *CYP3A5*1* allele (open circle) and *CYP3A5*3/*3* genotype (filled circle). The groups and number of patients are as follows: the graft liver (a–d), *CYP3A5*1* allele ($n=155$) and *CYP3A5*3/*3* ($n=251$); the small intestine (e–h), *CYP3A5*1* allele ($n=114$) and *CYP3A5*3/*3* ($n=197$). The horizontal bar indicates the median mRNA expression level for each genotype.

analysis, ABO blood group match was divided into two groups, either compatible, meaning that donor and recipient had identical or the compatible blood groups, or incompatible, meaning that they had incompatible blood groups. This result was similar to that of the analysis that examined only the *CYP3A5* genotype on the CYP3A subfamilies' mRNA expression (Fig. 1). The effect of the *CYP3A5**3 genotype on CYP3A7 mRNA expression in the liver was greater in ABO-incompatible individuals than in ABO blood-compatible individuals.

### Influence of donor or recipient *CYP3A5**3 genotype on the C/D ratio of tacrolimus during the 5 weeks following liver transplantation

Using a large number of cases ($n=407$), we examined whether the *CYP3A5**3 polymorphism influenced the C/D ratio of tacrolimus. A temporary intravenous injection of high-dose steroid was administered to treat acute cellular rejection. Because the steroid induced an increase in the intestinal mRNA expression of CYP3A4, C/D ratios determined during the 4 days after the end of

**Fig. 2**




The influence of graft liver or intestinal *cytochrome P450 (CYP) 3A5* polymorphism on the concentration/dose (C/D) ratio of tacrolimus in living-donor liver transplant recipients over 5 weeks. The mean tacrolimus C/D ratios for 1–7 (a, f), 8–14 (b, g), 15–21 (c, h), 22–28 (d, i), and 29–35 (e, j) days after transplantation for each *CYP3A5* genotype were compared. The bar indicates the median tacrolimus C/D ratio for each group and boxes represent the 25th and 75th percentiles of the data. The whiskers represent the lowest and highest values that fall within 1.5 × the interquartile range of the lower quartile and the upper quartile, respectively. The numbers of graft liver samples (a–e) and native intestine samples (f–j) with each *CYP3A5* genotype were 18 and 26, respectively, with *CYP3A5**1/*1; 137 and 137, respectively, with *CYP3A5**1/*3; and 252 and 244, respectively, with *CYP3A5**3/*3. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, between groups.

treatment with high-dose steroid were excluded from the analysis of the relationship between the tacrolimus C/D ratio and *CYP3A5**3 genotype [17]. The average C/D ratios for every week after surgery were calculated in each patient. Figure 2a–e shows that donor livers with *CYP3A5**1/*3 were associated with a significantly lower C/D ratio than those with *CYP3A5**3/*3 for all periods except postoperative days 22–28 ($P < 0.05$, Kruskal–Wallis test). As shown in Fig. 2f–j, recipients who had the *CYP3A5**3/*3 genotype had a significantly higher C/D ratio than those who had *CYP3A5**1/*1 for all periods (postoperative days 1–7; $P < 0.001$, postoperative days 8–35; $P < 0.01$, Kruskal–Wallis test).

