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Fig. 1A) [9,36]. Although the frequency of this substitution is very low (0.002), there is the possibility the −7833C>T variant may influence the transcription of the *MDR1* gene.

MDR1 mRNA expression was also induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (aryl hydrocarbon receptor (AhR) ligand) in three of seven primary human hepatocytes [37], an indication of the interindividual variation in AhR-induced expression, although the AhR responsive element (xenobiotics responsive element; XRE) in *MDR1* has not been identified. The −1211T>C (rs28746504, −1910 in Taniguchi et al., [17] and −1017 in Takane et al. [29]) SNP has been found in Japanese and Caucasian populations at allelic frequencies of 0.08–0.1 (Japanese) and 0.016 (Caucasian). The T-to-C substitution at nucleotide −1211 results in an XRE-like sequence (possible XRE, TGGTGTG>TGGCGTG, Fig. 3A) that we hypothesize may cause the interindividual variability in the response to TCDD. In addition to AhR-induced MDR1 mRNA expression, hypoxia-inducible factor-1 (HIF-1) regulates *MDR1* gene transcription [38]. The −1211T>C substitution also results in an HIF-1-responsive element (hypoxia responsive elements; HRE)-like sequence (possible HRE, GTGTG>GCGTG, Fig. 3A). However, it was unknown if HIF-1 could bind the candidate HRE in addition to the known HRE located between −49 and −45 (−53 and −49 in this study) in the promoter region of *MDR1*.

In order to clarify the functional significance of variants in the transcriptional regulatory region of *MDR1* gene, the effect of two SNPs (−7833C>T and −1211T>C) on the binding properties of nuclear receptors and on the transcriptional activity of *MDR1* was evaluated using electrophoretic mobility shift assay (EMSA) and a luciferase-reporter gene assay, respectively. The −7833C>T substitution resulted in a decrease in nuclear receptor binding and transcriptional activation *in vitro*, while the −1211T>C substitution did not produce an effect. These results suggest that the −7833C>T substitution influences the regulation of MDR1 mRNA levels through PXR, CAR, TRβ, and VDR.

2. Materials and methods

2.1. Chemicals

Rifampicin and 3-methylcholanthrene (3MC) were purchased from Wako Pure Chemicals (Osaka, Japan). $CoCl_2$, 3,3′,5-triiodo-L-thyronine (T3), and 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals, except T3 and $CoCl_2$, were dissolved in dimethyl sulfoxide (DMSO). T3 and $CoCl_2$ were dissolved in 0.2 M NaOH and water, respectively.

2.2. Plasmid constructs

The expression plasmids, pDEST12.2-hPXR, pEF6/V5-hVDR, pCMVTNT-hVDR, and pcDNA3.1-TRβ, were previously constructed in our laboratory [34,35,39]. The expression plasmid encoding human RXRα cDNA (pcDNA3.1-hRXRα) was a generous gift from Dr. Shuichi Koizumi (Yamanashi University, Japan). pCMVTNT-hTRβ was constructed by ligating the *Eco*RI/*Not*I fragment from pcDNA3.1-TRβ into the pCMVTNT expression plasmid (Promega, Madison, WI, USA), which was digested with *Eco*RI and *Not*I. The pEGFP-hCAR was a generous gift from Dr. Hideto Jinno (National Institute of Health Sciences, Japan) [40]. The nucleotide at the position 540 of pEGFP-hCAR differed from the reference sequence (NM_005122) (540C>T, synonymous substitution) [40]. Thus, wild-type sequence was introduced into the pEGFP-hCAR using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). pEGFP-hCAR was then digested with *Xho*I and *Eco*RI, and the resulting fragment was ligated into the pcDNA3.1/Zeo expression plasmid (Invitrogen, Carlsbad, CA, USA), which was digested with *Xho*I and *Eco*RI. This expression plasmid (pcDNA3.1-hCAR) was digested with *Xho*I and *Pme*I, and the resulting fragment was ligated into the pCMVTNT expression plasmid, which was digested with *Xho*I and *Sma*I (pCMVTNT-hCAR). The pDEST12.2-hPXR, pEF6/V5-hVDR, pcDNA3.1-TRβ, and pcDNA3.1-hCAR plasmids were used for transfections. The pDEST12.2-hPXR, pCMVTNT-hVDR, pCMVTNT-hTRβ, pCMVTNT-hCAR, and pcDNA3.1-hRXRα plasmids were used for *in vitro* synthesis.

The luciferase reporter gene plasmids, pMD10082L, pMD*824Δ90L, and pMD457L were previously constructed in our laboratory [34]. The pMD2912L plasmid was constructed by deleting the *Nsi*I fragment from pMD10082L. Mutations were introduced at positions −7833 and −1211 of pMD*824Δ90L-WT and pMD2912L-WT to create pMD*824Δ90L-VT and pMD2912L-VT, respectively, using a QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's instructions, with the following oligonucleotides:

−7833C>T_SNP: 5′-GCTCCTGGGAGAGAGTTTATTTGAGATTAAA-CAAG-3′
−1211T>C_SNP: 5′-CAGGAGAATGGCGTGAACCCGGGAG-3′.

The pGL2A8-2504 [41] plasmid, which contains the XRE of the Syrian hamster CYP2A8, was digested with *Kpn*I and *Hind*III. The resulting fragment was ligated to the *Kpn*I/*Hind*III site of the firefly luciferase rapid response reporter vector pGL4.12 (Promega). This plasmid (pGL4.12-2A8) was used as a positive control.

2.3. Electrophoretic mobility shift assay (EMSA)

TNT T7 Quick Coupled Transcription/Translation Systems (Promega) were used for *in vitro* synthesis of human RXRα protein from pcDNA3.1-hRXRα, according to the manufacturer's instructions. TNT SP6 Quick Coupled Transcription/Translation Systems (Promega) were used for *in vitro* synthesis of human VDR, TRβ, CAR, and PXR proteins from pCMVTNT-hVDR, pCMVTNT-hTRβ, pCMVTNT-hCAR, and pDEST12.2-hPXR, respectively, according to the manufacturer's instructions. The plus strand sequences of probes used in the EMSAs are shown in Fig. 1A. The oligonucleotides were purchased from Sigma Genosys (Hokkaido, Japan) and equal amounts of complimentary strands were annealed. The reaction mixture was prepared as follows: a 2.5 μL aliquot of the *in vitro* translated proteins (nuclear receptor alone, or mixed at a ratio of 1:1) or unprogrammed reticulocyte lysate was incubated for 20 min at room temperature with 1 μL of 5× binding buffer (15 mM $MgCl_2$, 0.5 mM EDTA, 2.5 mM dithiothreitol (DTT), 50% glycerol and 100 mM HEPES, pH 7.75), 0.5 μL of 1 mg/mL poly(dI–dC) (GE Healthcare, Little Chalfont, UK), and 0.5 μL of 0.33 μM 5'-fluorescein isothiocyanate (FITC)-labeled double stranded oligonucleotide probe. The protein–DNA complexes were resolved by electrophoresis on 6% non-denaturing Long Ranger gels (Lonza, Rockland, ME) run in 0.5×TBE (44.5 mM Tris, 44.5 mM boric acid, and 1.25 mM EDTA) at 500 V constant voltage, and visualized and quantified on a slab gel DNA sequencer (DSQ-2000L; Shimadzu Co., Kyoto, Japan).

2.4. Cell culture

Caco-2 cells, a human colon adenocarcinoma cell line, were obtained from the American Type Culture Collection (Manassas, VA, USA). Caco-2 cells were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin G/100 μg/mL streptomycin (Gibco-Invitrogen, Carlsbad, CA, USA), and 1× MEM non-essential amino acids solution (Gibco-Invitrogen) at 37 °C under 5% CO_2–95% air.

2.5. Transfection and luciferase reporter gene assays

Caco-2 cells were seeded into 96-well plates (1.7×10^4 cells/well), grown overnight, and transiently transfected using HilyMax (at a ratio of DNA to HilyMax of 1:5; Dojindo Laboratories, Kumamoto, Japan)

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according to the manufacturer's instructions with 10 ng/well of the indicated expression plasmid, 100 ng/well of the indicated luciferase reporter plasmid, and 10 ng/well of the *Renilla* luciferase reporter plasmid, pGL4.74 [hRluc/TK] (Promega) to normalize the transfection efficiency. After 24 h, the medium was replaced by phenol red-free DMEM (Gibco-Invitrogen) supplemented with 10% dextran-coated charcoal-stripped FBS (Hyclone Laboratories, Logan, UT, USA) containing 1 μM 3MC, 150 μM $CoCl_2$, 25 nM 1,25-$(OH)_2D_3$, 50 nM T3, 10 μM rifampicin, or vehicle (DMSO, water, or 0.2 mM NaOH) for 3.5 h except for $CoCl_2$. $CoCl_2$ treatment was performed for 6 h. Firefly and *Renilla* luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions and a luminometer (Wallac 1420 ARVO sx Multilabel Counter, Perkin-Elmer Life Sciences, Boston, MA, USA). Firefly luciferase activity was normalized to *Renilla* luciferase activity (Luc activity). The inducibility (fold induction) was calculated as the ratio of luciferase activity of ligand-treated cells to that of control cells. As for CAR, fold transactivation was calculated as the ratio of luciferase activity of CAR-transfected cells to that of pcDNA3.1 transfected cells. The results were presented as the mean ± standard deviation (S.D.) of at least four independent experiments. The statistical analysis was performed using two-tailed, unpaired t-tests with a significance level of $P<0.05$.

3. Results

3.1. C-to-T substitution at −7833 of MDR1 decreases binding affinity of nuclear receptors to ER8 and/or DR4(II)

Nuclear receptors, such as VDR, TRβ, CAR, and PXR reportedly upregulate *MDR1* expression through the response region located 7880 to 7810 bp upstream of the *MDR1* gene. This region contains several half-sites, a pair of which composes a DR or ER (Hs1–8 in Fig. 1A). The previously reported variation −7833C>T is located within the Hs6 half-site (Fig. 1A). The effect of the C-to-T substitution at −7833 on the binding affinity of nuclear receptors to the response region was examined with EMSA using *in vitro* translated VDR, TRβ, PXR, CAR and RXRα. ER8 and DR4(II) oligonucleotide probes containing either −7833C (WT) or −7833T (VT) were used because Hs6 is involved in both ER8 and DR4(II) (Fig. 1A). The probes used for the EMSA are summarized in Fig. 1A. As described in previous reports, VDR/RXRα forms DNA–protein complexes with ER8-WT and DR4(II)-WT, with a weaker binding affinity for DR4(II) than for ER8. Introduction of the variant into the probes (DR4(II)-VT and ER8-VT) decreased the formation of VDR/RXRα and probe complexes (Fig. 1B). As described previously, the DNA-TRβ/RXRα complex was formed using DR4(II)-WT, but not with ER8-WT (Fig. 1C). The DR4(II)-TRβ/RXRα complex significantly decreased when the variant type probe (DR4(II)-VT) was used. It has been reported that CAR binds to Hs6 as a monomer [33]. Consistent with this report, the DNA–CAR monomer complex was formed with either ER8-WT or DR4(II)-WT, and the C-to-T substitution at −7833 (ER8-VT and DR4(II)-VT) caused a decrease in band formation (Fig. 1D). The CAR/RXRα DNA–protein complex was also formed when either ER8-WT or DR4(II)-WT was used as a probe, although the binding affinity of ER8-CAR/RXRα was weak (Fig. 1E). Complex formation between CAR/RXRα and the probes decreased when the WT probes were replaced with ER8-VT and DR4(II)-VT (Fig. 1E). PXR/RXRα DNA–protein complexes formed using both ER8-WT and DR4(II)-WT produced weak bands, which disappeared when ER8-VT and DR4(II)-VT were used as probes (Fig. 1F). These results indicate that the C-to-T substitution at −7833 leads to decreased binding affinity of ER8 and/or DR4(II) for VDR/RXRα, TRβ/RXRα, CAR, CAR/RXRα, and PXR/RXRα.

