No Contribution of the ABCB11 p.444A Polymorphism in Japanese Patients with Drug-Induced Cholestasis


Department of Gastroenterology (T.K., S.H., Y.A., K.A., K.T., K.S., R.O., S.I., T.M.), Department of Molecular Life Science, Division of Basic Medical Science and Molecular Medicine (H.I.), and Institute of Medical Science (A.O.), Tokai University School of Medicine, Isehara, Japan; Department of Gastroenterology, Internal Medicine, Kitasato University School of Medicine, Sagamihara, Japan (T.N.); Department of Internal Medicine, National Defense Medical College, Tokorozawa, Japan (K.T., R.H., S.M.); Division of Gastroenterology and Hepatology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan (H.E., H.S.); Department of Gastroenterology, Juntendo University Urayasu Hospital, Urayasu, Japan (T.K.); International University of Health and Welfare, Research Centre of Clinical Medicine, Sanno Hospital, Tokyo, Japan (Y.H.); Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, Kawasaki, Japan (C.O.); Ikegami General Hospital, Tokyo, Japan (M.W.); Division of Medicinal Safety Science, National Institutes of Health Sciences, Tokyo, Japan (M.T., Y.S., K.M.); and Department of Medicine, Teikyo University School of Medicine, Tokyo, Japan (H.T.)

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ABSTRACT

European studies have revealed that the ABCB11 c.1331T>C (V444A) polymorphism (rs2287622) C-allele frequency is higher among patients with drug-induced cholestasis. Given the low incidence of this disease, however, this association has not been sufficiently elucidated. We aimed to investigate the significance of this polymorphism in Japanese patients. We determined ABCB11 V444A polymorphism frequencies and HLA genotypes in two independent drug-induced cholestasis cohorts. Expression and taurocholate transport activity of proteins from 444A variants were analyzed using Madin-Darby canine kidney II cells. In cohort 1 (n = 40), the V444A polymorphism C-allele frequency (66%) was lower than that in controls (n = 190, 78%), but this difference was not significant (P = 0.09). In cohort 2 (n = 119), comprising patients with cholestatic (n = 19), hepatocellular (n = 74), and mixed (n = 26) liver injuries, the C-allele frequency was lower among patients with cholestatic liver injury (68%) than among those with hepatocellular (75%) or mixed liver injury (83%), although this difference was not significant. In cohort 1, HLA-A*0201 was observed more frequently in patients (22%) than in controls [11%; P = 0.003; odds ratio, 2.4 (95% confidence interval, 1.4–4.0)]. Taurocholate transport activity of 444A-encoded protein was significantly lower than that of 444V-encoded protein (81% of 444V, P < 0.05) because of the reduced protein stability. In conclusion, ABCB11 444A had slightly reduced transport activity, but it did not contribute to the occurrence of drug-induced cholestasis in Japanese patients. Therefore, genetic susceptibility to acquired cholestasis may differ considerably by ethnicity.

Introduction

Drug-induced cholestasis is a rare but sometimes fatal disease for which the underlying mechanisms remain unknown. Previous European studies have revealed that the c.1331T>C (V444A) polymorphism (rs2287622) of ABCB11, a gene that encodes the bile salt export pump, is associated with drug-induced cholestasis because the C-allele was observed more frequently in patients with drug-induced cholestasis compared with that in controls (76% patients versus 57% controls, P < 0.05) (Lang et al., 2007). This trend has also been documented in patients with intrahepatic cholestasis of pregnancy (ICP) (67% patients versus 54% controls, P < 0.001) (Dixon et al., 2009). These observations suggest that this substitution from valine to alanine at amino acid position 444 of ABCB11 plays an important role in the occurrence of acquired intrahepatic cholestasis. Dixon et al. (2014) further studied 563 ICP patients of western European origin and identified rs3815676 and rs7577650 in ABCB11 and rs2109505 in ABCB4 as significantly associated single-nucleotide polymorphisms (SNPs). Although rs2287622 (1331T>C) in ABCB11 is significantly associated with ICP, a comprehensive analysis revealed that a more significant marker, rs7577650, drives this association (Dixon et al., 2014). The association of these SNPs with cholestatic disorder has not