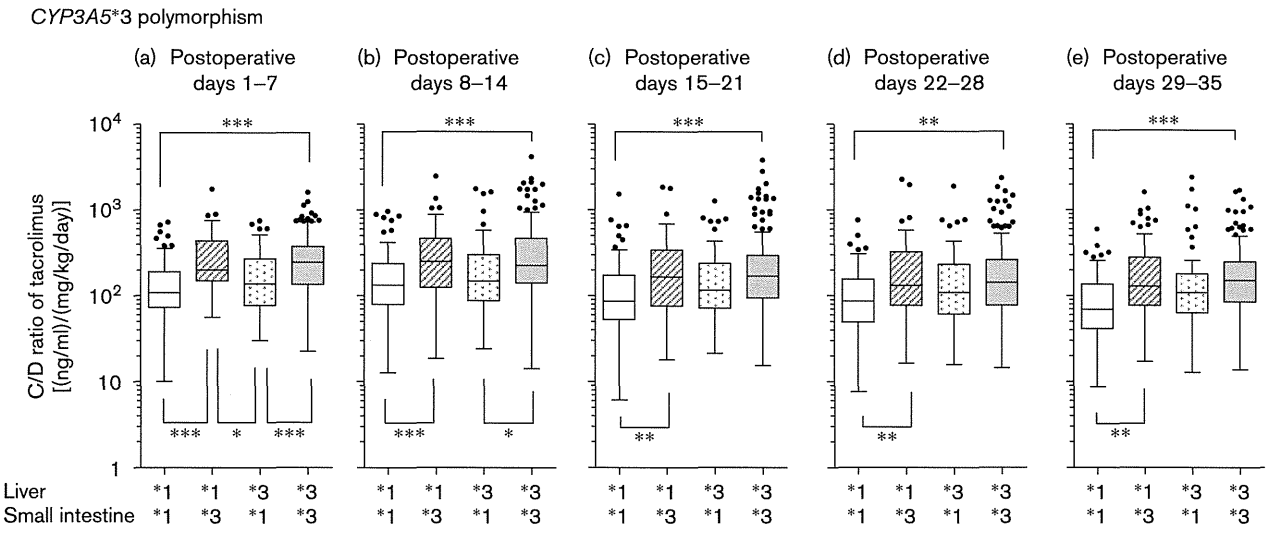
### Influence of the combination of donor and recipient *CYP3A5**3* genotype on the C/D ratio of tacrolimus during the 5 weeks following liver transplantation

Among the patients who received living-donor liver transplantation, several combinations of the graft liver (donor) SNPs and native intestine (recipient) SNPs were present, as indicated in Table 2. The effects of the combination of *CYP3A5* genotypes on the tacrolimus C/D ratio during the 5 weeks following surgery are shown in Fig. 3. The L*1/S*1 combination was associated with a significantly lower C/D ratio than the L*1/S*3 and L*3/S*3 combinations throughout all 5 postoperative weeks. Next, we examined the influence of CYP3A5 mRNA expression on the tacrolimus C/D ratio. The patients were divided into four groups on the basis of the median CYP3A5 mRNA expression levels in the liver and intestine, respectively (see Supplemental digital content 3, *http://links.lww.com/FPC/A736*, showing the relationship between the tacrolimus C/D ratio and CYP3A5 mRNA expression levels in the liver and intestine). The group with high CYP3A5 expression both in the liver and in the intestine had a lower C/D ratio than the group with low CYP3A5 expression in the liver and high expression in the intestine for only 2 weeks after surgery. The C/D ratio of tacrolimus in the group with high CYP3A5 expression in the liver and low expression in the intestine was higher than that in the group with high CYP3A5 expression both in the liver and in the intestine for only 7 days after transplantation.

### Effect of the *CYP3A5**1* allele on the frequency of acute cellular rejection between postoperative days 14 and 23 in living-donor liver transplant patients