3.2. C-to-T substitution at −7833 of MDR1 affects the inducibility by 1,25-$(OH)_2D_3$, T3, CAR, and PXR, but does not affect the inducibility by rifampicin

The EMSA experiments revealed that the C-to-T substitution at −7833 results in decreased binding affinity of VDR/RXRα, TRβ/RXRα, CAR, CAR/RXRα, and PXR/RXRα for ER8 and/or DR4(II). To investigate the effect of the substitution on VDR-, TRβ-, CAR-, and PXR-induced *MDR1* transcription, −7833C>T was introduced into pMD*824Δ90L reporter plasmid, which contains the nuclear receptor-response region (Fig. 2A), and a luciferase reporter assay was performed using the Caco-2 intestinal epithelial cell line. In order to exclude the interaction of other nuclear receptors, we used the Caco-2 because Caco-2 cells express the nuclear receptors at relatively lower levels. As described in previous reports [34,35], 1,25-$(OH)_2D_3$ (VDR ligand) or triiodothyronine (T3, TRβ ligand) increased the luciferase activity in Caco-2 cells that were co-transfected with VDR or TRβ expression plasmid (Figs. 2B and C) although VDR or TRβ alone could not activate the transcriptional activity without the ligand (data not shown). The C-to-T substitution resulted in significantly reduced transcriptional activation by 1,25-$(OH)_2D_3$ (Fig. 2B, $P=0.0015$, unpaired t-test, two-tailed). The substitution also led to a slight decrease in T3-induced transcriptional activation, but this decrease was not significant (Fig. 2C, $P=0.0688$, unpaired t-test, two-tailed). Co-transfection of a CAR expression plasmid led to increased transcriptional activity of the reporter gene compared with the mock transfection control group (fold transactivation in Fig. 2D) due to the constitutive activity of CAR. The C-to-T substitution also partially reduced constitutive CAR transactivity (Fig. 2D, $P=0.0326$, unpaired t-test, two-tailed). Rifampicin increased transactivation of the reporter gene following transfection with the PXR expression plasmid. The −7833C>T substitution did not affect the fold induction produced by rifampicin (Fig. 2E). However, the substitution reduced both the rifampicin-dependent and -independent transcriptional activation by PXR (Fig. 2F). These results indicated that −7833C>T in the *MDR1* gene reduced the transcriptional activation which is induced by 1,25-$(OH)_2D_3$

Fig. 2. Effect of −7833C>T on the transactivation of *MDR1* by VDR, TRβ, CAR, and PXR. (A) The C-to-T mutation at −7833 was introduced into a reporter gene plasmid containing the 5′ upstream region from −7880 to −7810 bp of *MDR1* (pMD*824Δ90L). Several half-sites (designated as Hs1 to Hs8) are boxed and arrows indicate the direction of the half-site. The C-to-T substitution at −7833 is located within Hs6. The numbers are in reference to the transcriptional start site at +1. In the variant construct (pMD*824Δ90L-VT), only the nucleotide that differs from the wild-type (pMD*824Δ90L-WT) is shown as a letter; asterisks represent unchanged nucleotides. (B) Caco-2 cells were co-transfected with the indicated luciferase reporter plasmid, VDR expression plasmid, and the *Renilla* luciferase reporter plasmid, and were subsequently treated with DMSO or 1,25-$(OH)_2D_3$. Firefly luciferase activity was normalized to *Renilla* luciferase activity, and the fold induction was calculated as the ratio of luciferase activity in 1,25-$(OH)_2D_3$-treated cells to that of DMSO-treated cells. Each value represents the mean ± S.D. of independent seven experiments. Statistical analysis was performed using a two-tailed, unpaired t-test, and a statistically significant difference, as compared with the wild-type, is indicated by an asterisk (**$P<0.005$). (C) The luciferase assay was performed as described in (B) except that the TRβ expression plasmid, T3, and 0.2 mM NaOH were used instead of the VDR expression plasmid, 1,25-$(OH)_2D_3$, and DMSO, respectively. The fold induction was calculated as the ratio of luciferase activity in T3-treated cells to that in 0.2 mM NaOH-treated cells. Each value represents the mean ± S.D. of independent six experiments. Statistical analysis was performed using the two-tailed, unpaired t-test, ($P=0.069$). (D) Luciferase activity was analyzed as described in the Materials and methods. Firefly luciferase activity was normalized to *Renilla* luciferase activity, and the fold transactivation was calculated as the ratio of luciferase activity of CAR-transfected cells to that of control plasmid-transfected cells. Each value represents the mean ± S.D. of independent four experiments. Statistical analysis was performed using the two-tailed, unpaired t-test, and a statistically significant difference, as compared with the wild-type, is indicated by asterisk (*$P<0.05$). (E) The luciferase assay was performed as described in (B) except that PXR expression plasmid and rifampicin were used instead of VDR expression plasmid and 1,25-$(OH)_2D_3$, respectively. The fold induction was calculated as the ratio of luciferase activity in rifampicin-treated cells to that in DMSO-treated cells. Each value represents the mean ± S.D. of independent five experiments. (F) These data are from the experiments described in (E), but are expressed in each treatment group. The firefly luciferase activity was normalized for transfection efficiency, using the activity of co-transfected *Renilla* luciferase, and represented as Luc activity.

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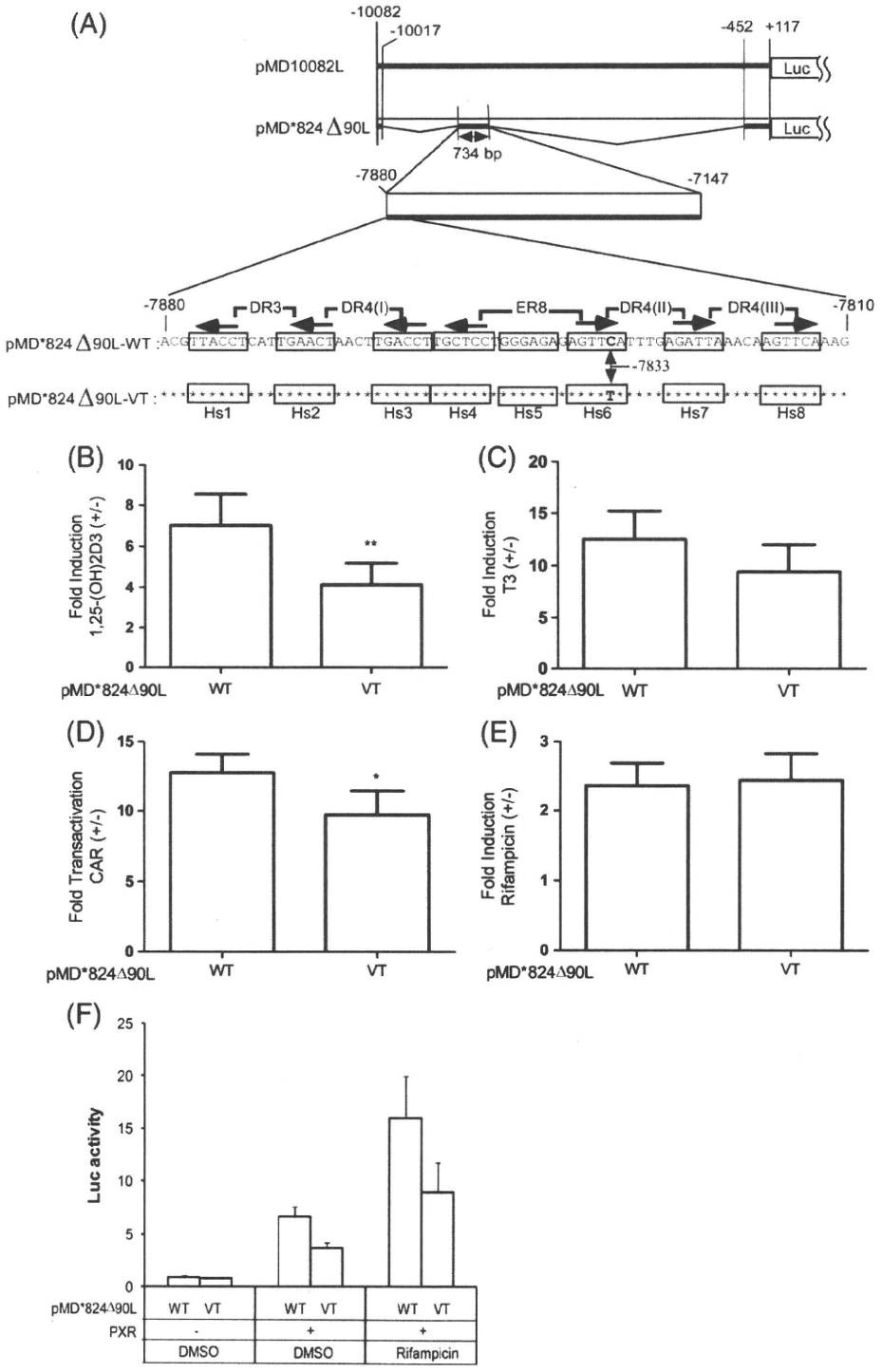
and T3. The −7833C>T also reduced CAR and PXR-dependent transcriptional activities.

3.3. XRE- and HRE-like sequences, resulting from a −1211T>C substitution in MDR1, are not functional

The T-to-C substitution at −1211 from the transcriptional start site of the human *MDR1* gene (−1211T>C, rs28746504) results in the formation of a XRE-like sequence (TGGTGTG>TGGCGTG, Fig. 3A). To examine the functionality of the XRE-like sequence, −1211T>C was introduced into a reporter plasmid containing the 5′ upstream region from −2912 to +117 bp of the *MDR1* gene and transfected into Caco-2 cells. The pGL4.12-2A8 construct (2A8), which contains the functional XRE, was used as the positive control [41]. As shown in Fig. 3B, 3MC (AhR ligand) did not affect transcriptional activity of Caco-2 cells transfected with either the wild-type (pMD2912L-WT) or mutated (pMD2912L-VT) construct, although the luciferase activity of the positive control reporter construct (2A8) was increased approximately 5-fold by 3MC, indicating that the XRE-like sequence containing −1211T>C is not functional.



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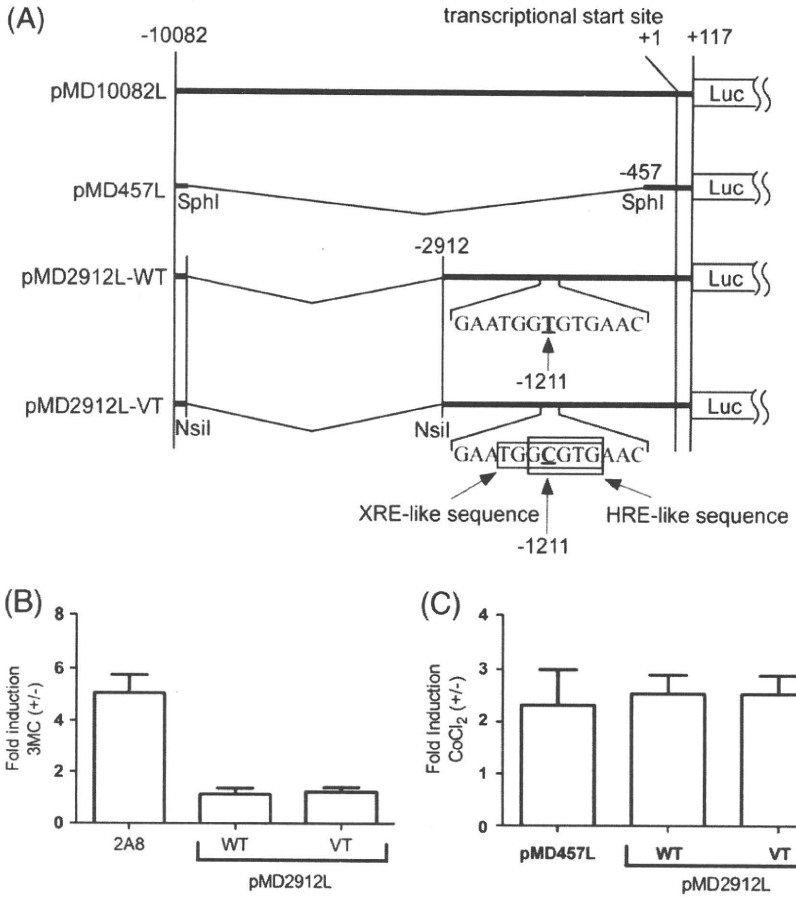

Fig. 3. Effect of −1211T>C on the transactivation of the *MDR1* gene by AhR and HIF. (A) Schematic representation of reporter plasmids containing the −1211T (pMD2912L-WT) or −1211C (pMD2912L-VT) allele. The pMD2912L plasmid was constructed by deleting the *Nsi*I fragment in pMD10082L. The numbers are in reference to the transcriptional start site at +1. Xenobiotic response element (XRE)-like and hypoxia response element (HRE)-like sequences (TGGCGTG and GCGTG, respectively) resulting from the T-to-C substitution at −1211 are boxed. pMD457L was constructed by deleting the *Sph*I fragment from pMD10082L. (B) Caco-2 cells were co-transfected with the indicated luciferase reporter plasmid and the *Renilla* luciferase reporter plasmid, and were subsequently treated with DMSO or 3MC as described in Materials and methods. Firefly luciferase activity was normalized to *Renilla* luciferase activity, and the fold induction was calculated as the ratio of luciferase activity in 3MC-treated cells to that in DMSO-treated cells. As a positive control, pGL4.12-2A8 (2A8), which contains the functional XRE of Syrian hamster CYP2A8, was used. Each value represents the mean ± S.D. of four independent experiments. (C) Caco-2 cells were co-transfected with the indicated luciferase reporter plasmid and the *Renilla* luciferase reporter plasmid, and then replaced by media with or without $CoCl_2$ as described in Materials and methods. Firefly luciferase activity was normalized to *Renilla* luciferase activity, and the fold induction was calculated as the ratio of luciferase activity in $CoCl_2$-treated cells to that of untreated cells. The pMD457L, which contains the functional HRE but does not contain the HRE-like sequence, was used as a control. Each value represents the mean ± S.D. of four independent experiments.