ABBREVIATIONS: ALP, alkaline phosphatase; BRIC, benign recurrent intrahepatic cholestasis; DILI, drug-induced liver injury; ICP, intrahepatic cholestasis of pregnancy; MDCK, Madin-Darby canine kidney; Ntcp, Na+-TC cotransporting polypeptide; Pc, corrected P; PFIC, progressive familial intrahepatic cholestasis; SNP, single-nucleotide polymorphism; TC, taurocholic acid; ULN, upper limit of normal.
been sufficiently investigated because of the low incidence of drug-induced cholestasis.

The effect of amino acid substitution at position 444 on the function of ABCB11 is controversial. An in vitro assay using vesicles isolated from S9 insect cells expressing ABCB11 revealed that the bile acid transport activity of 444A is equivalent to that of the wild-type protein (444V) (Lang et al., 2007). Conversely, hepatic expression of the ABCB11 protein was slightly but not significantly reduced in individuals harboring the 444A polymorphism (Meier et al., 2004). Therefore, the effects of this SNP on transport activity and protein expression remain unresolved.

Recently, an association between HLA genotypes and drug-induced liver injury (DILI) was reported. For example, the HLA-B*5701 genotype was significantly associated with fluvoxacillin-induced liver injury, with a remarkably high odds ratio of 80.6 (Daly et al., 2009). Marginal associations with DRB1*15 and DQB1*06 were reported in patients with cholestatic and mixed DILI (Andrade et al., 2004). These results indicate the involvement of an immune-mediated mechanism in the pathogenesis of DILI. However, potential cholestatic-associated HLA genotypes have not been thoroughly investigated.

In this study, we analyzed three ABCB11 SNPs (rs3815676, rs7577650, and rs2287622) and HLA genotypes in Japanese patients with drug-induced cholestasis. In addition, we determined the bile acid transport activity and expression levels of the ABCB11 444A protein in Madin-Darby canine kidney (MDCK) II cells.

Materials and Methods

Patients. Forty Japanese patients with drug-induced cholestasis who were referred to Tokai University Hospital or its affiliated hospitals between April 2004 and March 2012 were enrolled (cohort 1). The inclusion criteria for cohort 1 were as follows: 1) alkaline phosphatase (ALP) levels >2 times the upper limit of normal (ULN); 2) R ≤ 2, with R defined as alamine aminotransferase/ULN divided by ALP/ULN; and (3) absence of other hepatobiliary diseases. The second cohort comprised 119 Japanese patients with DILI who were referred to Teikyo University Hospital or its affiliated hospitals between May 2009 and December 2013 (cohort 2). The inclusion criteria for cohort 2 were as follows: 1) alanine aminotransferase levels >150 IU/L or ALP levels >2 times the ULN and 2) absence of other hepatobiliary diseases. Liver injuries were classified as “hepatoellular,” “mixed,” or “cholestatic,” according to the consensus criteria (Standardization of...1990; Aithal et al., 2011). Namely, patients with R ≤ 2 were classified as having cholestatic liver injury, whereas those with R ≥ 5 were classified as having hepatocellular liver injury. Patients with a R > 2 but < 5 were classified as having mixed liver injury.

All patients provided written informed consent. The Ethics Committees of Tokai University (cohort 1) and Teikyo University (cohort 2) approved this study.

