PVR, and CO) at baseline and week 12 of administration are shown in Table 6. The mean PAP and PVR at week 12 of administration decreased as compared with baseline, and CO increased as compared with baseline. The mean PAP and PVR decreased as compared with baseline, and CO increased as compared with baseline.

Among the subjects, only 1 subject showed deterioration in WHO functional class from class II at baseline to class III at week 12 of administration; 6 subjects improved (5 from class III to class II and 1 from class III to class I). Other 13 subjects sustained their class at baseline.

Hemodynamic parameters other than mPAP, PVR, and CO were also assessed. In the subjects, consequently, the change (mean±SD) in PVR index in last observation carried forward (LOCF) at week 12 of administration from baseline was –382.00±491.80 dyne·s/cm^5/m^2; therefore, PVR decreased. Furthermore, the actual value (mean±SD) of PVR index in the LOCF at week 12 of administration was 1,199.31±660.73 dyne·s/cm^5/m^2.

The changes (mean±SD) in Borgs dyspnea score in the LOCF at weeks 8 and 12 of administration from baseline were –0.84±1.89 and –0.95±1.94, respectively. Therefore, the scores decreased as compared with the baseline value (mean±SD: 3.10±1.45).

Plasma BNP concentrations showed an average decrease of 78.00 pg/ml at week 4 of administration as compared with the baseline value (mean±SD: 216.52±204.70 pg/ml) and also sustained decreases also at weeks 8 and 12 of administration.

Pharmacokinetics

The pharmacokinetics of sildenafil and its metabolite at steady state in the repeated oral administration of 20 mg t.i.d. was examined in 7 subjects. Consequently, the mean T_{max} of sildenafil was approximately 1.1 h after administration. The mean values (coefficients of variation) of C_{max}, AUC_{0-8}, $C_{ss,av}$, and C_{trough} of sildenafil at steady state were 164.88 ng/ml (45.4%), 545.14 ng·h/ml (54.1%), 68.14 ng/ml (54.1%), and 19.608 ng/ml (63.4%), respectively. Therefore, relatively large interindividual variations were observed. Furthermore, sildenafil underwent the first-pass effect and rapidly produced its metabolite, and the mean T_{max} value was approximately 1.6 h after administration. The mean values (coefficients of variation) for C_{max} and AUC_{0-8} of the metabolite were 87.27 ng/ml (35.1%) and 365.85 ng·h/ml (51.0%), respectively.

Safety

There were 36 episodes of undeniable causality in 16 cases (76.2%) among 21 subjects. There were no cases of serious and severe adverse events of undeniable causality. However, 2 subjects temporarily reduced the dose of the drug or discontinued its administration because of adverse events of undeniable causality. The major adverse events of undeniable causality were headache (10 cases, 22.7%) and flushing (8 cases, 18.2%); all the events were mild or moderate.

Therefore, there were no safety concerns about laboratory values, vital signs, and electrocardiographic findings.

Discussion

In the present multicenter, collaborative, open-label trial, PAH patients taking sildenafil showed sustained improvement in the 6-min walking distance at weeks 8 and 12 of administration without any serious adverse events causally related to sildenafil administration.

PAH is considered to be caused by pulmonary endothelial dysfunction, by the imbalance between vasoconstrictive factors such as endothelin and thromboxane, and vasorelaxant factors such as prostacyclin and nitric oxide, followed by pulmonary arterial vasoconstriction, and by the proliferation of the vascular wall including endothelium, smooth muscle and adventitia, resulting in increased resistance of pulmonary blood flow through pulmonary arterioles.[18–20] At present, there are 3 categories of effective drugs for PH which present one of the following pharmacological mechanisms. Endothelin receptor antagonists (eg, bosentan) suppress vasoconstriction induced by a vasoconstrictive factor endothelin, dilate pulmonary arteries, and regresses the proliferation of vascular wall cells.[21] Prostacyclin and nitric oxide–which are secreted from the pulmonary artery endothelium, relax smooth muscle cells, and inhibit the proliferation and promote apoptosis of vascular walls– are depleted in PAH patients. Cyclic adenosine monophosphate stimulated by prostacyclin in smooth muscle cells has a potent vasodilating and antiproliferative ability, and a synthetic prostacyclin, epoprostenol, is the first effective drug in the treatment of PH. Nitric oxide stimulates cGMP, which in turn produces cGMP with potent vasodilating and antiproliferative ability. cGMP is degraded by PDE-5 which has been reported to be increased in PAH.[22] Sildenafil inhibits PDE-5 and increases guanosine monophosphate, and alleviates PH.