The T-to-C substitution at −1211 also results in the formation of an HRE-like sequence (GTGTG>GCGTG, Fig. 3A). The effect of the −1211T>C mutation on hypoxia responsiveness was measured by transfecting Caco-2 cells with the pMD2912L-WT or pMD2912L-VT. The cells were cultured with or without $CoCl_2$, a hypoxia-mimetic agent [38]. As shown in Fig. 3C, $CoCl_2$-dependent inducibility of the wild-type construct (pMD2912L-WT) was similar to that of the variant construct (pMD2912L-VT). Moreover, the inducibility of the wild-type construct, which includes the HRE-like sequence and the known HRE between −53 and −49, was also similar to that of pMD457L, which contains the reported functional HRE alone [38]. Therefore, the −1211T>C substitution did not affect the hypoxic response, and the HRE-like sequence is also non-functional.

4. Discussion

Many studies have tried to clarify the relationship between P-gp expression levels and SNPs located in the coding and transcriptional regulatory regions of *MDR1* [2,19,21–23,28,42]. However, most of these studies have not explored the functionality of these SNPs based on the nuclear receptor-responsive elements that play important roles in the expression of MDR1 mRNA. Therefore, the proposed functions of SNPs in the 5′-flanking region, such as −129T>C, remain controversial [27,30,31]. In this study, we analyzed the SNP located in the nuclear receptor-responsive region of *MDR1* and the SNP that results in conversion to a potential nuclear receptor-responsive element. The −7833C>T substitution, located in the nuclear receptor-responsive region, was found to significantly decrease the binding affinity of VDR/RXRα, TRβ/RXRα, CAR, CAR/RXRα, and PXR/RXRα (Fig. 1). In addition, the −7833C>T substitution reduced the transcriptional activation induced by 1,25-$(OH)_2D_3$ and T3, and suppressed CAR and PXR-dependent transcriptional activities *in vitro* (Fig. 2).

Recently, we reported that the relative binding ability of VDR/RXRα to the elements located in this region is: DR4(I)>DR3>ER8(MdC3)>DR4(III)>DR4(II), and DR4(I), with ER8(MdC3), and DR4(III) the main players in VDR-mediated MDR1 induction [35]. Since −7833C>T is involved in ER8(MdC3) (Fig. 1A), and ER8(MdC3) contributes to VDR-mediated MDR1 induction [35], it is possible that the −7833C>T substitution decreases VDR-mediated MDR1 mRNA induction through ER8(MdC3). We also previously indicated that TRβ/RXRα bound to several DRs in the order: DR4(I)>DR4(II)>DR3≈DR4(III), and that every direct repeat contributes to TRβ-

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mediated MDR1 induction [34]. Since −7833C>T is located in DR4(II) (Fig. 1A), and TRβ/RXRα can bind to DR4(II) at a relative high affinity [34], it is reasonable that the −7833C>T substitution could affect TRβ-mediated MDR1 mRNA induction though DR4(II). Burk et al. reported that CAR binds DR4(I) and DR4(III) as a heterodimer with RXRα, and to the 5′ half-site of DR4(II) (designated as Hs6 in Fig. 1A) as a CAR monomer, and suggested that DR4(I) and the 5′ half-site of DR4(II) (Hs6) were important elements for the CAR-mediated MDR1 induction [33]. In this study, the CAR monomer formed a complex with Hs6 both in ER8(MdC3) and DR4(II), and CAR/RXRα formed a complex with DR4(II) (Figs. 1D and E). These results and those of Burk indicate that Hs6 contributes to the CAR-mediated MDR1 mRNA induction. Since −7833C>T is found in Hs6 and DR4(II) (Fig. 1A), the −7833C>T substitution could decrease CAR-mediated MDR1 mRNA induction (Fig. 2D) even if the CAR monomer or CAR/RXRα contributes to MDR1 mRNA induction. Geick et al. reported that PXR/RXRα bind to DR4(I), DR4(II), and DR4(III) in the nuclear receptor-responsive elements of *MDR1*, with the highest affinity for DR4(III). DR4(I) is involved in rifampicin-mediated induction by PXR [32]. They suggest that DR4(II) does not contribute to the rifampicin-mediated induction by PXR even if the PXR/RXRα can bind DR4(II). In our study, PXR/RXRα formed a weak complex with DR4(II), and the −7833C>T substitution decreased the binding affinity of PXR/RXRα to DR4(II) (Fig. 1F). The −7833C>T substitution also decreased the transcriptional activation by PXR both in the presence and absence of rifampicin (Fig. 2F), but fold induction of MDR1 mRNA by rifampicin was not affected (Fig. 2E). These results suggest that DR4(II) does not contribute to rifampicin-activated MDR1 mRNA induction by PXR, which was consistent with previous observations [32]. PXR-dependent *MDR1* gene transcription without rifampicin was observed not only in this study, but also in other reports [32,43,44], and PXR-dependent CYP3A4 gene transcription without rifampicin also has been reported [32,43,44]. Since the −7833C>T substitution decreased PXR-dependent basal activation without rifampicin (Fig. 2F), DR4(II), which contains −7833C>T, may be involved in PXR-dependent basal MDR1 expression, not ligand-dependent expression. Although −7833C>T affected nuclear receptor (VDR, TRβ, CAR, and PXR)-mediated *MDR1* gene transactivation, it suppressed only about one third of total transactivity in each nuclear receptor (Fig. 2). We propose that this partial inhibition may be due to the multiple responsive elements that mediate MDR1 mRNA induction. Since xenobiotics and endogenous substrates-activated P-gp inductions play key roles in physiological functions [1,4,11,45], nuclear receptors might stimulate *MDR1* transcription through the multiple responsive elements.

Since the T-to-C substitution at nucleotide −1211 forms an XRE-like sequence (Fig. 3A) and AhR-induced MDR1 expression shows interindividual variation in human hepatocytes [37,46], we hypothesized that the −1211T>C substitution could affect the interindividual variation in MDR1 mRNA expression. However, AhR-dependent *MDR1* activation was not observed with either −1211T or −1211C (Fig. 3B), indicating that the −1211T>C substitution does not form a functional XRE. The −1211T>C substitution also forms an HRE-like sequence (Fig. 3A), and the −129T>C substitution, which is completely linkage disequilibrated with −1211T>C in Japanese [27,31], reportedly affects the expression level of MDR1 mRNA in intestine and colon cancer cells. Since the concentrations of oxygen in the intestine and cancer cells are relatively low compared to other tissues [30], it is possible that the −1211T>C substitution affects the HIF-induced MDR1 expression in a synergistic or inhibitory manner. However, the −1211T>C substitution could not affect the MDR1 expression, which was induced by a chemically-induced hypoxic condition, indicating that the −1211T>C substitution could not form the functional HRE (Fig. 3C).

In summary, we have demonstrated that the −7833C>T substitution in the *MDR1* gene decreases the binding affinities of nuclear receptors, VDR/RXRα, TRβ/RXRα, CAR, CAR/RXRα, and PXR/RXRα, to their responsive elements located around −7833. We also showed that the C-to-T substitution at −7833 reduces transcriptional activation of *MDR1* by VDR, TRβ, CAR, and PXR. However, another SNP at −1211 (T>C), which forms XRE-like and HRE-like sequences, failed to affect the AhR-dependent and hypoxia-induced *MDR1* transcriptional activation. Although the frequency of −7833C>T substitution in the *MDR1* gene is relatively low, knowledge of the −7833C>T substitution in *MDR1* is crucial for subjects who hold the −7833T allele because the pharmacokinetics of P-gp substrates may differ from wild-type profile. Further study, especially clinical studies, is necessary to confirm the functional significance of the −7833C>T substitution in the interindividual differences in P-gp expression level.

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Short Communication

*CYP3A4*16* and *CYP3A4*18* Alleles Found in East Asians Exhibit Differential Catalytic Activities for Seven CYP3A4 Substrate Drugs[S]

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ABSTRACT:

CYP3A4, the major form of cytochrome P450 (P450) expressed in the adult human liver, is involved in the metabolism of approximately 50% of commonly prescribed drugs. Several genetic polymorphisms in *CYP3A4* are known to affect its catalytic activity and to contribute in part to interindividual differences in the pharmacokinetics and pharmacodynamics of CYP3A4 substrate drugs. In this study, catalytic activities of the two alleles found in East Asians, *CYP3A4*16* (T185S) and *CYP3A4*18* (L293P), were assessed using the following seven substrates: midazolam, carbamazepine, atorvastatin, paclitaxel, docetaxel, irinotecan, and terfenadine. The holoprotein levels of CYP3A4.16 and CYP3A4.18 were significantly higher and lower, respectively, than that of CYP3A4.1 when expressed in Sf21 insect cell microsomes together with human NADPH-P450 reductase. CYP3A4.16 exhibited intrinsic clearances (V_{max}/K_m) that were lowered considerably (by 84–60%) for metabolism of midazolam, carbamazepine, atorvastatin, paclitaxel, and irinotecan compared with CYP3A4.1 due to increased K_m with or without decreased V_{max} values, whereas no apparent decrease in intrinsic clearance was observed for docetaxel. On the other hand, K_m values for CYP3A4.18 were comparable to those for CYP3A4.1 for all substrates except terfenadine; but V_{max} values were lower for midazolam, paclitaxel, docetaxel, and irinotecan, resulting in partially reduced intrinsic clearance values (by 34–52%). These results demonstrated that the impacts of both alleles on CYP3A4 catalytic activities depend on the substrates used. Thus, to evaluate the influences of both alleles on the pharmacokinetics of CYP3A4-metabolized drugs and their drug-drug interactions, substrate drug-dependent characteristics should be considered for each drug.

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Introduction

CYP3A4, the major form of cytochrome P450 (P450) expressed in the adult human liver, is involved in the metabolism of approximately 50% of commonly prescribed drugs (Guengerich, 1999). CYP3A4 is capable of oxidizing a wide range of structurally diverse drugs as well as endogenous compounds. For example, many anticancer drugs, such as docetaxel, paclitaxel, etoposide, tamoxifen, irinotecan, vinblastine, and cyclophosphamide, are known to be metabolized by CYP3A4.

The expression and catalytic activity of CYP3A are highly variable among individuals, and this variability is partially attributable to genetic factors (Ozdemir et al., 2000). Several *CYP3A4* genetic polymorphisms are known to affect the metabolism of CYP3A4 substrate drugs (www.cypalleles.ki.se/cyp3a4.htm). In addition, *CYP3A4* alleles were reported to exhibit large ethnic differences in their distribution. In the Japanese, four alleles with amino acid alterations, *CYP3A4*6* (D277EfsX8), *CYP3A4*11* (T363M), *CYP3A4*16* (T185S), and *CYP3A4*18* (L293P), are found at frequencies of <0.001, 0.002, 0.014 to 0.05, and 0.013 to 0.028, respectively (Lamba et al., 2002; Yamamoto et al., 2003; Fukushima-Uesaka et al., 2004). Of these alleles, *CYP3A4*16* has also been detected in Korean (allele frequency, 0.002) and Mexican populations (allele frequency, 0.05) and *CYP3A4*18* is distributed commonly among East Asians such as Chinese (allele frequency, 0.008–0.01), Koreans (allele frequency, 0.012–0.017), and Malaysians (allele frequency, 0.021) (Wen et al., 2004; Hu et al., 2005; Lee et al., 2007; Ruzilawati et al., 2007; Kang et al., 2009).