Analysis of ABCB11 SNPs. V444A Polymorphism (rs2287622), rs3815676, and rs7577650 and HLA Genotypes. ABCB11 SNPs, including the V444A polymorphism, rs3815676 and rs7577650, were determined by polymerase chain reaction followed by direct sequencing, and the results were compared with those of 190 Japanese controls. The following primers were used: V444A sense, 5'-CTTTGGGGGCTACTACATAAC-3'; V444A antisense, 5'-CTGACTGAAAATTGTGTGCTAG-3'; rs3815676 sense, 5'-ATGGTTGAAGAGCAGGAGG-3'; rs3815676 antisense, 5'-TGCTCTATGATACACCATGCTGA-3'; rs7577650 sense, 5'-CTTCAAGTGCCACTAGGATG-3'; and rs7577650 antisense, 5'-GCGAA-AATATTAGAAGATCTAC-3'. HLA genotypes, including HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DPB1, were analyzed using the LumineX method (Itoh et al., 2005), and the results were compared with those of pooled Japanese controls (n = 3002).

Reagents and Vector Construction. [3H]-Taurocholic acid (TC), TC, and cycloheximide were purchased from PerkinElmer Life Sciences (Boston, MA), Calbiochem (San Diego, CA), and Sigma-Aldrich (St. Louis, MO), respectively. A pEZ-M16-bile salt export pump vector that expressed a fusion protein human ABCB11 with a C-terminal enhanced yellow fluorescent protein tag was constructed after codon optimization. Vectors expressing ABCB11 variants were constructed by site-directed mutagenesis (QuickChange XL site-directed mutagenesis kit; Stratagene, La Jolla, CA), and mutagenesis was verified by DNA sequence analysis.

ABCB11 Activity Assay. TC transport activity was evaluated, as described previously (Mochizuki et al., 2007). In brief, MDCK II cells were replated on Transwell membrane inserts (no. 3470; diameter, 6.5 mm; pore size, 0.4 µm; Corning Costar, Cambridge, MA) at a concentration of 5 × 10^5 cells/membrane. After 24 hours, the cells were transfected with vectors expressing Na+-TC cotransporting polypeptide (Ntcp) (Sun et al., 1998), ABCB11, or both using Lipofectamine 2000 (Life Technologies Japan, Tokyo, Japan). To evaluate TC transport activity, ABCB11 variants were used instead of the wild type. After 2 days, the cells were incubated in wells with 10 µM TC (containing 1 µM [3H]-TC) in the basal compartments. After incubation for 1 hour at 37°C, radioactivity in the apical medium (0.1 ml) was determined using a scintillation counter (Beckman Coulter, Tokyo, Japan; model LS 1801). Transcellular TC flux was calculated from the radioactivity in the apical medium. ABCB11 activity was determined from the permeability–surface area product for TC transport across the apical membrane, which was calculated as follows: (rate of transcellular TC flux) / (intracellular TC concentration).

Immunoblot Analysis. MDCK II cells were grown on 100-mm plates and transfected, as described above. After 48 hours, the cells were washed three times with PBS, incubated for 20 minutes at 4°C in 200 µL lysis buffer (50 mM Tris, pH 7.5, 2 mM CaCl2, 1% Triton X-100) containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets; Roche Diagnostics, Indianapolis, IN), and harvested by scraping with a rubber policeman. Cell lysates were transferred to 1.5-ml tubes and centrifuged for 15 minutes (1500g; 4°C). Supernatants were transferred to new tubes, and protein concentrations were determined according to the method of Lowry et al. (1951). Laemmli buffer was added to a final concentration of 1×, and the samples were incubated for 30 minutes at room temperature. Proteins were separated by SDS-PAGE on 7.5% gels, transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA), and probed with an antibody against green fluorescent protein (Life Technologies) or β-actin (Sigma-Aldrich). The antibody against green fluorescent protein also recognizes enhanced yellow fluorescent protein. The membranes were incubated with appropriate horseradish peroxidase–coupled secondary antibodies (Cell Signaling Technology, Beverly, MA), followed by an enhanced chemiluminescent reagent (PerkinElmer Life Sciences). Blot images were captured using the ATTO Light Capture system (ATTO, Tokyo, Japan), and band density was quantified using a CS analyzer (ATTO).