Six-minute walking distance is a reliable index of functional capacity in patients with PAH and has been widely used as the primary endpoint in most clinical trials designed for patients with PAH.[23–25] In the present subjects, the 6-min walking distance increased by 87.5 m and 84.2 m at weeks 8 and 12 of administration, respectively; these values were greater than those reported with other therapeutic drugs for PAH [eg, epoprostenol (47 m),[23] bosentan (44 m),[24] and beraprost (63 m)[25]].

The efficacy and safety of sildenafil for PAH patients have been demonstrated in uncontrolled[18–20] and controlled[8,16] clinical trials. The controlled clinical trial with the largest number of patients was the Sildenafil Use in Pulmonary Arterial Hypertension (SUPER) study, in which 278 patients were enrolled; the mean 6-min walk distance and mean PVR significantly increased by 45 m and decreased by 122 dyne·s/cm^5, respectively, as compared with the placebo group.[9] Our study afforded comparable results in efficacy. Safety profiles also were almost equivalent between the SUPER study and ours. Namely, any laboratory changes of clinical concern were not observed in these studies. One patient each in the placebo group and the sildenafil 20 mg group died from right heart failure and from acute embolism and urosepsis, respectively, in the SUPER study in contrast to the present trial in which no deaths occurred. The SUPER study reported 1 serious adverse event of undeniable causality in one patient receiving 20 mg of sildenafil in contrast to none in the present trial. The incidences of headache in the SUPER study and ours were 46.0% and 22.7%, respectively.

Changes in hemodynamic parameters (mean PAP, PVR, and CO) at week 12 of administration improved as compared with baseline and indicated a decrease in the mean PAP and an increase in CO. However, no statistically significant changes were found in systemic arterial pressure and heart rate. Therefore, improvement in CO did not involve increases in systemic artery pressure and heart rate. The improvement in CO was also corroborated by an increase in mixed venous oxygen saturation. Decreases in right atrial pressure, systolic pulmonary arterial pressure, and diastolic pulmonary arterial pressure suggested the overall improvement in right heart

function by the administration of sildenafil. The secondary endpoints–changes in other hemodynamic parameters, changes in Borg dyspnea scores, and changes in plasma BNP concentrations–also improved. These results indicated the efficacy of sildenafil which was orally administered to PAH patients at a regimen of 20 mg t.i.d. for 12 weeks.

Study Limitations

The principal limitation of the present trial is the fact that it was not a double-blind, controlled study, because of ethical considerations. Therefore, the possibility of investigator or selection bias cannot be excluded completely with regard to the endpoints examined, especially functional capacity. Another limitation of the present trial was that it did not enroll any PAH patients with WHO class IV. This has possibly favored the clinical outcomes of enrolled subjects with respect to their background at baseline. Although the number of enrolled subjects was as low as at 21, the present study is the first systematically designed study that provides clinical evidence for the efficacy, safety, and pharmacokinetic profile of sildenafil in Japanese patients with PAH.

Conclusions

Sildenafil 20 mg t.i.d. was effective for patients with PAH through improvements in the 6-min walking distance, hemodynamic parameters, Borg dyspnea scores, and plasma BNP concentration after 12-week oral administration. Furthermore, sildenafil showed relatively large interindividual variations in pharmacokinetic parameters, was well tolerated by the patients, and did not elicit any concerns about safety based on the results from laboratory tests, vital signs, and electrocardiography.