*CYP3A4*16* and *CYP3A4*18* are reported to affect both in vitro and in vivo catalytic activities toward several substrates and to be involved in the interindividual differences in the pharmacokinetics and pharmacodynamics of CYP3A4 substrate drugs. CYP3A4.16 exhibited an approximately 50% reduction in intrinsic clearance (V_{max}/K_m) for testosterone (TST) 6β-hydroxylation activity in vitro (Murayama et al., 2002). We recently demonstrated the substrate-dependent altered kinetics of CYP3A4.16 for midazolam (MDZ) and carbamazepine (CBZ) (Maekawa et al., 2009). The intrinsic clearance for 1′-hydroxymidazolam (1′-OH-MDZ), 4-hydroxymidazolam (4-

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ABBREVIATIONS: P450, cytochrome P450; APC, 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin; TST, testosterone; MDZ, midazolam; CBZ, carbamazepine; 1′-OH-MDZ, 1′-hydroxymidazolam; ATV, atorvastatin; PTX, paclitaxel; DTX, docetaxel; IRN, irinotecan; TFN, terfenadine; 4-OH-MDZ, 4-hydroxymidazolam; 3′-*p*-OH-PTX, 3′-*p*-hydroxypaclitaxel; 2-OH-ATV, 2-hydroxyatorvastatin; 4-OH-ATV, 4-hydroxyatorvastatin; NPC, 7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin; OR, NADPH P450 reductase.

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OH-MDZ), and CBZ 10,11-epoxide formation decreased by 50, 30, and 74%, respectively, compared with the wild type. In vivo, heterozygous *CYP3A4*16* patients administered paclitaxel (PTX) showed significantly reduced 3′-*p*-hydroxypaclitaxel (3′-*p*-OH-PTX)/PTX area under the plasma concentration-time curve ratios (Nakajima et al., 2006). In addition, decreased metabolism of irinotecan (IRN) to the inactive metabolite 7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino] carbonyloxycamptothecin (APC) was observed with *CYP3A4*16* (Sai et al., 2008).

In contrast to *CYP3A4*16*, *CYP3A4*18* seems to be bidirectional in terms of its catalytic activity toward different substrates, although different evaluation systems were used for each study. For example, the CYP3A4.18 protein exhibited increased activity for TST and chlorpyrifos (Dai et al., 2001), but not for nifedipine (Lee et al., 2005) in vitro. On the other hand, for the conventional probe drug MDZ, CYP3A4.18 showed decreased metabolism in vitro but not in vivo (Lee et al., 2007). Kang et al. (2009) demonstrated that *CYP3A4*18* is the gain-of-function allele for metabolism of several CYP3A4 substrates, including sex steroids like estrogens, leading to a relative sex-hormone deficiency that may predispose older women to osteoporosis.

In this study, to evaluate the effects of *CYP3A4*16* and *CYP3A4*18* on the catalytic activity toward structurally diverse substrates, recombinant wild-type (CYP3A4.1) and variant CYP3A4 enzymes (CYP3A4.16 and CYP3A4.18) were expressed using baculovirus-insect cell systems. The seven substrates used in the investigation were MDZ, CBZ, atorvastatin (ATV), PTX, docetaxel (DTX), IRN, and terfenadine (TFN) (Supplemental Fig. S1).

Materials and Methods

Materials. Purified human cytochrome b_5 was purchased from either Invitrogen (Carlsbad, CA) or Oxford Biomedical Research (Rochester, MI). MDZ and PTX were obtained from Wako Pure Chemicals (Osaka, Japan). 1′-OH-MDZ and 4-OH-MDZ were obtained from BD Gentest (Woburn, MA). CBZ, CBZ 10,11-epoxide, 3′-*p*-OH-PTX, and TFN and its metabolite *t*-butylhydroxyterfenadine were purchased from Sigma-Aldrich (St. Louis, MO). A second TFN metabolite, α,α-diphenyl-4-piperidinomethanol, was obtained from Fine & Performance Chemicals Ltd (Middlesbrough, UK). ATV, its metabolites 2-hydroxyatorvastatin (2-OH-ATV) and 4-hydroxyatorvastatin (4-OH-ATV), and DTX and its metabolite, DTX hydroxy *tert*-butyl carbamate (M2), were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). IRN and its CYP3A4 metabolites, APC, and 7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin (NPC), were kindly supplied by Yakult (Tokyo, Japan). All other chemicals and solvents used were of the highest commercially available grade or analytical grade.

Expression of Recombinant Wild-Type and Mutant CYP3A4 Proteins. Insect cell microsomes coexpressing CYP3A4 (wild type or variants) and NADPH P450 reductase (OR) were prepared according to methods described previously (Maekawa et al., 2009). The cytochrome P450 content and OR activity in microsomes were measured (Phillips and Langdon, 1962; Omura and Sato, 1964), and Western blotting of CYP3A4 and OR was performed as described previously (Maekawa et al., 2009).

Assay for CYP3A4 Activity. To compare alterations in kinetic parameters among substrates, three batches of wild-type and two variant enzyme preparations were used for all kinetic studies. Kinetic analysis on all seven CYP3A4 substrates was performed under proper conditions for the incubation time and P450 concentrations such that linear relationships for metabolite formation were obtained.

Catalytic activities for MDZ 1′- and 4-hydroxylations and CBZ 10,11-epoxide formation were measured as described previously (Maekawa et al., 2009), with slight modifications. For other substrates (ATV, PTX, DTX, IRN, and TFN), the incubation conditions were similar to those used for MDZ and CBZ. For all substrates, CYP3A4s from insect microsomes and purified cytochrome b_5 were mixed together (CYP3A4/b_5 ratio, 1:4), and protein concentrations and the OR/P450 ratio in the CYP3A4 wild-type and variant reaction mixtures were adjusted to be equivalent by adding both control (uninfected) microsomes and microsomes expressing solely OR. MDZ (0.2–200 μM), CBZ (10–500 μM), ATV (5–120 μM), PTX (1–50 μM), DTX (0.25–64 μM), IRN (5–400 μM), or TFN (0.0125–160 μM) was added into aliquots of the above-mentioned enzyme preparations. The reaction was started by adding NADPH generation system and terminated by adding appropriate stop solutions containing suitable internal standard for the measurement of each metabolite. Samples were mixed well and then spun at 13,000*g* for 3 to 5 min.

Metabolite analyses for MDZ, CBZ, ATV, and PTX were carried out on a tandem quadrupole mass spectrometer (Micromass Quattro Premier XE; Waters, Milford, MA) interfaced with an Acquity UPLC System (Waters) equipped with an Acquity BEH C18 column (1.7 μm, 2.1 × 30 mm; Waters) kept at 50°C. Two solutions (solution A, 10 mM ammonium acetate; solution B, 90% acetonitrile containing 10 mM ammonium acetate) were used as the mobile phase. Metabolites were eluted by linear gradient, increasing solution B. Detections were performed by monitoring the transitions of *m/z* 342 to 203 (1′-OH-MDZ), *m/z* 342 to 234 (4-OH-MDZ), *m/z* 253 to 180 (CBZ 10,11-epoxide), *m/z* 575 to 440 (2-OH-ATV and 4-OH-ATV), and *m/z* 870 to 122 (3′-*p*-OH-PTX).

For IRN, TFN, and DTX, a time-of-flight mass spectrometer (Micromass LCT Premier XE; Waters) interfaced with an Acquity UPLC System, equipped with Acquity BEH C18 column (1.7 μm, 2.1 × 100 mm; Waters), and kept at 40°C was used for metabolite analyses. The mobile phase consisted of a mixture of acetonitrile/methanol/distilled water containing 0.1% (v/v) formic acid (14:14:72 for IRN, 21:21:58 for TFN, and 15:45:40 for DTX) delivered isocratically at a flow rate of 0.3 ml/min. Detections were performed by monitoring the $M+H^+$ ions, *m/z* 824.3493 ± 0.02 (*t*-butyl hydroxyl DTX), 519.2243 ± 0.02 (NPC), 619.2768 ± 0.02 (APC), 488.3165 ± 0.02 (*t*-butyl hydroxyl TFN), and 268.1701 ± 0.02 (α-α-diphenyl-4-piperidinomethanol).

Kinetic parameters were calculated using the computer program designed for nonlinear regression analysis (MULTI program) (Yamaoka et al., 1986). Kinetic parameters for MDZ 4-hydroxylation, ATV 2- and 4-hydroxylation, PTX 3′-*p*-hydroxylation, IRN oxidation to NPC, and DTX *t*-butyl hydroxylation were determined by the hyperbolic Michaelis-Menten model (eq. 1). The substrate inhibition model (eq. 2) was used for MDZ 1′-hydroxylation, TFN *C*-hydroxylation, and TFN *N*-demethylation, where K_s is the substrate inhibition constant. In the case of the 10,11-epoxidation of CBZ, kinetic parameters were determined by the modified two-site equation ($V_{max1} = 0$) (Korzekwa et al., 1998) (eq. 3).

$$V = V_{max}S/(K_m + S) \quad (1)$$

$$V = V_{max}S/(K_m + S + S^2/K_s) \quad (2)$$

$$V = (V_{max2}S^2/K_{m1}K_{m2})/(1 + S/K_{m1} + S^2/K_{m1}K_{m2}) \quad (3)$$

Kinetic data were determined as the mean ± S.D. for three microsomal preparations derived from separate baculovirus infections, and statistical analysis was conducted by Dunnett's multiple comparison test in SAS (SAS Institute, Cary, NC). A p value of <0.05 was set as a statistically significant difference.

Results and Discussion

Expression of Wild-Type and Variant CYP3A4s in Insect Cells. Wild-type (CYP3A4.1) and variant proteins (CYP3A4.16 and CYP3A4.18) were coexpressed with human OR in Sf21 insect cells. Typical CO difference spectra with a maximum absorbance at 450 nm were obtained for all microsomal fraction preparations (Supplemental Fig. S2). CYP3A4.18 exhibited a larger peak at 420 nm than either CYP3A4.1 or CYP3A4.16. In three independent expression experiments, holoenzyme contents in the variant CYP3A4.16 (230.8 ± 25.2 pmol/mg microsomal protein) and CYP3A4.18 microsomes (51.3 ± 3.2 pmol/mg microsomal protein) were significantly higher and lower ($p < 0.05$), respectively, than that in the wild-type

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TABLE 1

Kinetic parameters for 10 catalytic reactions using seven substrates by CYP3A4.1, CYP3A4.16, and CYP3A4.18

Data are represented by mean ± S.D. of three different expression experiments.

	K_m	V_{max}	Intrinsic Clearance (V_{max}/K_m)	K_s
	μM	*pmol/min/pmol P450*	*μl/min/pmol P450*	*μM*
MDZ 1′-hydroxylation				
CYP3A4.1	1.9 ± 0.1	28.1 ± 2.8	14.8 ± 1.5	407 ± 32
CYP3A4.16	2.6 ± 0.1***	15.0 ± 3.5**	5.8 ± 1.7***	986 ± 302*
CYP3A4.18	2.0 ± 0.1	17.5 ± 3.2*	8.8 ± 1.6**	713 ± 168
MDZ 4-hydroxylation				
CYP3A4.1	23.1 ± 5.2	12.9 ± 0.1	0.58 ± 0.14	
CYP3A4.16	51.5 ± 3.5***	11.7 ± 1.4	0.23 ± 0.04*	
CYP3A4.18	22.3 ± 3.5	9.2 ± 2.0*	0.42 ± 0.11	
CBZ epoxidation[a]				
CYP3A4.1	21.9 ± 5.2 (K_{m1}) 165 ± 15 (K_{m2})	15.5 ± 2.5	0.095 ± 0.018	
CYP3A4.16	48.0 ± 3.7***(K_{m1}) 603 ± 204** (K_{m2})	11.0 ± 1.0	0.020 ± 0.008***	
CYP3A4.18	20.0 ± 2.7 (K_{m1}) 172 ± 32 (K_{m2})	15.7 ± 4.5	0.090 ± 0.011	
ATV 2-hydroxylation				
CYP3A4.1	24.2 ± 7.6	6.6 ± 1.1	0.29 ± 0.08	
CYP3A4.16	87.4 ± 22.6**	8.2 ± 1.9	0.10 ± 0.02*	
CYP3A4.18	20.2 ± 6.8	3.8 ± 1.3	0.20 ± 0.11	
ATV 4-hydroxylation				
CYP3A4.1	19.6 ± 4.0	16.1 ± 5.0	0.84 ± 0.29	
CYP3A4.16	65.4 ± 19.3**	8.4 ± 1.4	0.14 ± 0.05*	
CYP3A4.18	16.1 ± 3.4	11.1 ± 4.2	0.71 ± 0.31	
PTX 3′-*p*-hydroxylation				
CYP3A4.1	2.9 ± 0.1	0.55 ± 0.06	0.19 ± 0.03	
CYP3A4.16	12.9 ± 2.4***	0.55 ± 0.13	0.04 ± 0.02***	
CYP3A4.18	2.7 ± 0.1	0.24 ± 0.07**	0.09 ± 0.03**	
DTX *t*-butyl hydroxylation				
CYP3A4.1	2.5 ± 0.1	0.38 ± 0.01	0.16 ± 0.01	
CYP3A4.16	1.8 ± 0.2**	0.26 ± 0.03**	0.14 ± 0.01	
CYP3A4.18	2.3 ± 0.2	0.24 ± 0.04**	0.10 ± 0.03*	
IRN oxidation to NPC				
CYP3A4.1	19.3 ± 2.7	1.4 ± 0.2	0.07 ± 0.02	
CYP3A4.16	34.0 ± 2.9**	0.9 ± 0.4	0.03 ± 0.01*	
CYP3A4.18	19.7 ± 2.8	0.7 ± 0.1*	0.04 ± 0.01*	
TFN *t*-butyl hydroxylation				
CYP3A4.1	3.4 ± 0.3	3.4 ± 0.6	1.0 ± 0.2	218 ± 5
CYP3A4.16	3.5 ± 0.5	2.1 ± 0.1*	0.6 ± 0.1*	51 ± 8
CYP3A4.18	6.0 ± 1.2*	3.1 ± 0.5	0.5 ± 0.1*	311 ± 131
TFN *N* -demethylation[b]				
CYP3A4.1	2.4 ± 0.5	2.2 ± 0.4	0.95 ± 0.25	629 ± 244
CYP3A4.16	2.1 ± 0.2	1.5 ± 0.3	0.72 ± 0.12	92 ± 8
CYP3A4.18	3.4 ± 0.3*	1.9 ± 0.2	0.57 ± 0.10	

* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the wild-type (Dunnett's multiple comparison test).
[a] For CBZ epoxidation, K_{m1} and K_{m2}, V_{max2}, and intrinsic clearance (V_{max2}/K_{m2}) values are indicated in each column.
[b] For TFN *N*-demethylation, kinetic profile of CYP3A4.18 was better fitted to the Michaelis-Menten model than to the substrate inhibition model.