Quantification of ABCB11 mRNA Expression in Transfected MDCK II Cells. MDCK II cells were transiently transfected with an empty vector or wild-type or variant ABCB11. Total RNA was extracted 24 hours after transfection. After digestion with DNase I, cDNA was obtained by reverse transcription with SuperScript III and random hexamers (Life Technologies). Quantitative real-time polymerase chain reaction was performed using TaqMan technology on an ABI 7700 sequence detection system (Applied Biosystems, Foster City, CA). The primer and 6-carboxyfluorescein dye-labeled probe for human ABCB11 were purchased from Applied Biosystems. ΔCt values were obtained by normalization to an endogenous reference (ribosomal RNA), and the abundance of ABCB11 mRNA relative to the wild type was determined by calculating 2^-ΔΔCt.

Determination of Protein Degradation Half-Life. Cells were incubated with cycloheximide (20 µg/ml) to inhibit further protein synthesis. After incubation for the indicated time periods, the cells were harvested, lyzed, and subjected to SDS-PAGE and immunoblot analysis, as described previously (Kagawa et al., 2008). Band density was quantified, as described above, and normalized so that the density at t = 0 was 100%. The log10 of the percentage of density was plotted versus time, and the half-life was calculated from the log10 of 50% for the protein.

Statistical Analysis. Differences in allelic frequencies and genotype distribution between the two groups were compared using Fisher’s exact test. The Bonferroni correction was used for genotyping results. Namely, corrected P (Pc) values were calculated by multiplying P values by the number of compared genotypes. The degree of linkage disequilibrium for ABCB11 SNPs was evaluated using Haploview 4.2 (Barrett et al., 2005). Wilcoxon signed-rank test and Friedman test were used to compare ABCB11 activity and protein expression levels. P values <0.05 were considered significant.
Results

ABCB11 V444A Polymorphism in Japanese Patients with Drug-Induced Cholestasis

**Cohort 1.** The median age of patients in cohort 1 was 67 years (range: 21–80 years), and the majority were males (67%). The main causative therapeutic drug group was antiplatelet agents (20%), followed by cardiovascular agents (15%), anticoagulants (10%), antidiabetic agents (10%), and antibiotics (8%). The CC, CT, and TT genotypes of the ABCB11 V444A polymorphism (Fig. 1) were present in 43%, 48%, and 10% of patients and 60%, 36%, and 4% of controls, respectively. The C-allele frequency was lower among patients (66%) than among controls (78%); however, this difference was not significant (Pc = 0.09).

We also performed genotyping for two ABCB11 SNPs (rs3815676 and rs7577650). No linkage disequilibrium was observed among the three studied SNPs. The rs3815676 C-allele frequencies were 0% and 6% among patients and controls for either SNP (Pc = 0.06 for rs3815676 and Pc = 1 for rs7577650).

Regarding HLA analysis (Fig. 2), HLA-A*0201 was observed more frequently in patients (22%) than in controls (11%) [P = 0.003; Pc = 0.045; odds ratio, 2.4; 95% confidence interval, 1.4–4.0]. There were no significant differences in the frequencies of other HLA genotypes.

**Cohort 2.** Cohort 2 comprised 74 patients (29 males, 39%) with hepatocellular injury, 19 patients (11 males, 58%) with cholestatic injury, and 26 patients (11 males, 42%) with mixed DILI. The median ages of patients in these subgroups were 56 years (range: 17–82 years), 69 years (range: 44–78 years), and 59 years (range: 27–78 years), respectively. In the hepatocellular group, the main causative therapeutic drug groups were anticancer agents (12%) and anti-inflammatory agents (12%), followed by antibiotics (11%), herbal medicines (11%), cardiovascular agents (9%), and psychiatric agents (7%). In the cholestatic group, the main groups were hormonal agents (16%), anti-inflammatory agents (16%), and cardiovascular agents (16%), followed by antibiotics (11%). In the mixed group, the main group was antibiotics (15%), followed by psychiatric agents (12%), cardiovascular agents (12%), anticancer agents (8%), immunosuppressive agents (8%), antiallergic agents (8%), and dietary supplements (8%).