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References

1. Ruiz MJ, Escribano P, Delgado JF, Jiménez C, Tello R, Gómez MA, et al. Efficacy of sildenafil as a rescue therapy for patients with severe pulmonary arterial hypertension and given long-term treatment with prostanoids: 2-year experience. *J Heart Lung Transplant* 2006; **25:** 1353–1357.
2. Kähler CM, Colleselli D. Pulmonary arterial hypertension (PAH) in connective tissue diseases. *Rheumatology* 2006; **45:** 11–13.
3. Humbert M, Sitbon O, Simonneau G. Treatment of pulmonary arterial hypertension. *N Engl J Med* 2004; **351:** 1425–1436.
4. D'Alonzo GE, Barst RJ, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, et al. Survival in patients with primary pulmonary hypertension: Results from a national prospective registry. *Ann Intern Med* 1991; **115:** 343–349.
5. Nakano T, Aoyanagi S, Kawai A, Kuriyama T, Kobayashi M, Saji T, et al. Guideline for treatment of pulmonary hypertension. *Jpn Circ J* 2001; **65**(Suppl. 5): 1077–1118.
6. Simonneau G, Robbins IM, Beghetti M, Channick RN, Delcroix M, Denton CP, et al. Updated clinical classification of pulmonary hypertension. *J Am Coll Cardiol* 2009; **54:** 43–54.
7. Japan Intractable Diseases Information Center. Numbers of Certificates of Specified Disease Treatment Beneficiaries in 2004. http://www.nanbyou.or.jp (accessed July 12, 2010).
8. Galiè N, Ghofrani HA, Torbicki A, Barst RJ, Rubin LJ, Badesch D, et al. Sildenafil citrate therapy for pulmonary arterial hypertension. *N Engl J Med* 2005; **353:** 2148–2157.
9. Guilluy C, Sauzeau V, Rolli-Derkinderen M, Guérin P, Sagan C, Pacaud P, et al. Inhibition of RhoA/Rho kinase pathway is involved in the beneficial effect of sildenafil on pulmonary hypertension. *Br J Pharmacol* 2005; **146:** 1010–1018.
10. Watanabe H. Rho-kinase activation in patients with pulmonary arterial hypertension. *Circ J* 2009; **73:** 1597–1598.
11. Do.e Z, Fukumoto Y, Takaki A, Tawara S, Ohashi J, Nakano M, et al. Evidence for Rho-kinase activation in patients with pulmonary arterial hypertension. *Circ J* 2009; **73:** 1731–1739.
12. Michelakis ED, Tymchak W, Noga M, Webster L, Wu XC, Lien D, et al. Long-term treatment with oral sildenafil is safe and improves functional capacity and hemodynamics in patients with pulmonary arterial hypertension. *Circulation* 2003; **108:** 2066–2069.
13. Galiè N, Seeger W, Naeije R, Simonneau G, Rubin LJ. Comparative analysis of clinical trials and evidence-based treatment algorithm in pulmonary arterial hypertension. *J Am Coll Cardiol* 2004; **43:** 81S–88S.11.
14. Rubin LJ. Pulmonary arterial hypertension. *Proc Am Thorac Soc* 2006; **3:** 111–115.
15. Zusman RM, Morales A, Glasser DB, Osterloh IH. Overall cardiovascular profile of sildenafil citrate. *Am J Cardiol* 1999; **83:** 35–44.
16. Dishy V, Sofowora G, Harris PA, Kandcer M, Zhan F, Wood AJ, et al. The effect of sildenafil on nitric oxide-induced vasodilation in healthy men. *Clin Pharmacol Ther* 2001; **70:** 270–279.
17. Krenzelok EP, Krenzelok E. Sildenafil: Clinical toxicology profile. *Clin Toxicol* 2000; **38:** 7645–7651.
18. Herrmann HC, Chang G, Klugherz BD, Mahoney PD. Hemodynamic effects of sildenafil in men with severe coronary artery disease. *N Engl J Med* 2000; **342:** 1622–1626.
19. Sastry BK, Narasimhan C, Reddy NK, Raju BS. Clinical efficacy of sildenafil in primary pulmonary hypertension: A randomized, placebo-controlled, double-blind, crossover study. *J Am Coll Cardiol* 2004; **43:** 1149–1153.
20. Breavo JA. Cyclic nucleotide phosphodiesterases: Functional implications of multiple isoforms. *Pysiol Rev* 1995; **75:** 725–748.
21. Budhiraja R, Tuder RM, Hassoun PM. Endothelial dysfunction in pulmonary hypertension. *Circulation* 2004; **109:** 159–165.
22. Michelakis ED, Wilkins MR, Rabinovitch M. Emerging concepts and translational priorities in pulmonary arterial hypertension. *Circulation* 2008; **118:** 1486–1495.
23. Voelkel NF, Cool C, Lee SD, Wright L, Geraci MW, Tuder RM. Primary pulmonary hypertension between inflammation and cancer. *Chest* 1998; **114:** 225S–230S.
24. Wheeler W, Hayes S, Nguyen N, Cilla AM, Rybowicz J, Jones CC, et al. Sildenafil: A possible treatment for acute pulmonary hypertension during cardiac surgery. *BUMC Proc* 2002; **15:** 13–15.
25. Black SM, Sanchez LS, Mata-Greenwood E, Bekker JM, Steinhorn RH, Fineman JR. sGC and PDE5 are elevated in lambs with increased pulmonary blood flow and pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol* 2001; **281:** L1051–L1057.
26. Barst RJ, Rubin LJ, Long WA, McGoon MD, Rich S, Badesch DB, et al. A comparison of continuous intravenous epoprostenol (prostacyclin) with conventional therapy for primary pulmonary hypertension. *N Engl J Med* 1996; **334:** 296–301.
27. Rubin LJ, Badesch DB, Barst RJ, Galie N, Black CM, Keogh A, et al. Bosentan therapy for pulmonary arterial hypertension. *N Engl J Med* 2002; **346:** 896–903.
28. Vizza CD, Sciomer S, Morelli S, Lavalle C, Di Marzio P, Padovani D, et al. Long term treatment of pulmonary arterial hypertension with beraprost, an oral prostacyclin analogue. *Heart* 2001; **86:** 661–665.