CYP3A4.1 microsomes (104.4 ± 23.9 pmol/mg microsomal protein). OR activity varied among the preparations but was not significantly different ($p > 0.05$) among CYP3A4.1 (1032.3 ± 88.2 nmol cytochrome *c* reduced/min/mg protein), CYP3A4.16 (659.4 ± 254.6 cytochrome *c* reduced/min/mg protein), and CYP3A4.18 (1019.1 ± 260.1 cytochrome *c* reduced/min/mg protein). On the other hand, total (apoenzyme and holoenzyme) CYP3A4 protein expression levels in insect cell microsomes were not significantly different ($p > 0.05$) between the wild type and variants by immunoblot analysis (data not shown).

Catalytic Activities of Wild-Type and Variant CYP3A4s. To characterize the substrate-dependent functional alterations of *CYP3A4*16* and *CYP3A4*18*, CYP3A4 catalytic activities of wild type and variants toward the seven substrates (MDZ, CBZ, ATV, PTX, DTX, IRN, and TFN) were measured. For four of the substrates, two different metabolites were detected: 1′- and 4-OH-MDZ from MDZ, 2- and 4-OH-ATV from ATV, APC and NPC from IRN, and *t*-butylhydroxy-TFN and α-α diphenyl-4-piperidinomethanol (azacyclonol) from TFN. Because the level of APC formed from IRN was too low to quantify precisely under our experimental conditions, kinetic analysis for IRN was performed only for NPC formation. The kinetic profiles are shown in Supplemental Fig. S3, and kinetic parameters are summarized in Table 1. The variant-to-wild-type ratios (percent) of intrinsic clearance values (V_{max}/K_m) are compared among substrates used (Fig. 1).

CYP3A4.16 showed significantly higher K_m values than CYP3A4.1 for seven catalytic reactions: MDZ 1′- and 4-hydroxylations ($p < 0.001$), CBZ 10,11-epoxidation ($p < 0.01$), ATV 2- and 4-hydroxylations ($p < 0.01$), PTX 3′-*p*-hydroxylation ($p < 0.001$), and IRN oxidation to NPC ($p < 0.01$). The V_{max} value of CYP3A4.16 was significantly lower (by 47%) ($p < 0.01$) for MDZ 1′-hydroxylation, but not for MDZ 4-hydroxylation (91% of the wild type), suggesting that catalytic site-dependent changes in V_{max} values occurred. The intrinsic clearance (V_{max}/K_m) of CYP3A4.16 was significantly reduced compared to that of CYP3A4.1 for the following catalytic reactions: MDZ 1′- and 4-hydroxylations (by 61 and 60%,

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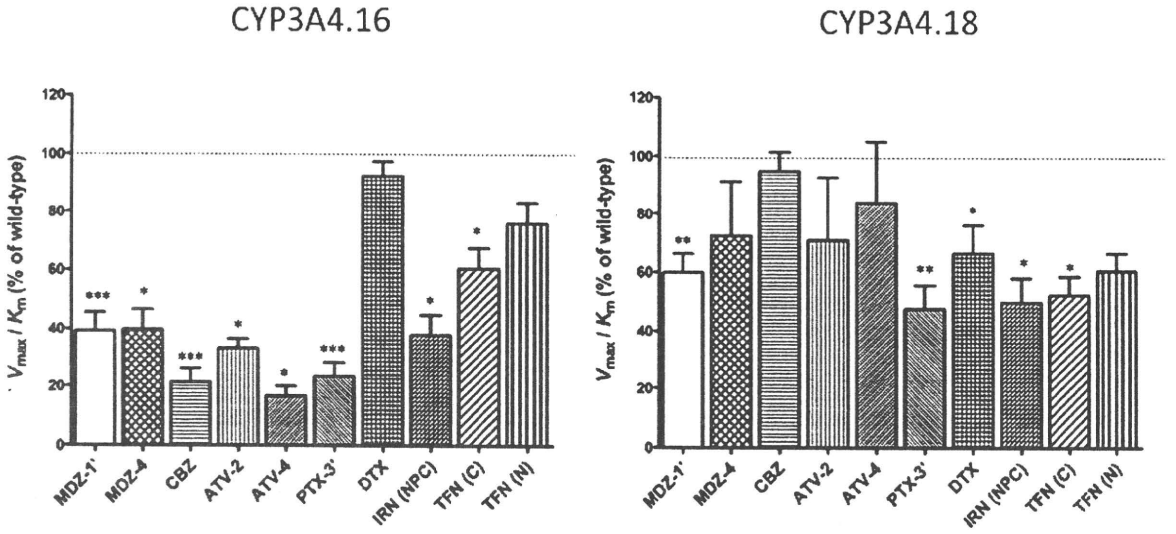

FIG. 1. The percent ratios of intrinsic clearance of variants to that of the wild type. Data are represented by mean ± S.D. of three different expression experiments. MDZ-1′, MDZ 1′-hydroxylation; MDZ-4, MDZ 4-hydroxylation; CBZ, CBZ 10,11-epoxidation; ATV-2, ATV 2-hydroxylation; ATV-4, ATV 4-hydroxylation; PTX-3′, PTX 3′-*p*-hydroxylation; DTX, DTX *t*-butyl hydroxylation; IRN (NPC), IRN oxidation to NPC; TFN (C), TFN *t*-butyl hydroxylation; TFN (N), TFN *N*-demethylation. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the wild-type (Dunnett's multiple comparison test).

$p < 0.001$ and $p < 0.05$, respectively), CBZ 10,11-epoxidation (by 79%, $p < 0.001$), ATV 2- and 4-hydroxylation (by 67 and 84%, respectively, $p < 0.05$), PTX 3′-*p*-hydroxylation (by 77%, $p < 0.001$), IRN oxidation to NPC (by 62%, $p < 0.05$), and TFN *t*-butyl hydroxylation (by 40%, $p < 0.05$). In contrast, for DTX hydroxylation and TFN *N*-demethylation, no significant differences ($p > 0.05$) in the intrinsic clearance values were observed between CYP3A4.1 and CYP3A4.16.

Our results were consistent with those by Miyazaki et al. (2008), who found that recombinant CYP3A4.16 expressed in *Escherichia coli* is markedly deficient in MDZ, TST, and nifedipine metabolisms with lower V_{max} and increased K_m relative to CYP3A4.1. Thr185 in the E helix is far away from the active site and is not located in the substrate recognition site. Further studies are necessary to elucidate the role of this residue in the binding of structurally diverse CYP3A4 substrates to the substrate recognition site.

In agreement with the lower in vitro catalytic activity of CYP3A4.16 toward PTX and IRN, *CYP3A4*16* heterozygous patients administered PTX or IRN were reported to show significantly reduced metabolite-to-substrate area under the plasma concentration-time curve ratios, which are parameters for in vivo CYP3A4 activity (Nakajima et al., 2006; Sai et al., 2008). As for substrates for which the clinical significance of *CYP3A4*16* has not been evaluated, this study demonstrated that ATV metabolism was markedly affected by CYP3A4.16. Because CYP3A4 (but not CYP3A5) is the major enzyme involved in the formation of the two ATV metabolites: 2- and 4-OH-ATV (Park et al., 2008), the clinical relevance of *CYP3A4*16* for efficacy and/or adverse reactions of ATV should be further investigated. In contrast, CYP3A4.16 retained its catalytic activity toward DTX, and thus it is predicted that this allele does not substantially influence the metabolism of DTX in vivo.

For CYP3A4.18, the reduced intrinsic clearances were observed for MDZ 1′-hydroxylation (by 40%, $p < 0.01$), PTX 3′-*p*-hydroxylation (by 52%, $p < 0.01$), DTX *t*-butyl hydroxylation (by 32%, $p < 0.05$), IRN oxidation to NPC (by 50%, $p < 0.05$), and TFN *t*-butyl hydroxylation (by 48%, $p < 0.05$) compared with CYP3A4.1. Except for TFN, the lowered V_{max} values for CYP3A4.18 resulted in lower activity in contrast to those for CYP3A4.16, which exhibited increased K_m values for most substrates. On the other hand, CYP3A4.18 had similar kinetic profiles to CYP3A4.1 in their values for K_m, V_{max}, and intrinsic clearance for oxidation of CBZ (Table 1; Supplemental Fig. S3), which has the lowest molecular weight among the seven substrates (Supplemental Fig. S1).

For the substrates MDZ, PTX, and IRN, it was reported that heterozygous *CYP3A4*1/CYP3A4*18* did not affect their pharmacokinetics (Nakajima et al., 2006; Lee et al., 2007; Sai et al., 2008). Because our in vitro results with CYP3A4.18 showed a partial decrease in V_{max} values for these drugs, an in vivo-in vitro correlation was not observed, at least for heterozygotes. Further studies are necessary to evaluate the clinical relevance of homozygous *CYP3A4*18*.

By molecular modeling studies, Kang et al. (2009) demonstrated that the L293P substitution at the beginning of the I helix caused significant secondary structural changes in the I helix and reduced protein stability. Our spectral analysis that CYP3A4.18 preparations contained more P420 than CYP3A4.1 might also be in agreement with their modeling. These possible conformational changes in CYP3A4.18 may affect substrate access depending on the substrate structure.

In conclusion, the substrate-dependent functional alterations of CYP3A4.16 and CYP3A4.18 were assessed toward seven structurally diverse substrates, MDZ, CBZ, ATV, PTX, DTX, IRN, and TFN. Compared to the wild type, CYP3A4.16 exhibited more than 60% reduced activity toward MDZ, CBZ, ATV, PTX, and IRN due to increased K_m values. In contrast, CYP3A4.18 showed a moderate reduction in its catalytic activity (by 34–52%) for MDZ, PTX, DTX, and IRN due to decreased V_{max} values. Thus, to evaluate the influences of both alleles on the pharmacokinetics of other CYP3A4-metabolized drugs and their drug-drug interactions, substrate drug-dependent characteristics should be elucidated for each drug.

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Review

Prediction of Severe Adverse Drug Reactions Using Pharmacogenetic Biomarkers

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Summary: Severe adverse drug reactions (ADRs) are a major issue for drug therapy because they can cause serious disorders and be life-threatening. Many severe ADRs appear to be idiosyncratic and unpredictable. Genetic factors may underlie susceptibility to severe ADRs, and identification of predisposing genotypes may improve drug therapy by facilitating prescreening of carriers for specific genetic biomarkers. In this review, we clarify the current status of ADRs in Japan from open ADR data sources. Then, we introduce recent progress in the field of pharmacogenetic biomarkers for severe cutaneous ADRs, liver injury, and statin-induced myopathy. Key challenges for discovery of predictable risk alleles for these severe ADRs are also discussed.