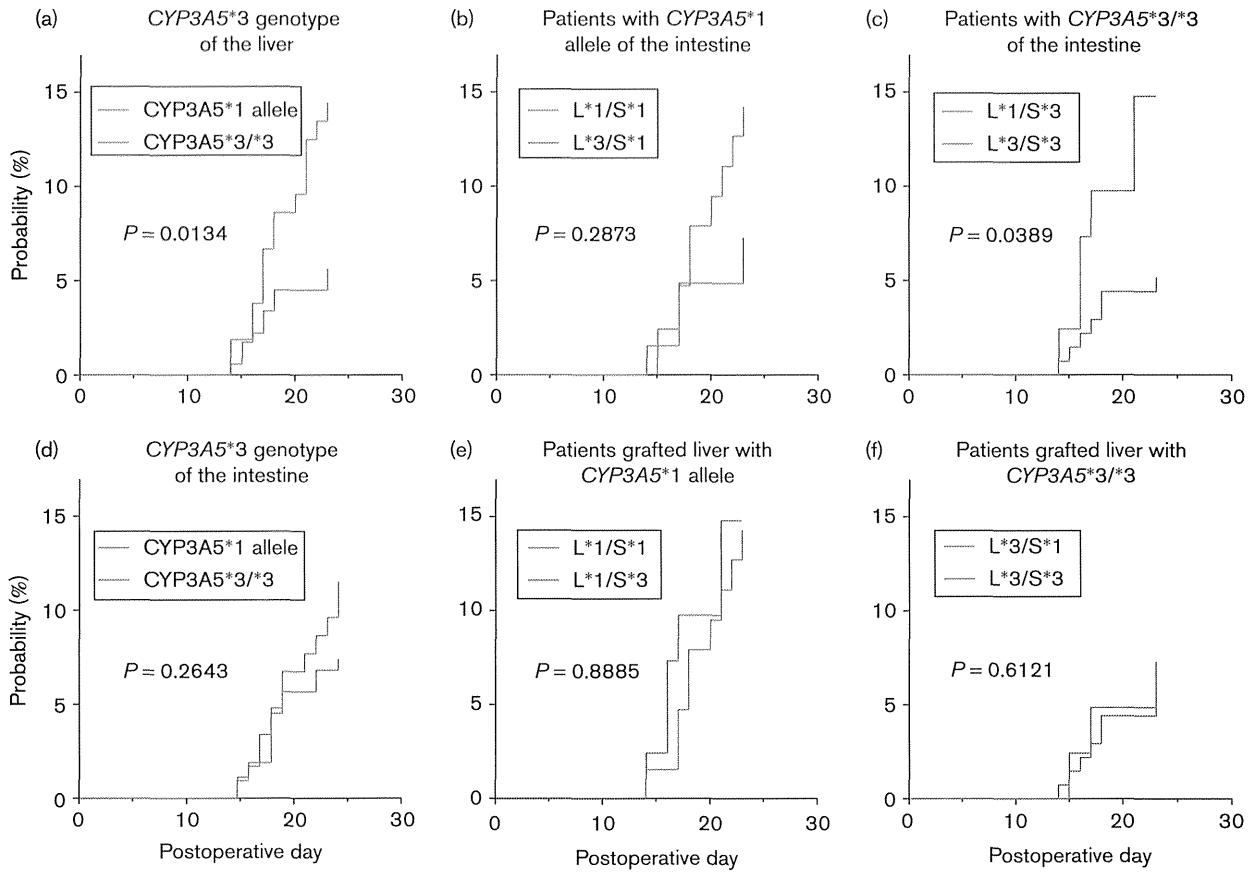
Acute cellular rejection events between postoperative days 14 and 23 in living-donor liver transplant patients were examined retrospectively. Cases of ABO blood type incompatibility and retransplantation were excluded. Four additional cases were excluded from analysis because of patient death within 14 days after surgery. The Kaplan–Meier curve in Fig. 4a shows that the *CYP3A5* genotype of the graft liver was a significant factor affecting the post-transplant risk of acute cellular rejection between postoperative days 14 and 23 [5.7% for *CYP3A5**3/*3 ($n = 177$) vs. 14.5% for the *CYP3A5**1

**Fig. 3**




The influence of combinations of graft liver and native intestinal *cytochrome P450 (CYP) 3A5* genotypes on the concentration/dose (C/D) ratio of tacrolimus for postoperative days 1–35 after living-donor liver transplantation. Cases were categorized into four groups on the basis of the graft and intestinal *CYP3A5* genotypes (*CYP3A5**1, *CYP3A5**1/*1 and *CYP3A5**1/*3; *CYP3A5**3, *CYP3A5**3/*3). The C/D ratios of tacrolimus in each group were compared for 5 weeks: 1–7 (a), 8–14 (b), 15–21 (c), 22–28 (d), and 29–35 (e) days after transplantation. The bar indicates the median tacrolimus C/D ratio for each group and boxes represent the 25th and 75th percentiles of the data. There were 93 groups that had both *CYP3A5**1 graft livers and native intestines, 62 that had *CYP3A5**1 graft livers and *CYP3A5**3 native intestines, 68 that had *CYP3A5**3 graft livers and *CYP3A5**1 native intestines, and 184 that had both *CYP3A5**3 graft livers and native intestines. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ between groups.