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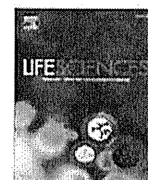

Expressions of multidrug resistance protein 1 and multidrug resistance-associated protein 1 in lung dendritic cells

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ABSTRACT

Aim: Multidrug resistance protein 1 (MDR1) and multidrug resistance-associated protein 1 (MRP1) are ATP-binding cassette transporters that mediate the efflux of a broad spectrum of substances and xenobiotics from cells in barrier organs. Interestingly, they are expressed in immune cells including some kinds of dendritic cells (DCs). In the present study, the expressions and activities of MDR1 and MRP1 in mouse lung DCs (LDCs) were investigated.
Main methods: We purified LDCs comprising ~98% MHC-Class II^{+}/CD11c^{+} cells using magnetic and flow cytometric cell sorting. The highly purified LDCs expressed MDR1 and MRP1 at both the mRNA and protein levels. The fluorescent probes rhodamine 123 and Fluo-3 were used as substrates in efflux assays to measure the transport activities of MDR1 and MRP1, respectively.
Key findings: MDR1 blockade by the specific inhibitor verapamil reduced the percentage of rhodamine 123^{low} cells in LDCs (from 31.8 ± 6.3% to 11.8 ± 2.8%, $p<0.02$). MRP1 blockade by the specific inhibitor MK-571 reduced the percentage of Fluo-3^{low} cells in LDCs (from 53.8 ± 1.7% to 26.8 ± 6.4%, $p<0.03$).
Significance: These data showed that LDCs exhibited MDR1- and MRP1-mediated efflux activities. MDR1 and MRP1 in LDCs may be involved in protective functions through their efflux activities.

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Introduction

The product of the human *ABCB1* gene and its mouse homologue *Abcb1a* and *Abcb1b* genes, multidrug resistance protein 1 (MDR1)/P-glycoprotein, is a membrane protein belonging to the large mammalian ATP-binding cassette (ABC) transporter family (Marzolini et al., 2004; van der Deen et al., 2005). MDR1 mediates the efflux of a broad spectrum of peptides, lipids and xenobiotics from cells in barrier organs, resulting in decreased intracellular concentrations of these substances. Multidrug resistance-associated protein 1 (MRP1) is another ABC transporter protein that eliminates toxins and xenobiotics, and protects the host as a physiological defensive barrier (van der Deen et al., 2005). These proteins were initially detected in tumor cell lines and mediate the efflux of anti-cancer drugs, thereby leading to multidrug resistance (van der Deen et al., 2005). In addition, they are expressed in normal hematopoietic cells such as T cells, B cells, natural killer cells and monocytes, and play physiologic roles in the immune system (Marzolini et al., 2004; van de Ven et al., 2009; van der Deen et al., 2005).

Antigen-presenting cells, comprising macrophages/monocytes, B cells and dendritic cells (DCs), capture, process and present foreign antigens to specific T cells along with appropriate costimulatory molecules and cytokines (Vermaelen and Pauwels, 2005). DCs are the only class of antigen-presenting cells that have the capacity to stimulate naive T cells and thereby initiate primary immune responses (Banchereau and Steinman, 1998). DCs comprise a heterogeneous cell population (Banchereau and Steinman, 1998) and transporters have been detected in some kinds of DCs, including MDR1 on Langerhans cells, a subtype of DCs in the skin, and MRP1 on bone marrow or monocyte-derived DCs (Randolph et al., 1998; Robbiani et al., 2000; Schroeijers et al., 2002).

The airway comes into direct contact with a variety of harmful, allergic and toxic substances. In addition to surfactants and the epithelial lining fluid, transporters in the conducting airway, alveolar epithelium and alveolar macrophages (AMs) act as efflux pumps to remove inhaled toxic materials (Cordon-Cardo et al., 1990; Endter et al., 2007; Scheffer et al., 2002). Lung DCs (LDCs) are localized in the airway and alveolar epithelium (Holt and Schon-Hegrad, 1987; Sertl et al., 1986), and are thought to have the capacity for antigen uptake and processing (Cumberbatch et al., 1991). Although transporters in LDCs may be involved in protective functions through their efflux activities, little is known about their distribution and function. This is partly because LDCs are a rare cell population and difficult to study because of the limited number of available cells (Vermaelen and

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Pauwels, 2005). However, recent advances in magnetic and flowcytometric cell sorting have enabled the isolation of highly purified LDCs. In the present study, we aimed to investigate the expressions and activities of the representative transporters MDR1 and MRP1 in highly purified LDCs dissociated from mouse lung tissues. We compared LDCs with AMs, which are already known to express these transporters (Scheffer et al., 2002).