Keywords: drug-induced liver injury; drug-induced myopathy; human lymphocyte antigen; Stevens-Johnson syndrome; toxic epidermal necrolysis

Introduction

Severe adverse drug reaction (ADR) is a major reason for failure of new drug development and withdrawal of approved drugs from the market. The classical pharmacological classification of ADRs by Rawlins and Thompson distinguished two types of severe ADRs.[1)] Type A reactions are dose-dependent and predictable on the basis of the drug's known pharmacological actions. Type A reactions are relatively common and include hypoglycemia induced by diabetic drugs and bleeding induced by warfarin, an oral anti-coagulant. By contrast, type B reactions are idiosyncratic, unpredictable from the pharmacological action of the drug, and are not necessarily dose-dependent. These type B reactions make up approximately 10–15% of all ADRs and include severe cutaneous disorders, such as Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), and drug-induced liver injury (DILI) caused by various drugs.

The aim of this review is to provide an update on the current understanding of pharmacogenetic analysis related to severe ADRs, especially severe cutaneous ADRs, DILI, and statin-induced myopathy; great progress has been recently observed in pharmacogenetic biomarkers of these ADRs, and this should facilitate early-stage detection of severe ADRs. Therefore, pharmacogenetic biomarkers of ADRs hold promise for reducing severe ADRs and pave the way for creating more affordable pharmaceuticals.

Domestic case reports for severe adverse drug reactions in Japan

Domestic cases of severe ADRs are reported to the Pharmaceuticals and Medical Devices Agency (PMDA) by pharmaceutical companies based on the Pharmaceutical Affairs Law in Japan. The ADR report in Japan includes information on suspicious drugs; ADR diagnoses, which are expressed using the Medical Dictionary for Regulatory Activities preferred terms (MedDRA-PT); and patient background such as gender, age, and concomitant use of other drugs. Because the quotation frequency of MedDRA-PTs in ADR reports reflects the number of ADR events, the quotation frequency provides basic information for estimating the event frequency of each ADR. Ac-

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The URL of the Drug Safety Information website of the Pharmaceuticals and Medical Devices Agency is "http://www.info.pmda.go.jp/fukusayou/menu_fukusayou_attention.html" and the URL of material from the Committee on Safety of Drugs of the Ministry of Health, Labour and Welfare is "http://www.mhlw.go.jp/shingi/yakuji.html#anzen".

Table 1. Accumulated number of each adverse drug reaction term in cases reported from April 2004 to February 2009

Classification of accumulated frequency of each ADR term	Number of ADR terms in each classification	Representative ADR terms concerning SJS/TEN, hepatotoxicity, and rhabdomyolysis*
>1,000	30	Hepatic function abnormal (4,866), Liver disorder (3,936), Rhabdomyolysis (1,648), Stevens-Johnson syndrome (1,202)
500–1,000	26	Toxic epidermal necrolysis (751), Jaundice (715)
100–499	221	Hepatitis fulminant (406), Hepatitis acute (388), Oculomucocutaneous syndrome (324), Hepatitis (310), Cholestasis (184)
<100	4,491	
Total	4,768	

Based on the open data source MHLW website: http://www.mhlw.go.jp/shingi/yakuji.html#anzen
*Accumulated frequency is listed in parentheses.

Table 2. Number of case reports concerning SJS/TEN for fiscal years 2005 to 2008

Year (fiscal) Molecular entities	2005			2006			2007			2008		
	SJS	TEN	Sum	SJS	TEN	Sum	SJS	TEN	Sum	SJS	TEN	Sum
Allopurinol	14	12	26	22	8	30	15	11	27	33	18	51
Carbamazepine	17	5	22	17	4	21	24	6	30	26	10	36
Diclofenac	9	5	14	8	2	10	6	1	7	7	9	16
Loxoprofen	9	2	11	11	5	16	7	7	14	12	9	21
Phenobarbital	5	5	10	2	4	6	6	0	6	8	4	12
Non-pyrines	6	4	10	2	4	6	1	9	10	2	4	6
Zonisamide	9	1	10	3	2	5	1	4	5	9	6	15
Acetaminophen	1	4	5	1	5	6	1	12	13	3	10	13
Mortality (rate, %)	13 (6)	41 (27)		14 (5)	41 (30)		17 (7)	36 (23)		12 (4)	50 (26)	
Total	223	151	374	271	136	407	260	156	416	289	189	478

Based on the open data source PMDA drug safety information website: http://www.info.pmda.go.jp/fukusayou/menu_fukusayou_attention.html

cording to material from the Committee on Safety of Drugs of the Ministry of Health, Labour and Welfare (MHLW, see footnote for URL address), the total number of MedDRA-PTs was 4,768 terms cited among 168,045 ADR events from domestic cases reported to PMDA from April 2004 to February 2009. As shown in **Table 1**, the number of MedDRA-PTs cited more than 100 times among 168,045 ADR events was 277. SJS/TEN, hepatotoxicity, and rhabdomyolysis are considered to be major ADRs in Japan because the frequently cited top 277 MedDRA-PTs included most ADR terms concerning SJS/TEN, hepatotoxicity, and rhabdomyolysis. In particular, accumulated quotation frequency of abnormal hepatic function (4,866) and liver disorder (3,936) ranked second and third, followed by interstitial pneumonia (5,190).

Major severe cutaneous ADRs, SJS and TEN, are life-threatening skin disorders which are often accompanied by high fever and systemic complications.[2,3] SJS/TEN incidence is generally very low and more than 100 different causative drugs have been reported.[4–6] We counted the event number for major suspected drugs in domestic cases concerning SJS and TEN from April 2005 to March 2009 based on the open data source of PMDA, and the results are shown in **Table 2**. Allopurinol, an anti-hyperuricemia drug, is the most frequently reported drug for SJS and TEN (**Table 2**). Many cases were reported with anticonvulsant drugs including carbamazepine (CBZ), phenobarbital, and zonisamide and non-steroidal anti-inflammation drugs (NSAID), which include diclofenac, loxoprofen, non-pyrines, and acetaminophen (**Table 2**). Although it is difficult to calculate the exact incidence of SJS and TEN in Japan, the Japanese mortality rates of SJS and TEN based on domestic cases were about 4–7% and 23–30%, respectively, which were in accordance with rates reported in other populations[7,8] (**Table 2**). Therefore, the mortality rate calculated by ADR reports can be a useful reference to estimate the mortality rate of severe ADRs such as SJS and TEN.

The liver is a common target for drug toxicity due to its pivotal role in drug metabolism. Moreover, any drug has the potential to cause liver injury.[9] Therefore, hepatotoxicity is the most common ADR, causing drug withdrawals and post-marketing regulatory decisions and

Table 3. Case reports concerning hepatotoxicity in fiscal year 2008

Molecular entities	Hepatic function abnormal	Liver disorder	Jaundice	Hepatitis fulminant	Hepatitis acute	Hepatitis	Cholestasis
Terbinafine HCl	52	47	3	0	5	11	2
Fluvastatin Na	33	21	0	0	0	1	0
Itraconazole	17	10	0	0	0	0	0
Loxoprofen Na	12	15	0	0	0	2	0
Carbamazepine	11	13	0	1	1	2	0
Rosuvastatin Ca	15	7	0	1	0	3	0
Tegafur/Uracil	12	10	1	3	1	0	0
Ticlopidine HCl	9	12	4	0	0	1	15
Gefitinib	16	2	0	1	0	0	0
Cyclosporine	11	3	3	0	0	0	1
Atorvastatin Ca	7	8	0	0	0	0	1
Fenofibrate	7	5	0	0	0	4	0
Acarbose	2	8	0	0	0	0	0
Tranilast	6	4	1	0	0	1	1
Aspirin	1	8	1	0	0	1	0
Non-pyrines (4)	3	5	0	0	0	0	0
Voglibose	3	4	1	0	1	3	0
Temozolomide	6	1	0	0	0	0	0
Cefcapene pivoxil HCl	3	3	2	2	0	0	0
Total	1,063	771	90	82	68	58	55

Based on the open data source PMDA drug safety information website: http://www.info.pmda.go.jp/fukusayou/menu_fukusayou_attention.html

is associated with significant mortality.[10] We counted the event number of major suspected drugs in domestic cases concerning various hepatotoxicities based on the open data source from PMDA from April 2008 to March 2009, and the results are shown in **Table 3**. Highly reported hepatotoxicities were abnormal hepatic function, liver disorder, jaundice, fulminant hepatitis, acute hepatitis, hepatitis, and cholestasis (**Table 3**). Although the difference between abnormal hepatic function and hepatic disorder is unclear, these MedDRA-PTs are exclusively used. The total number of case reports for hepatotoxicity was 2,509. Other hepatotoxicity related reports (more than 10 cases) not in cluded in **Table 3** were hepatic failure (44), hepatocellular injury (41), cholelithiasis (21), acute hepatic failure (19), autoimmune hepatitis (19), hyperbilirubinemia (17), veno-occlusive liver disease (15), cholecystitis (14), mixed liver injury (12), cholecystitis acute (11), jaundice cholestatic (10), and cholangitis (10). There were 89 other hepatotoxicity-related reports (less than 9 cases) not included in **Table 3**. The estimated frequency of reported hepatotoxicity ranges from 1 per 10,000 to 1 per 10 million patient-years of exposure[11] (**Table 3**).

Rhabdomyolysis, one of the most serious myopathies, is characterized by the leakage of muscle cell content, including electrolytes, myoglobin, and other sarcoplasmic proteins [*e.g.*, creatine kinase, aldolase, lactate dehydrogenase, alanine aminotransferase (AST), and aspartate aminotransferase (AST)] into the circulation.[12] Lipid-lowering drugs (*e.g.*, statins and fibrates) are well known causes of rhabdomyolysis, and reports produced by the US Food and Drug Administration (FDA) showed that the rate of fatal rhabdomyolysis was 0.15 per 1 million statin

Table 4. Case reports concerning rhabdomyolysis for fiscal years 2004 to 2008

Molecular entities	Fiscal year				
	2004	2005	2006	2007	2008
Atorvastatin	51	41	48	31	28
Bezafibrate	16	22	17	16	11
Pravastatin	21	24	19	9	11
Simvastatin	21	15	5	8	2
Levofloxacin	9	10	10	9	4
Fluvastatin	13	8	7	4	6
Omeprazole	10	8	10	3	4
Propofol	8	14	2	5	8
Rosuvastatin	–	0	4	15	14
Risperidone	7	5	5	5	5
Fenofibrate	10	9	3	1	5
Pitavastatin	4	3	10	3	9
Total	389	351	359	291	332

Based on the open data source PMDA drug safety information website: http://www.info.pmda.go.jp/fukusayou/menu_fukusayou_attention.html

prescriptions dispensed.[13,14] **Table 4** shows suspicious drugs in Japan, which are similar to US data, and the reported range of case numbers is 300 to 400 per year for the last 5 years (April 2004–March 2009).

Genomic analysis of severe adverse drug reactions

Severe ADRs affect only a minority of patients taking drugs. However, hereditary forms of severe ADRs and cases occurring in identical twins have been reported, implying involvement of certain genetic factors in predisposing individuals to such severe ADRs.[15,16] The genetic basis of ADRs can be categorized into two broad groups. The first group involves genes that drive pharmacological mechanisms (drug targets, drug metabolizing enzymes, and drug transporters).[17] Common mechanisms underlying these severe ADRs are unusual drug accumulation in the target organ due to polymorphisms in drug metabolizing enzyme and drug transporter genes, and unusual sensitivity in the target organ due to changes in drug target genes.[18] The second category involves the immune system in a drug-induced allergic reaction. One important molecule for ADRs associated with immune reactions is the human lymphocyte antigen (HLA), which plays a key role in initiation of immune responses and killing target cells by presenting antigens to the T-cell receptor.[19] The *HLA* gene region codes for three classical class I (HLA-A, HLA-B, and HLA-C) and three class II (HLA-DR, HLA-DP, and HLA-DQ) antigens. Class I antigens are recognized by cytotoxic $CD8^+$ T cells and class IIs by $CD4^+$ T cells. Of the highly polymorphic *HLA* genes, *HLA-B* is the most polymorphic with over 800 variants reported in the human genome.[20] *HLA* genes within each class encode structurally similar but distinct HLA proteins that bind and present HLA-type-specific antigenic peptides to T-cell receptors.[19,20] HLA disease associations that are related to genes with immunological and inflammatory functions have been identified in many autoimmune and inflammatory conditions.

In addition to these susceptible genes, recent advances in molecular biology have led to analysis of the association of whole genome polymorphisms with ADRs.[21,22] For example, recent high-density DNA microarrays can analyze more than one million genomic biomarkers at the same time. Therefore, association analysis has been conducted by both candidate gene and genome-wide analysis.