Regarding ABCB11 V444A polymorphism (Fig. 3), the CC, CT, and TT genotypes were observed in 53%, 32%, and 16% patients in the cholestatic group and in 54%, 42%, and 4% patients in the hepatocellular group, respectively. The C-allele frequency was 68% in the cholestatic group, a lower rate than that observed in the hepatocellular (75%) and mixed (83%) groups and 190 controls (78%). However, these differences were not statistically significant. We also compared the C-allele frequencies in the combined cholestatic patients (cohort 1 and 2) and controls, but the difference was not significant (67% versus 78%, Pc = 0.06). The HLA and 2 ABCB11 SNP (rs3815676 and rs7577650) genotyping results were not available for this cohort.

Bile Acid Transport Activity and Protein Expression of ABCB11 444A

The bile acid transport activity of ABCB11 was determined in MDCK II cells cotransfected with Ntcp and either wild-type or variant ABCB11 (Fig. 4A). Slightly but significantly lower TC transport activity was exhibited by 444A (81% of the wild-type activity, P < 0.05 versus wild type). We also compared this activity with that of a benign recurrent intrahepatic cholestasis (BRIC) 2 mutant, A570T, and a progressive familial intrahepatic cholestasis (PFIC) 2 mutant, D482G. A570T exhibited 23% activity, which was much lower than that of both the wild type and 444A (P < 0.05). The activity of D482G was 5.0%, which was the lowest among the studied variants (P < 0.05).

Next, we examined the protein expression of these variants in MDCK II cells. The expression of 444A was 69% of the wild-type expression (Fig. 4B); this difference was significant (P < 0.05). The expression of A570T was 6.6% of the wild-type expression, and was significantly lower than that of 444A and the wild type (P < 0.05) and higher than that of D482G (2.2% of the wild type, P < 0.05). The protein expression almost paralleled the activity level of each variant. These variations in protein expression among wild-type and variant ABCB11s were not attributable to differences in the transcriptional level because the ABCB11 mRNA expression levels were similar (Fig. 4C).

Furthermore, we explored the biochemical half-life of the ABCB11 protein to determine whether protein stability correlated with protein expression. The half-life of 444A was 3.0 hours, which was slightly shorter than that of the wild-type protein (3.3 hours) and longer than those of A570T (2.5 hours) and D482G (2.2 hours; Fig. 5).

Discussion

In the present study, we demonstrated that the ABCB11 SNP variant V444A exhibited slightly but significantly reduced transport activity (81% of the wild type). However, the 444A allele frequencies were not higher in two cohorts of Japanese patients with drug-induced cholestasis and DILI. The 444A protein expression level was 69% of the wild type, with a biochemical half-life of 3.0 hours, which was slightly shorter than that of the wild-type protein (3.3 hours) and longer than those of A570T (2.5 hours) and D482G (2.2 hours; Fig. 5).

Fig. 1. Genotype distribution of ABCB11 SNP rs2287622 in cohort 1.
valine to alanine at position 444 generates a slightly unstable ABCB11 protein and results in reduced transport activity. The PFIC2 mutant D482G and the BRIC2 mutant A570T exhibited markedly (5% of the wild type) and moderately (23% of the wild type) reduced transport activities, respectively. The reduced activities of these mutants were again attributable to reduced protein stability (Kagawa et al., 2008); the half-lives of D482G and A570T were 2.0 hours and 2.5 hours, respectively, which were much shorter than that of the wild type. PFIC2 results in a severe phenotype that requires liver transplantation, whereas BRIC2 manifests as occasional cholestasis triggered by viral infection or pregnancy and subsides spontaneously. Our results coincide with the previously described phenotypic differences between PFIC2 and BRIC2 (Lam et al., 2007; Kagawa et al., 2008).