Materials and methods

Mice

Experiments were performed on 10-week-old male BALB/c mice (Nippon SLC, Shizuoka, Japan) maintained under specific pathogen-free conditions. All the mouse experiments were performed in compliance with the animal care guidelines of our institution.

Preparation of AMs and LDCs

AMs were harvested by whole-lung lavage using 5 ml of ice-cold PBS. The purity of the AMs was routinely >98%, as determined by their morphology (Naito et al., 2008). For LDC isolation, lung single-cell suspensions were prepared using a gentleMACS Dissociator (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions (Jungblut et al., 2009). Briefly, lung tissues were perfused with 5 ml of PBS through the right ventricle, digested in gentleMACS tubes (Miltenyi Biotec) containing 1 mg/ml of collagenase/dispase (Roche, Mannheim, Germany) and 0.05 mg/ml DNase I (Sigma, St. Louis, MO) for 1 h at 37 °C and then dissociated. To purify LDCs, $CD11c^+$ cells were enriched by positive selection after incubation with allophycocyanin-conjugated anti-CD11c monoclonal antibody-conjugated magnetic microbeads (Miltenyi Biotec) using a MACS cell sorter (Miltenyi Biotec). The enriched population was further sorted with phycoerythrin-conjugated anti-MHC II and allophycocyanin-conjugated anti-$CD11c^+$ antibodies (Miltenyi Biotec) by flow cytometry using a FACSAria (BD Biosciences, San Jose, CA). To check the phenotype of the DCs, fluorescein isothiocyanate-conjugated anti-CD40, anti-CD80 and anti-CD86 monoclonal antibodies (BD Biosciences) were used.

RNA isolation and quantitative real-time RT-PCR analysis

Total RNA was extracted from the AMs and LDCs using the acid guanidinium thiocyanate-phenol-chloroform extraction method. First-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexanucleotides (Invitrogen). Quantitative real-time RT-PCR amplifications were performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Carlsbad, CA) and TaqMan™ universal PCR master mix (Applied Biosystems) according to the manufacturer's instructions. Primers and probes for the MDR1 genes *Abcb1a* and *Abcb1b*, MRP1 gene *Abcc1* and *glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) were obtained from Applied Biosystems (Assay ID Mm00440761 m1, Mm01324120 m1, Mm00456156 m1 and Mm99999915 g1, respectively). Murine *Gapdh* expression was evaluated as an endogenous control to allow normalization among the samples. The thermal cycling conditions were as follows: hold at 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. All reactions were performed in triplicate and the amplification data were analyzed with StepOne Software 2.0™ (Applied Biosystems). The data were obtained as the relative expression levels between the genes of interest and the *Gapdh* transcript levels.

Flow cytometric analysis

For flow cytometric analysis, DCs were subjected to fixation and permeabilization using an Inside Stain Kit (Miltenyi Biotec). The cells were then incubated with 2 µg/ml of anti-MDR1 monoclonal antibody C219 (Covance, Dedham, MA), anti-MRP1 monoclonal antibody MRPr1 (Covance), isotype-matched IgG1 or isotype-matched IgG2b (Beckman Coulter, Brea, CA) for 30 min on ice (Robbiani et al., 2000). After washing, the bound antibodies were detected with a fluorescein isothiocyanate-conjugated secondary antibody. Flow cytometry was performed using the FACSAria. FlowJo software (Tree Star Inc., Ashland, OR) was used for data analysis. The results were expressed as the mean fluorescence intensity, which was determined by subtracting the mean fluorescence of the C219 or anti-MRP1 monoclonal antibody from that of their isotype-matched antibody. And the cells were imaged with a FluoView™ FV1000 Confocal Scanning Laser Microscope (Olympus, Tokyo, Japan) to study the localizations of the transporters.