Severe cutaneous adverse drug reactions

Carbamazepine (CBZ) is one of the most widely used aromatic anticonvulsants and is often used as a pain-relief drug for prosopalgia. CBZ is metabolized by mainly hepatic CYP3A4, CYP2B6, and CYP2C8, which generate various potentially reactive metabolites, such as CBZ-10,11-epoxide; 3-hydroxy-CBZ; 2-hydroxy-CBZ; and CBZ-2,3-epoxide.[23,24] CBZ is generally well tolerated but also is associated with idiosyncratic adverse reactions such as SJS/TEN. A high frequency of CBZ-induced SJS/TEN was reported in Han Chinese (0.25% in new exposures to CBZ) compared to Caucasians (0.014% in new exposures to CBZ).[25–29] Furthermore, CBZ-induced SJS/TEN has been reported in identical twins.[15] These studies suggest that susceptibility to such reactions may be genetically determined. Since most reactive CBZ metabolites are detoxified to non-toxic dihydrodiols by liver microsomal epoxide hydrolase 1 (EPHX1) or to glutathione conjugates by glutathione S-transferase μ 1 (GSTM1),[30,31] some researchers have attempted to find the defective alleles of *EPHX* and *GST* genes in patients with SJS/TEN; however, these attempts have failed to find associations, indicating that reactive metabolite generation from CBZ is not sufficient to cause SJS/TEN.[32,33] Recently, Chung *et al.* reported a tight association between CBZ-induced SJS/TEN and *HLA-B*1502* allele in Han Chinese[25] (**Table 5**). They showed that all 44 Han Chinese patients with CBZ-induced SJS/TEN carried the *HLA-B*1502* allele and its odds ratio was 2,505 (95% confidence interval, 195 to 27,483, $Pc = 2.02 \times 10^{-32}$). The finding was further confirmed by the same group in

Table 5. Association between severe cutaneous adverse drug reaction and *HLA* type

Drug	HLA	Population	OR or (N)[a]	Pc[b]	Ref
Carbamazepine	B*1502	Han Chinese	2505	2.0×10^{-32}	25)
Carbamazepine	B*1502	Asian ancestry	(4/4)	–	37)
Carbamazepine	B*1502	European	(0/8)	–	37)
Carbamazepine	B*1502	Thai	25.5	0.0005	106)
Carbanazepine	B*1502	Japanese	(0/7)	–	36)
Carbamazepine	B*1502	Han Chinese	1357	1.6×10^{-41}	34)
Phenytoin	B*1502	Thai	18.5	0.005	106)
Antiepileptic	B*1502	Han Chinese	17.6	0.001	35)
Allopurinol	B*5801	Han Chinese	580	4.7×10^{-24}	44)
Allopurinol	B*5801	European	80	$<10^{-6}$	5)
Allopurinol	B*5801	Japanese	40.8	$<10^{-4}$	36)
Abacavir	B*5701	Australian	117	$<10^{-4}$	48)
Abacavir	B*5701	British	24	$<10^{-4}$	47)
Abacavir	B*5701	Australian	960	$<10^{-4}$	50)
Abacavir	B*5701	White	(36/65)	–	49)
		Black	(0/9)	–	
		Other	(1/10)	–	
Abacavir	B*5701	Japanese	(0/7)	–	40)
Nevirapine	DRB1*0101 and high CD4	Caucasian Australian	18	0.0006	59)
Nevirapine	Cw*0802-B*1402	Sardinian	15	0.05	60)
Nevirapine	B*3505	Thai	18.96	4.6×10^{-6}	62)
Nevirapine	Cw8	Japanese	6.2	0.03	61)

[a] OR is odds ratio and (N) is sensitivity (carrier cases/all cases).
[b] Pc indicates corrected P value.

an additional study that included patients who were Han Chinese or Chinese descendants from Taiwan, Hong Kong, China, and the USA[34] (**Table 5**). The involvement of *HLA-B*1502* was also detected in SJS/TEN caused by other aromatic anti-epileptic drugs, such as phenytoin in Han Chinese[35] (**Table 5**). However, such strong association between *HLA-B*1502* and CBZ-induced SJS/TEN has not been detected in Caucasian and Japanese SJS/TEN patients[36,37] (**Table 5**). *HLA-B*1502* is present at a higher allele frequency in South-east Asian populations than in Caucasian and Japanese populations.[38] *HLA-B*1502* was not detected in 486 healthy Japanese subjects[39] and in 935 USA Caucasians (http://www.allelefrequencies.net/), while the allele frequency in Han Chinese is 8.6%.[25] The low frequency of *HLA-B*1502* in Caucasian and Japanese populations may account for the fact that no association between *HLA-B*1502* and CBZ-induced SJS/TEN was observed in Caucasians and Japanese.[36,40] Alternatively, these results suggest that *HLA-B*1502* is involved in the mechanism, but is not sufficient for CBZ-induced SJS/TEN. There could be other co-factors, such as virus infection or other variants of genes, for example, *CYP3A4*, *CYP2B6*, *CYP2C8*, *EPHX1*, and *GSTM1*; T cell receptors; genes related to apoptosis; or genes for co-stimulatory molecules involved in the interaction between antigen-presenting cells and T cells.

Allopurinol is a xanthine oxidase inhibitor that prevents the production of uric acid and is commonly used for hyperuricemia and gout.[41] Allopurinol is metabolized by xanthine oxidase to oxipurinol, which forms ribonucleotide adduct and ribonucleoside adduct.[42] Allopurinol has been reported to be a causative drug of a variety of delayed cutaneous adverse reactions, such as SJS/TEN.[43] Recently, a strong association of *HLA-B*5801* with allopurinol-induced severe cutaneous adverse reactions (drug-induced hypersensitivity syndrome and SJS/TEN) was found in Han Chinese in Taiwan[44] (**Table 5**). They showed that the *HLA-B*5801* allele was present in all patients (51/51) with allopurinol-induced severe cutaneous adverse reactions, but only in 15% of tolerant patients (20/135). The odds ratio was 580 (95% confidence interval, 34 to 9781, $Pc = 4.7 \times 10^{-24}$). Although the association was confirmed in Caucasians[5] and Japanese,[36] the odds ratio in Han Chinese (580) was much higher than that in Caucasians (80) and Japanese (40) (**Table 5**). Approximately 9 to 11% of Han Chinese are carriers of the allele, and its prevalence is generally lower in Caucasian (1 to 6%), Japanese (0.68%), and African (2 to 4%) populations.[43,44] These reports suggest that *HLA-B*5801* might be a genetic biomarker for allopurinol-induced severe cutaneous adverse reactions; however, the extent of the association showed ethnic differences.

Abacavir is a potent nucleoside analog reverse transcriptase inhibitor that is used in combination with other drugs to treat human immunodeficiency virus infection. The most serious adverse reaction of abacavir that limits its use in therapy is a hypersensitivity reaction which includes the combination of fever, skin rash, constitutional symptoms, gastrointestinal tract symptoms, and respiratory symptoms.[45] Hypersensitivity to abacavir occurs in approximately 5 to 8% of patients treated with abacavir, typically within 1 to 6 weeks of the initial dose.[45] Abacavir is metabolized by type 1 alcohol dehydrogenase to an aldehyde-reactive metabolite.[46] The initial association between *HLA-B*5701* and abacavir-induced hypersensitivity reaction was elucidated by two independent research groups in 2002[47,48] (**Table 5**). The association was reported only in Caucasians and not in Africans or Japanese[40,49] (**Table 5**) because the allelic frequency of *HLA-B*5701* in Caucasians is approximately 8%, but low in individuals of African or Asian descent.[47,48] Fine recombinant genetic mapping has identified a significant linkage disequilibrium of the haplotypic M493T polymorphism of heat shock protein-Hom (Hsp70-Hom M493T) and *HLA-B*5701* in abacavir-induced hypersensitivity reaction cases in Western Australians, which enhanced the discrimination of hypersensitive subjects from the tolerant control (odds ratio, 3,893; $Pc < 0.00001$) when compared to *HLA-B*5701* only (odds ratio, 960; $Pc < 0.00001$).[50] The Hsp70-Hom M493T polymorphism may facilitate the loading of abacavir- or its metabolite-hapten endogenous peptide onto *HLA-B*5701*.[51] A large randomized, controlled clinical trial assessing the clinical effectiveness of *HLA-B*5701* screening in Caucasians (PREDICT-1 study),[52] and a case-control study of *HLA-B*5701* in both Caucasians and African Americans (SHAPE study),[53] were highly supportive of the use of *HLA-B*5701* screening in clinical practice to exclude *HLA-B*5701* carriers from patients treated with abacavir. However, 7 Japanese patients, who were all *HLA-B*5701*-negative, had abacavir-induced hypersensitivity reactions.[40] Thus, the genetic screening of *HLA-B*5701* is unlikely to be effective for the Japanese.

Nevirapine is a non-nucleoside reverse transcriptase inhibitor that is used in combination with antiretroviral therapy.[54] The major treatment-limiting toxicity associated with nevirapine use is skin rash and hypersensitivity, which emerge in 5% of patients who have initiated nevirapine therapy.[55] Nevirapine is metabolized by CYP3A4 predominantly, and to a lesser degree by CYP2B6 and other CYP isoforms, to several hydroxylated metabolites: 12-hydroxynevirapine has been implicated as a putative nevirapine metabolite causing hypersensitivity reactions.[56] $CD4^+$ T cells have been shown to be involved in the nevirapine-induced hypersensitivity reaction.[57,58] Thus, a high level of $CD4^+$ T cells (more than 25% above normal) is one risk factor for nevirapine-induced hypersensitivity reaction.[55] In addition to $CD4^+$ T cell levels, associations between several types of HLA and

nevirapine-induced hypersensitivity reactions have been reported in different countries or populations. For example, *HLA-DRB1*0101* (Western Australian),[59] *HLA-Cw* 0802-B* 1402* haplotype (Sardinian patients, people in Italian autonomous regions),[60] *HLA-Cw8* (Japanese),[61] and *HLA-B* 3505* (Thai)[62] have been reported (**Table 5**). These results imply that the primary determining *HLA* allele may be different among populations in the nevirapine-induced hypersensitivity reaction.

Drug-induced liver injury

Several patterns of DILI exist, with the most useful classification being hepatocellular, cholestatic, or a combination of both.[63] Hepatocellular injury involves marked elevation of serum ALT and AST levels, usually preceding an increase in total bilirubin and no increase or only modest increases in alkaline phosphatase (ALP) levels.[9] In cholestatic injury, increases in ALP levels predominate and precede increases in ALT and AST.[9] The patterns in which a drug causes liver injury are regarded as being either predictable (dose-dependent) or unpredictable (idiosyncratic).[64] Acetaminophen-induced hepatotoxicity has been considered the classic example of a dose-related hepatotoxin,[65] although few other drugs fit this pattern.[64] Rather, the majority of drugs that are capable of producing liver injury do so in an unpredictable fashion with variable latency periods.[9,66]

Single nucleotide polymorphisms (SNPs) in drug metabolizing enzymes and drug transporters, which regulate the metabolism and disposition of drugs, represent the best studied set of pharmaceutically important genetic markers of DILI. *N*-Acetyltransferase (NAT) functions by acetylating drugs, therefore causing active drug metabolites to be detoxified. Deficient alleles of *NAT* (such as *NAT1* 14* and **15* and *NAT2* 5*, **6*, and **7*), which reduce detoxification activity, increase the toxicity of drugs including isoniazid, sulfonamides, and procainamides.[67,68] The allele distribution of the Caucasian population differs from that reported in the Japanese population.[67,69] Another enzyme pathway of importance is that of glutathione in the detoxification of reactive metabolites. Genetically determined deficiencies in glutathione synthetase and GST have been associated with increased hepatotoxicity of certain drugs, including acetaminophen, metronidazole, and nitrofurantoin.[70,71] Frequencies of GST-deficient alleles show ethnic differences. For example, the homozygous deletion genotype frequency in *GSTM1* ranges from 0.38 to 0.67 in Caucasians, from 0.33 to 0.63 in East Asians, and from 0.22 to 0.35 in Africans and African-Americans.[72] Pacific Islanders have the highest reported frequency of homozygous deletion genotypes (0.64–1.0) of any group studied.[73,74] Troglitazone is a 2,4-thiazolidinedione anti-diabetic drug with insulin-sensitizing activities.[75,76] Troglitazone-associated idiosyncratic hepatic dysfunction and hepatic failure were reported after introduction of the drug into the market.[77-79] Yamamoto *et al.* reported that CYP3A4 catalyzed troglitazone into an epoxide of a quinone metabolite which may be eliminated by GSTs and EPHX.[80] To address the susceptible genetic factors responsible for the hepatotoxicity associated with the drug, Watanabe *et al.* performed a genetic polymorphic analysis by a target gene approach in troglitazone-treated Japanese patients with type 2 diabetes mellitus[81] (**Table 6**). They observed a correlation between hepatic failure and both *GSTT1* and *GSTM1* null genotypes. They reported that the odds ratio was 3.692 and its 95% confidence interval was 1.354 to 10.066 (Pc = 0.008). A more recent example of drug hepatotoxicity resulting from genetic polymorphisms of drug metabolizing enzymes and drug transporters is that of diclofenac, a non-steroidal anti-inflammatory drug that is among the most common drugs to cause idiosyncratic hepatotoxicity. Diclofenac-induced hepatotoxicity occurs at a rate of 6 per 100,000 users, and 8 to 20% of the patients who develop jaundice die of liver failure. It has been concluded that diclofenac hepatotoxicity is associated with the possession of variant UDP-glucuronosyltransferase 2B7 (*UGT2B7* 2*), ATP-binding cassette transporter C2 (*ABCC2*, −24C > T), and *CYP2C8* haplotypes[82] (**Table 6**).