The effects of the V444A variant on bile acid transport activity and expression remain controversial. Using SF9 insect cell vesicles (in contrast to the mammalian cell expression system used in the present study), Lang et al. (2007) showed that the transport activity of 444A was similar to that of 444V. In the mammalian cell line HeLa, Ho et al. (2010) did not find any significant differences in bile acid transport activity and protein expression between 444A and 444V. In contrast, 444V was expressed to a greater extent than 444A in CHO-K1 cells (Byrne et al., 2009). Meier et al. (2006) studied the hepatic expression of ABCB11 protein in 110 individuals undergoing liver resection and demonstrated a trend toward reduced expression in individuals harboring the 1331C-allele (444A) polymorphism compared with those harboring the 1331T-allele (444V). Semiquantitative evaluation revealed a 1.15-fold higher hepatic ABCB11 protein expression level in individuals with the 1331CC genotype and a 0.9-fold lower level in those with the 1331TT genotype when compared with those with the 1331TC genotype. Therefore, individuals with the 1331CC genotype had an ABCB11 expression of 80% compared with those harboring the 1331TT genotype. Our in vitro study reinforced the results of Byrne et al. (2009) and Meier et al. (2006) and identified decreased protein stability as a cause of reduced protein expression. Although some missense mutations, such as the D482G, enhanced aberrant splicing shown using a minigene system, this SNP (1331T>C) did not alter the pre-mRNA splicing pattern (Byrne et al., 2009). A major concern is whether a 444A protein with slightly reduced transport activity can cause acquired cholestasis.

We analyzed the frequency of the ABCB11 V444A polymorphism C-allele in two independent cohorts of patients with cholestatic DILI. In cohort 1, the frequency was 66%, which was lower than that in the controls (78%), although this difference was not statistically significant. The C-allele frequency in our Japanese population was consistent with previous reports (73.3% (Kim et al., 2009) and 80.4% (Lang et al., 2007)).
and higher than reported values in Caucasians [59.5% (Lang et al., 2006) and 55% (Meier et al., 2006)]. In cohort 2, the C-allele frequencies in patients with cholestatic, hepatocellular, and mixed DILI were 68%, 75%, and 83%, respectively. Therefore, a trend toward a lower frequency of this SNP C-allele was observed in patients with cholestatic DILI relative to controls or other types of DILI.

Fig. 4. Bile acid transport activity and protein expression of wild-type and variant ABCB11. (A) Bile acid transport activity of wild-type and variant ABCB11. MDCK II cells grown on Transwell membrane inserts were cotransfected with Ntcp and β-galactose or Ntcp alone and either wild-type or variant ABCB11. After 2 days, [3H]TC (1 μM) and cold TC (10 μM) were placed in the basal compartment and the cells were incubated for 1 hour at 37°C, after which transcellular TC flux was determined according to the radioactivity in the apical medium. ABCB11 activity was determined according to the permeability–surface area product for TC transport across the apical membrane (PSapical), which was calculated as follows: (rate of transcellular TC flux)/(intracellular TC concentration). These activities were normalized to the wild-type activity, which was set at 100%. The plotted points and vertical bars represent the means ± S.E. of six independent experiments performed in duplicate. *P < 0.05 compared with the wild type, †P < 0.05 compared with V444A, and ‡P < 0.05 compared with A570T. (B) Expression of wild-type and variant ABCB11. MDCK II cells were transiently transfected with wild-type or variant ABCB11 and subsequently lysed. Cell lysates (5 μg each) were separated by SDS-PAGE and subjected to immunoblot analysis using an antibody against green fluorescent protein. β-Actin was used as a control. Top: Representative immunoblot analysis results are shown. Bottom: ABCB11 protein expression levels calculated relative to β-actin and normalized to the wild-type expression, which was set at 100%. Values represent means ± S.E. of four independent experiments performed in duplicate. *P < 0.05 compared with the wild type, †P < 0.05 compared with V444A, and ‡P < 0.05 compared with A570T. (C) Expression of wild-type and variant ABCB11 mRNA. MDCK II cells were transiently transfected with an empty vector or wild-type or variant ABCB11. Total RNA was extracted 24 hours after transfection. cDNA was obtained by reverse transcription with SuperScript III and random hexamers, and quantitative real-time polymerase chain reaction was performed using TaqMan technology on an ABI 7700 sequence detection system. ΔCt values were obtained by normalizing target mRNA to an endogenous reference (ribosomal RNA), and the abundance of ABCB11 mRNA relative to the wild type was determined by calculating 2^−ΔΔCt. Values represent means ± S.E. of four independent experiments.