ABC transporter activities in LDCs and AMs

The fluorescent probes rhodamine 123 and Fluo-3 were used as substrates in efflux assays to measure the transport activities of MDR1 and MRP1, respectively (Kyle-Cezar et al., 2007; Robey et al., 1999). LDCs and AMs (1×10^5 cells/ml) were loaded with 1 µg/ml of rhodamine 123 (Invitrogen) or 5 µg/ml of Fluo-3 (Invitrogen) for 30 min at 37 °C. After washing with cold PBS, the cells were incubated with and without 50 µM of the MDR1 inhibitor verapamil (Sigma) or 75 µM of the MRP1 inhibitor MK-571 (Enzo Life Science, Plymouth, PA) for 1 h at 37 °C. The cells were then washed, and the intracellular rhodamine 123 or Fluo-3 retention levels were estimated in the FL1 channel by flow cytometry using the FACSAria. The regions of cells with low fluorescence intensity of rhodamine 123 (rhodamine 123^{low} cells) or Fluo-3 (Fluo-3^{low} cells) represented the cells in which dye efflux was mediated by MDR1 and MRP1, respectively (Donnenberg et al., 2001). A total of 1.0×10^4 cells were acquired based on their forward and side scatters.

Statistical analysis

All data were expressed as means ± SEM and analyzed using JMP version 5.0.1J software (SAS Institute Japan, Tokyo, Japan). An unpaired t-test was used. Values of $p<0.05$ were considered significant.

Results

Isolation of DCs

The purified LDC preparations comprised ~98% MHC-Class II^+/$CD11c^+$ cells as determined by flow cytometry. The cells showed the characteristic morphology of DCs, comprising adherence, formation of homotypic aggregates and presence of cytoplasmic protrusions. In our experiments, the yields of mouse LDCs were $5.4\pm1.2\times10^4$ cells/mouse.

Expressions of MDR1 and MRP1 on LDCs

A real-time quantitative RT-PCR assay was performed to detect the *Abcb1a*, *Abcb1b* and *Abcc1* gene expressions in LDCs. The *Abcb1a* and *Abcb1b* genes encode MDR1 while the *Abcc1* gene encodes MRP1. Because these gene expressions have already been confirmed in AMs (Scheffer et al., 2002), the mRNA levels in AMs were set to 1.0. When the mRNA levels in LDCs were obtained relative to those in AMs, *Abcb1a* mRNA expression was 3.3 ± 0.6. The *Abcb1b* and *Abcc1* mRNA expressions were 16.0 ± 0.7 and 4.2 ± 0.7, respectively, showing significantly more abundant expressions on LDCs compared with

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AMs ($p<0.05$). In addition, the MDR1 and MRP1 protein expressions were examined by immunolabeling. As shown in Fig. 1, MDR1 and MRP1 were clearly detected in LDCs using the C219 and MRPr1 antibodies, respectively. The mean fluorescence intensities of the C219 and MRPr1 monoclonal antibodies were increased compared with those of their isotype-matched control monoclonal antibodies (Fig. 1A, B). The fluorescent detection of cell surface transporters was confirmed by confocal laser scanning microscopy (Fig. 1C, D).