Table 6. Association between drug-induced liver injury and genetic polymorphisms

Drug	Gene variant	Population	OR[a]	Pc[b]	Ref
Troglitazone	GSTM1/T1	Japanese	3.69	0.008	81)
Ticlopidine	HLA-A*3303	Japanese	36.5	7.32×10^{-7}	86)
Diclofenac	UGT2B7*2	Unknown	8.5	0.03	82)
	ABCC2(C-24T)	Unknown	5.0	0.005	
	CYP2C8 haplotypes	Unknown	–	0.04	
Flucloxacillin	HLA-B*5701	European	45.0	8.7×10^{-33}	21)

[a] OR is the odds ratio.
[b] Pc indicates corrected P value.

Immune-mediated mechanisms via the reactive metabolite binding to macromolecules are believed to be associated with idiosyncratic DILI. *HLA* has been considered to be involved in T-cell mediated cytotoxic reactions and drug-induced allergic reactions. Therefore, *HLA* might be another type of candidate genetic biomarker of DILI.[64,66] Ticlopidine, an anti-platelet agent, which has been widely used for the secondary prevention of atherothrombosis,[83] has shown severe hepatotoxicity, mainly of the cholestatic type,[84] and there appears to be an increased rate of hepatic adverse reactions in Japanese compared with Caucasian patients.[85] Hirata *et al.* explored genetic risk factors for ticlopidine-induced hepatotoxicity using 22 Japanese patients with ticlopidine-induced hepatotoxicity and 85 Japanese patients who tolerated ticlopidine therapy without ex-

periencing adverse reactions and they found a significant correlation between ticlopidine-induced hepatotoxicity and *HLA-A*3303*[86] (**Table 6**). Allelic frequency of *HLA-A*3303* is 7.54% in Japanese, 0.6% in Caucasians, and 4.5% in African-Americans (http://www.allelefrequencies.net/). They reported that 12 patients (86%) among 14 patients who showed ticlopidine-induced cholestatic hepatotoxicity had *HLA-A*3303* and the odds ratio was 36.50 (95% confidence interval, 7.25 to 183.82). Another example of the correlation between *HLA* and DILI is that of flucloxacillin, which is widely used in many European countries and Australia for treatment of staphylococcal infection. Its use has been associated with a characteristic cholestatic hepatitis that is more common in females, the elderly, and patients with prolonged treatment courses.[87-89] In the United Kingdom, the incidence of flucloxacillin-related DILI has been estimated at 8.5 in every 100,000 new users in days 1 to 45 after starting treatment.[89] Daly *et al.* conducted a genome-wide association study using 51 cases (white European ancestry) of flucloxacillin-related DILI and 282 controls matched for sex and ancestry and found the strongest correlation between flucloxacillin-related DILI and a genetic marker (rs2395029) in complete linkage disequilibrium with *HLA-B*5701*[21] (**Table 6**). They reported that the odds ratio was 45 (95% confidence interval, 19.4 to 105). Among 51 cases, 43 patients (84%) carried the risk allele (G), which has a frequency of approximately 5% in the population controls and in European populations generally.

Statin-induced myopathy

In rare cases, statins can cause muscle pain or weakness in association with elevated creatine kinase levels (*i.e.*, myopathy), and occasionally this leads to muscle breakdown and myoglobin release (*i.e.*, rhabdomyolysis), with a risk of kidney failure and death.[90] The mechanisms by which statins cause myopathy remain unknown but appear to be related to statin concentrations in blood and muscle.[90]

Morimoto *et al.* studied genetic factors contributing to the risk of stain-induced myopathy and showed that the frequencies of *OATP-C* 15* [tagged by 388A>G (N130D) and 521T>C, V174A], a mutant allele of *OATP-C* (OATP1B1, *SLC21A6/SLCO1B1*) was significantly higher in Japanese patients with myopathy who were receiving pravastatin or atorvastatin than in patients without myopathy[91] (**Table 7**). They also found another *OATP-C* mutant allele, 1628T>G (L543W), which is located in exon 12 of *SLC21A6/SLCO1B1* in a Japanese patient with pravastatin-induced myopathy[92] (**Table 7**). They examined the transporting activity for pravastatin and other substrates and found that the activity decreased significantly in HEK293 cells expressing mutant proteins with V174A and L543W compared to those in cells expressing *OATP-C* 1a*, the reference allele of *OATC-C*.[93,94] OATP-C has been shown to mediate the hepatic uptake of statins.[95] From these results, they speculated that patients who are carrying these defective *OATP-C* mutant alleles have increased plasma concentrations of these statins and are thus more susceptible to the myotoxic effects of these statins compared to non-carrier patients treated with pravastatin and atorvastatin.[92] In fact, Ide *et al.* recently reported that *OATP-C* 15* significantly influenced the relative bioavailability [F(rel)] of pravastatin; F(rel) was increased 1.50- and 1.95-fold in heterozygous and homozygous participants, respectively, for the *OATP-C* 15* allele in comparison with participants without the allele from a covariate analysis of population pharmacokinetic analysis.[96]

Table 7. Candidates of *OATP-C* genomic biomarkers for statin-induced myopathy

Variant	*In vitro* activity	Statin	Ref
521T>C	Decrease	Pravastatin or atorvastatin	91)
521T>C	Decrease	Simvastatin	22)
1628T>G	Decrease	Pravastatin	91, 94)

The SEARCH Collaborative Group, which aims to determine whether a daily dose of 80 mg of simvastatin safely produces greater benefit than a daily dose of 20 mg, found 98 definite or incipient cases of myopathy among 6,031 participants who were assigned to receive 80 mg of simvastatin.[22] All participants were from the United Kingdom, but their ethnicity was not specified. They performed a genome-wide association study using approximately 300,000 genomic markers in 85 subjects with definite or incipient myopathy and 90 controls, and found a single strong association of myopathy with the rs4149056 (521T>C) SNP located within *SLCO1B1* (**Table 7**). They reported that the odds ratio for myopathy was 4.5 (95% confidence interval, 2.6 to 7.7) per copy of the C allele, and 16.9 (95% confidence interval, 4.7 to 61.1) in CC as compared with TT homozygotes. They concluded that this variant of *SLCO1B1* is strongly associated with an increased risk of statin-induced myopathy.

These studies suggest that variant *OATP-C* decreased the hepatic uptake of statin and increased blood and muscle concentrations of statin. The increase of the blood and muscle concentrations of statin may cause myopathy or rhabdomyolysis. Genotyping these *SLCO1B1* variants might help to achieve the benefits of statin therapy more safely.

Use of pharmacogenetic biomarkers in clinical practice

In principle, identifying genetic risk factors for severe ADRs, particularly type B reactions, could significantly

decrease the incidence rate of ADRs and improve the process of drug development.[97] Among these type B ADRs which we consider in this review, the usefulness of abacavir *HLA*-genetic biomarker (*HLA-B*5701*) has been confirmed in Caucasians from several prospective studies, such as the PREDICT-1 study.[52] The association of CBZ-induced SJS/TEN and an *HLA*-genetic biomarker (*HLA-B*1502*) in Han Chinese is extremely high compared with other drugs.[25] Therefore, *HLA-B*1502* screening is recommended for CBZ in clinical practice by the US FDA, and *HLA-B*5701* screening is recommended for abacavir by the US FDA and European Medical Agency. Before treatment with CBZ or abacavir, *HLA* analysis should be performed to exclude *HLA-B*1502* or *HLA-B*5701* unless the patient is from a population who shows extremely low frequency of these *HLA* types. Such exclusion of patients from treatment with causative drugs would markedly reduce the possibility of severe ADRs and prevent overestimation of severe cutaneous ADRs that could otherwise result in excessive discontinuation of treatment.[34,98,99] In Japan, package inserts of CBZ and abacavir describe these research results.

Perspective on pharmacogenetic biomarkers

An unresolved issue for genetic biomarkers is ethnic differences, since these *HLA* markers show ethnic specificity. For example, an *HLA* marker of abacavir (*B*5701*) or CBZ (*B*1502*) is present only in Caucasians or Han Chinese (and South East Asians), respectively, and its usefulness has not been shown in other populations such as Japanese.[37,40] On the other hand, the association between allopurinol treatment and *HLA-B*5801* was observed not only in Han Chinese but also in Caucasians and Japanese, although the odds ratios were lower than that of CBZ.[5,36,44] Therefore, it is absolutely necessary to explore the *HLA* marker for each population against each drug and also to find the universal genetic biomarker (if one exists) of severe ADRs for clinical practice. As shown for nevirapine, the association between the rash with hepatitis and *HLA-DRB1*0101* was observed in Western Australian patients with $CD4^+$ T-cell levels greater than 25% above normal levels.[59] This case suggests that the combination of *HLA* genetic biomarkers and other biomarkers might be useful to predict ADRs for some drugs. A prospective study or comparative study with other populations is necessary for *HLA* biomarkers of ticlopidine- and flucloxacillin-related DILI and *SLCO1B1* biomarkers of statin-induced myopathy.

In most cases of allergic reactions, such as SJS/TEN and DILI, *HLA*-drug toxicity associations are thought to arise as a result of the interaction of a specific *HLA* allele with the drug or its metabolite, causing an immune reaction to be triggered.[38,100,101] As shown in the abacavir-induced hypersensitivity reaction, the drug metabolite may play an important role in the allergic reaction process,[102] suggesting that sequential reactions from drug metabolism to the immune mechanism can exist in the allergic process. Thus, drug toxicities that are driven primarily by the immune response may require bioactivation of the drug to a specific metabolite to evoke the specific immune response that will lead to the generation of an adverse reaction.[38,100,101] These complex mechanisms may be involved in most cases of allergic reactions because reactive metabolites have been detected not only in abacavir but also in nevirapine, CBZ, and allopurinol.

Most of the currently available genetic biomarkers are limited in relation to *HLA*, drug metabolizing enzymes, and drug transporters.[17] Considering that the technology to identify genetic variants across the whole genome is advancing rapidly, many more significant genetic factors for ADRs are likely to be identified in the future. In such whole-genome case-control analysis, there might be critical points to resolve. The first problem is the size of case and control groups. Accrual of large numbers of cases is necessary for genome-wide association study of genetic factors underlying severe ADRs, even though the number of patients with specific types of ADRs is small.[103] Control subjects for such studies should be matched for drug exposure, concomitant use of other drugs that could affect the pharmacokinetics and pharmacodynamics of the drug in question, and subject background such as age, gender, and ethnicity.[103] The second problem is objective diagnosis of ADRs. Because one drug could induce many ADR phenotypes in which the mechanism may be different, standardization of diagnosis is necessary.[40,103] These critical points affect the sufficient statistical power to detect the genetic biomarker. In order to resolve these problems, several regional networks to study severe ADRs have been established, including our research group in Japan (for SJS/TEN, DILI, and myopathy in Japanese),[36] European collaboration for studying the genetic basis of adverse drug reactions (EUDRAGENE for six severe ADRs in multiple European populations),[104] the United States Drug Induced Liver Injury Network (DILIN, for DILI),[105] and the International Serious Adverse Event Consortium (SAEC, for SJS/TEN and DILI in global populations).[21] These networks involve scientists in regulatory agencies, healthcare systems, and pharmaceutical industries as well as academia. Moreover, for the goal of standardizing phenotypes and comparing ethnicity regarding genetic risk factors for severe ADRs, these networks may form a global consortium together with new networks from other communities in the future.

Conclusions

Specific types of *HLA* which showed strong association with severe cutaneous ADRs and DILI have been found as candidate pharmacogenetic biomarkers for each ADR. The *HLA* type was different for different causative drugs,

and the allelic frequency of *HLA* genetic polymorphisms showed ethnic differences. The genetic polymorphism of drug transporter gene *SLCO1B1* has been shown to be associated with statin-induced myopathy. It is necessary to conduct prospective studies to establish valid pharmacogenetic biomarkers for severe ADRs. A large research network for the collection of DNA samples from patients with ADRs is also necessary to explore a variety of pharmacogenetic biomarkers for ADRs.

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