**Fig. 4**




Effect of the graft liver or intestinal *cytochrome P450* (*CYP*) *3A5**3 polymorphism on the time to onset of acute cellular rejection between postoperative days 14 and 23. The Kaplan–Meier curve shows the association between the time to occurrence of acute cellular rejection and *CYP3A5**3 genotype in the liver (donors; a, b, c) or intestine (recipients; d, e, f). In the groups that had the *CYP3A5**1 allele of the intestine, the difference between the *CYP3A5**1 allele and the *CYP3A5**3/*3 of the liver was compared (b, L*1/S*1; $n=64$, L*3/S*1; $n=41$). In the groups that had *CYP3A5**3/*3 of the intestine, the difference between the *CYP3A5**1 allele and the *CYP3A5**3/*3 of the liver was compared (c, L*1/S*3; $n=41$, L*3/S*3; $n=136$). In the groups that had the *CYP3A5**1 allele of the liver, the difference between the *CYP3A5**1 allele and the *CYP3A5**3/*3 of the intestine was compared (e, L*1/S*1; $n=41$, L*1/S*3; $n=64$). In the groups that had *CYP3A5**3/*3 of the liver, the difference between the *CYP3A5**1 allele and the *CYP3A5**3/*3 of the intestine was compared (f, L*3/S*1; $n=41$, L*3/S*3; $n=136$).

allele, including both *CYP3A5**1/*1 and *CYP3A5**1/*3 ($n=105$); $P=0.0134$ by log-rank test]. However, the *CYP3A5* genotype of the native intestine had almost no effect on the frequency of acute cellular rejection (7.4% for *CYP3A5**3/*3 vs. 11.5% for the *CYP3A5**1 allele; $P=0.2643$ by log-rank test; Fig. 4d). Next, we examined the influence of the *CYP3A5**3 genotype in the graft liver or native intestine on the frequency of acute cellular rejection when the *CYP3A5**3 genotype in the native intestine or graft liver was identical, respectively. In the groups that have the *CYP3A5**3/*3 genotype in the intestine, the *CYP3A5**1 allele in the liver has a significantly higher frequency of acute cellular rejection than *CYP3A5**3/*3 in the liver (Fig. 4c). In the groups that have the *CYP3A5**1 allele in the intestine, a similar result was found as in the group that had the *CYP3A5**3/*3 genotype in the intestine without statistical significance (Fig. 4b). However, there was no significant difference between L*1/S*1 and L*1/S*3 and between L*3/S*1 and L*3/S*3 ($P=0.8885$ and 0.6121, respectively; Fig. 4e and f). Next, the influence of sex (donor or recipient) or a combination of sex (recipient and donor) on the frequency of acute cellular rejection between postoperative days 14 and 23 was compared using a Kaplan–Meier curve (see Supplemental digital content 4, *http://links.lww.com/FPC/A737*, presenting time to acute cellular rejection and sex or sex combination). The donor and recipient sex or sex combination had no effect on the frequency of acute cellular rejection. In addition, multivariate analysis was carried out using a Cox regression model to identify the independent effect of the grafted-liver *CYP3A5* polymorphism on acute cellular rejection in comparison with sex (donor and recipient) and intestinal *CYP3A5* polymorphism (Table 3). Donor sex, recipient sex, and intestinal *CYP3A5* polymorphism were not significant indicators of acute cellular rejection and not

**Table 3 Cox proportional hazards regression analysis of risk factors for acute cellular rejection in living-donor liver transplantation patients**

| Factors | Relative hazard[a] | 95% CI | *P* value |
|---|---|---|---|
| Analysis with all factors included[b] | | | |
| *CYP3A5**1 allele in the liver | 2.592 | 1.085–6.191 | 0.032 |
| *CYP3A5**1 allele in the intestine | 1.038 | 0.437–2.463 | 0.933 |
| Sex of recipient | 0.596 | 0.262–1.359 | 0.219 |
| Sex of donor | 0.996 | 0.446–2.227 | 0.933 |
| Analysis with *CYP3A5* genotype in the liver | | | |
| *CYP3A5**1 allele in the liver | 2.629 | 1.181–5.853 | 0.018 |

CI, confidence interval.
[a]Relative hazards were calculated as the antilogs of the regression coefficients in the proportional hazards regression analysis.
[b]All risk factors shown were included in the Cox proportional hazards regression model.

associated with acute cellular rejection, whereas liver *CYP3A5**1 genotype was a strong risk indicator for acute cellular rejection after living-donor liver transplantation (relative risk 2.629; $P=0.018$). Moreover, the relationship between *CYP3A5**3 genotype and sex was examined in patients with and without acute cellular rejection using Fisher's exact test. In patients with acute cellular rejection, there were no sex-related differences in the frequency of *CYP3A5* genotypes in the liver or any sex-related differences in intestinal *CYP3A5* genotype frequency ($P=0.0992$ and 0.2377, respectively). Similarly, in patients without acute cellular rejection, there were no sex-related differences in the frequency of *CYP3A5* genotypes in the liver ($P=0.2955$) or the intestine ($P=0.6992$).