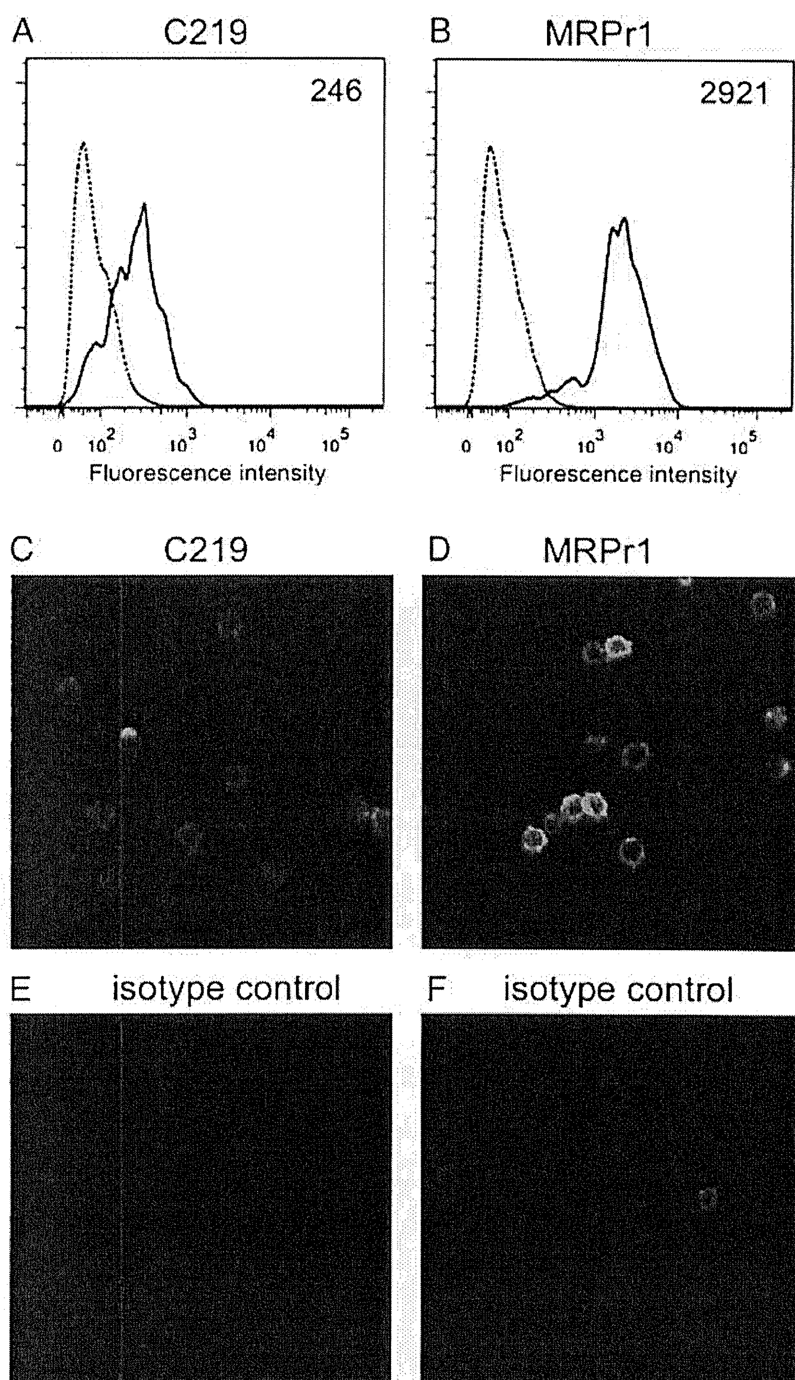

Fig. 1. MDR1 and MRP1 expressions in lung dendritic cells (LDCs). (A, B) Flow cytometric analyses of MDR1 and MRP1 proteins were performed using the C219 and MRPr1 monoclonal antibodies (filled profiles), respectively, or their isotype-matched control monoclonal antibodies (open profiles). The numbers shown in the top right corners are the mean fluorescence intensities determined by subtracting the mean fluorescence of the C219 or anti-MRP1 monoclonal antibody from that of their isotype-matched antibody. The results shown are representative of three independent experiments. (C–F) Fluorescent detection of cell surface transporters by confocal laser scanning microscopy. The fluorescent the C219 (C) and MRPr1 (D) monoclonal antibodies are present on the cell surface. Staining with isotype-matched control monoclonal antibodies (E, F) is shown.

Functional activities of MDR1 and MRP1 on LDCs

The functional activities of MDR1 and MRP1 were assessed by transporter-mediated substrate efflux assays using fluorescent transport substrates. We loaded purified cells with the MDR1 substrate rhodamine 123 or MRP1 substrate Fluo-3 followed by incubation in the presence or absence of transporter-specific inhibitors and examined whether the inhibitors affected the intracellular rhodamine 123 and Fluo-3 contents. Because the regions of cells with low fluorescent dye intensities (rhodamine 123^{low} cells and Fluo-3^{low} cells) are decreased when the transporter functions are inhibited, the differences in the numbers of rhodamine 123^{low} cells and Fluo-3^{low} cells between the presence and absence of the inhibitors reflect the transporter activities. MDR1 blockade by the specific inhibitor verapamil reduced the percentage of rhodamine 123^{low} cells in LDCs (from 31.8±6.3% to 11.8±2.8%, $p<0.02$, Fig. 2). The specific MRP1 inhibitor MK-571 reduced the percentage of Fluo-3^{low} cells in LDCs (from 53.8±1.7% to 26.8±6.4%, $p<0.03$, Fig. 2). The efflux functions in LDCs through MDR1 and MRP1 were similar to those in AMs (Fig. 3). Cellular immunophenotyping revealed that treatment with verapamil or MK-571 resulted in significant downregulation of CD80 expression in LDCs ($p<0.05$, Table 1).

Discussion

We have shown that the transporters MDR1 and MRP1 are expressed in highly purified mouse LDCs. In directional transport experiments with fluorescent substrates, rhodamine 123^{low} cells and Fluo-3^{low} cells were reduced in LDCs treated with verapamil and MK-571, respectively. Because verapamil and MK-571 inhibit the efflux functions of MDR1 and MRP1, respectively, our data suggest that MDR1 and MRP1 on LDCs are functionally active.