Fig. 5. Biochemical half-lives of wild-type and variant ABCB11 in MDCK II cells. MDCK II cells were transiently transfected with wild-type or variant ABCB11. After 24 hours, the cells were further cultured in the presence of cycloheximide (20 μg/ml) and lysed after 0, 2, 4, and 8 hours. Cell lysates were separated by SDS-PAGE and subjected to immunoblot analysis using an antibody against green fluorescent protein. Loaded protein amounts: 5 μg. Top: Representative immunoblot analysis results are shown. Bottom: Band densities measured and normalized so that the density at time t = 0 is set at 100% are shown. The log10 of the density percentage was plotted versus time, and the half-life was calculated from the log10 of 50%. Values represent means ± S.E. of four independent experiments performed in duplicate.
These results are in sharp contrast to those reported by previous studies conducted in European populations. Previously, the C-allele was observed more frequently among patients with drug-induced cholestasis \([n = 23; 76\% \text{ of patients versus } 57\% \text{ of controls}, P < 0.05] (\text{Lang et al.}, 2007)\). Similar differences were observed in studies of ICP \([n = 491, 67\% \text{ of patients versus } 54\% \text{ of controls}, P < 0.001] (\text{Dixon et al.}, 2009)\); \(n = 42, 76\% \text{ of patients versus } 51\% \text{ of controls}, P = 0.0007] (\text{Meier et al.}, 2008)\). A Spanish study reported that the C-allele was associated with an increased risk of developing DILI; however, this association was observed only among those with hepatocellular liver injury (\text{Urzurrut et al.}, 2013). Recently, \text{Dixon et al.} (2014) demonstrated through a conditional analysis that rs3815676 and rs5777650, rather than rs2287622 \((1331T>C)\), associated significantly with ICP in western Europeans. However, we could not find significant associations of these two intronic SNPs with cholestatic DILI in Japanese populations.

It is difficult to explain why our results obtained in Japanese patients disagree from those obtained in Caucasian populations. We also cannot explain the reason for the increased expression of 444V (T-allele) relative to that of 444A (C-allele), with a predominance of 444V in our cohorts. One possible explanation is linkage disequilibrium with underlying causative risk alleles, which might differ by ethnicity.