There was no significant difference between the mean trough concentrations of tacrolimus between postoperative days 10 and 23 (in patients without acute cellular rejection, median 7.9 ng/ml) and between postoperative day 10 and the day on which acute cellular rejection occurred (in patients with acute cellular rejection, median 8.6 ng/ml; $P=0.2981$).

## Discussion

The CYP3A enzyme subfamily consists of four forms in humans: CYP3A4, CYP3A5, CYP3A7, and CYP3A43. This enzyme subfamily participates in the metabolism of a wide range of endogenous compounds, drugs, and toxins, including tacrolimus and cyclosporine. It is well acknowledged that CYP3A4 is the main form of CYP3A in the liver and intestine, but genetic polymorphism that affects the expression of this protein is still unclear, especially in Asians, including Japanese. In contrast, the expression of CYP3A5 is polymorphic and is correlated with the *CYP3A5**3 allele, which was first identified by Kuehl *et al.* [18] and Hustert *et al.* [19]. Initially, CYP3A7 was reported to be expressed primarily in the fetal liver, but the expression of CYP3A7 mRNA has recently been reported in some adult livers [20,21]. In addition, CYP3A43 is expressed at low levels in adult human liver and has been cloned [22,23]. Because no relationship has been reported between *CYP3A5* genotype and the expressions of other CYP3A subfamilies and all *CYP3A* genes are closely coded in the human genome around 7q22, we examined whether the *CYP3A5**1 allele was related to CYP3A4, CYP3A5, CYP3A7, and CYP3A43 mRNA levels in graft livers and native intestines. In agreement with our previous finding [5], the level of CYP3A5 mRNA expression was associated with the *CYP3A5* genotype in both the graft liver and the native intestine, but the levels of CYP3A4 and CYP3A43 mRNA expression were not (Fig. 1). Interestingly, the mRNA expression of CYP3A7 was higher in livers with the *CYP3A5**1 allele than in those without it ($P=0.0004$; Fig. 1c), but was similar in small intestines with the *CYP3A5**1 allele and those with the *CYP3A5**3/*3 genotype (Fig. 1g). CYP3A4 and CYP3A5 mRNA expression levels were almost 100 times those of CYP3A7 and CYP3A43 expression levels. CYP3A7 has lower metabolic capacity for some drugs than CYP3A4 or CYP3A5 [24] and the physiological function of CYP3A43 is unclear. Therefore, the contribution of CYP3A7 and CYP3A43 toward tacrolimus pharmacokinetics might be small in both the liver and the small intestine of liver transplant patients. The expressional relationship between *CYP3A5**1 SNP and CYP3A7 mRNA is an interesting subject for future study. There are few reports describing the relationship between age and the mRNA levels of detoxification enzymes (e.g. CYP3As and multidrug resistance protein 1) in the intestine, although it was reported that the intestinal mRNA levels of multidrug resistance protein 1 in children had no clear maturation trajectory [25]. We examined the relationship between the mRNA expression levels of CYP3As in the liver and donor age and between the mRNA expression levels of CYP3As in the intestine and recipient age in living-donor liver transplantation (Supplemental digital content 1, *http://links.lww.com/FPC/A734*). There was no significant coefficient of correlation between each hepatic mRNA expression level of CYP3As and age of the donor in living-donor liver transplantation ($r=-0.0771$ to 0.0246). In the small intestine, there was no correlation between each mRNA expression level of CYP3As and age of the recipient, except CYP3A4 mRNA. Although there was a statistical significance ($P=0.0012$), the coefficient of correlation was quite small ($r=-0.1825$). Although our data on hepatic mRNA expression were only obtained in adults, neither intestinal nor hepatic mRNA expression of CYP3As showed any relationship with age.

In the present study, we examined the association of *CYP3A5**3 genotypes with the level of CYP3A5 mRNA expression in the intestine and graft liver, and the effects of genotype on the C/D ratio of tacrolimus in a larger group of living-donor liver transplant patients than was examined in our previous study [6]. The level of CYP3A5 mRNA expression was greater in intestines and graft livers that had *CYP3A5**1/*1 and *CYP3A5**1/*3 than in