The present study showed that the relative transport activity levels of D482G, a PFIC2 mutant, and A570T, a BRIC2 mutant, were approximately 20\% and 5\% of the wild-type activity, respectively, whereas 444A exhibited approximately 80\% activity. PFIC2 and BRIC2 occur in homozygous or compound heterozygous carriers of the PFIC2 and BRIC2 mutations, respectively, suggesting that hereditary cholestasis induction requires profoundly impaired ABCB11 activity. Therefore, it remains unknown whether heterozygous or homozygous 444A, exhibiting approximately 80\% activity, can cause acquired cholestasis. We observed HLA-A*0201 more frequently among patients (22\%) than among controls (11\%) \((P = 0.003; \text{OR} = 0.045)\); odds ratio, 2.4 \((95\% \text{ confidence interval}, 1.4-4.0)\); Fig. 2. To date, several HLA class I and class II alleles have been reported to associate with the development of DILI. However, only one report documented an association of HLA-A*0201 with amoxicillin/clavulanic-related DILI in a genome-wide association study of 201 patients of European ethnic origin \((39\% \text{ were cholestatic type}) (\text{Lucena et al.}, 2011)\). In that study, HLA-A*0201 (odds ratio, 2.2) and DRB1*1501-DQB1*0602 (odds ratio, 3.3) were identified as risk factors. The DRB1*1501-DQB1*0602 association has also been reported in previous studies \((\text{Hautekeete et al.}, 1999; \text{O'Donohue et al.}, 2000)\). A few HLA alleles were shown to associate strongly with particular types of DILI: HLA-B*5701 with fluvoxacin-induced DILI in northern Europeans \((\text{odds ratio, 80.6}) (\text{Daly et al.}, 2009)\) and HLA-A*3303 with ticlopidine-induced cholestatic DILI in Japanese individuals \((\text{odds ratio, 36.5}) \text{(Hirata et al.}, 2008)\). These observations strongly indicate an immune-mediated mechanism in the occurrence of DILI. Further large-scale studies are needed to elucidate the association of HLA-A*0201 with cholestatic DILI.

Antibiotics were not the leading DILI-causing agents in our cohorts, in contrast with previous studies \((\text{Chalasani et al.}, 2008; \text{Takikawa et al.}, 2009; \text{Bjornsson et al.}, 2013)\). We were unable to explain the reason for this difference; however, this type of bias can occur by chance in small cohorts such as ours.

Our study had some limitations. Although we studied two independent cohorts, their sizes were relatively small and might have led to sampling errors. We also did not analyze the ABCB11 mutations that cause PFC12 and BRIC2 \((\text{Pauli-Magnus et al.}, 2005; \text{Byrne et al.}, 2009)\). Heterozygous carriers of these mutations can potentially develop drug-induced cholestasis, although such cases have not been reported. In addition, we did not study the nonsynonymous mutations of ABCB11 and ABCB4 or polymorphisms of ABCB2 that were previously reported in a particular case of DILI. These include 2026G>T \((\text{D676Y})\) and 2563G>A \((\text{G855R})\) in ABCB11 \((\text{Lang et al.}, 2007)\), 2290A>C \((\text{I764L})\) and 3245T>A \((\text{L1082Q})\) in ABCB4 \((\text{Lang et al.}, 2007)\), and haplotypes containing g.-1774delG or g.-1549G>A, g.242>T, c.334-94C>T, and c.3972C>T in ABC2 \((\text{Choi et al.}, 2007)\). In conclusion, although the instability of the ABCB11 444A protein led to slightly reduced bile acid transport activity, this did not contribute to the occurrence of drug-induced cholestatic liver injury in Japanese patients.

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Authorship Contributions

\text{Participated in research design: Kagawa, Takikawa.}

\text{Conducted experiments: Kagawa, Hirose, Arase, Oka, Anzai, Tsunaya, Shiraishi, Orii, Ieda, Nakazawa, Tomita, Hokari, Miura, Ebiniuma, H. Saito, Kitamura, Horie, Okuse, Wasada, Inoko, Maekawa, Takikawa.}

\text{Performed data analysis: Kagawa, Oka, Inoko, Tohkin, Y. Saito, Maekawa, Takikawa.}

\text{Wrote or contributed to the writing of the manuscript: Kagawa, Oka, Inoko, Y. Saito, Maekawa, Takikawa, Mine.}

References


Address correspondence to: Dr. Tatehiro Kagawa, Department of Gastroenterology, Tokai University School of Medicine, Isehara 259-1193, Japan. E-mail: kagawa@tokai.ac.jp