DCs are present at various portals of entry for ambient particles, such as the skin, airway and intestinal tract. However, few studies have evaluated the expressions of MDR1 and MRP1 on DCs. Randolph et al. (1998) reported that Langerhans cells express MDR1 and showed that these DCs have efflux activity using an MDR1 substrate. Apart from this primary function, the transporters on DCs act for efficient migration toward lymph nodes by controlling the intracellular accumulation of key signaling lipids (Randolph et al., 1998; Robbiani et al., 2000). Blocking MDR1 function diminishes the extrusion of DCs from cultured skin explants (Randolph et al., 1998). MRP1 is expressed in mouse bone marrow-derived DCs and human monocyte-derived DCs (Robbiani et al., 2000; Schroeijers et al., 2002). Functionally, MRP1 is necessary for entry of DCs into their afferent lymphatic and differentiation process (Robbiani et al., 2000; van de Ven et al., 2006) and its overexpression in DCs is thought to facilitate the release of proinflammatory mediators (Robbiani et al., 2000). DCs comprise a heterogeneous cell population (Banchereau and Steinman, 1998) and the contributions of MDR1 or MRP1 to DC functions may be dependent on the DC origin. These two transporters are not redundant and they possibly function in different pathways or at different points (Randolph, 2001). It is assumed that each transporter works differently according to the type and origin of the antigen-presenting cells. Therefore, it is meaningful to investigate the expressions and activities of these transporters in the lung. In the present study, we initially confirmed that MDR1 and MRP1 were expressed in highly purified LDCs consisting of more than 98% MHC-Class II^{+}/$CD11c^{+}$ cells.

The functional activities of MDR1 and MRP1 were assessed using fluorescent transport substrates. Ex vivo models for efflux measurements of fluorescent substrates with and without inhibitors have been used as validated surrogate markers to investigate transporter functions (Donnenberg et al., 2001). In the present study, the proportions of verapamil-inhibited rhodamine 123^{low} cells and MK-571-inhibited Fluo-3^{low} cells among LDCs were similar to those among AMs, indicating that MDR1 and MRP1 have efflux activities on LDCs. The reduction of MDR1 transporter activity by verapamil was more marked than the reduction of MRP1 transporter activity by MK-571. To investigate whether the phenotype of the LDCs was changed by the transporter inhibitors, cellular immunophenotyping was carried out with anti-CD40, anti-CD80 and anti-CD86 antibodies.

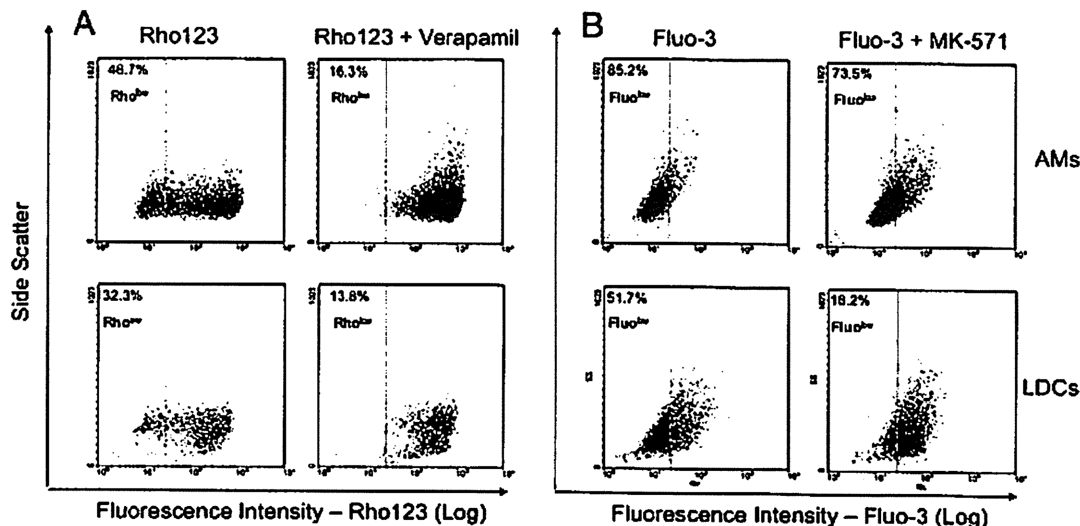

Fig. 2. Effects of transporter-specific inhibitors on the cell proportions with low fluorescent dye contents. Representative results of five independent analyses are shown. The percentages of cells with low fluorescent dye contents are shown in the top left corners. (A) Purified alveolar macrophages (AMs) and lung dendritic cells (LDCs) at 1.0×10⁵ cells/ml were loaded with 1 μg/ml of the MDR1 substrate rhodamine 123 followed by incubation in the presence or absence of 50 μM of the MDR1-specific inhibitor verapamil. The region of cells with low rhodamine 123 fluorescence intensity (left side region: Rho^{low}), determined by the cut-off fluorescence level to contain >99% of cells when no fluorescent substrate was added, indicates the cells that efflux the dye via MDR1. The representative dot plots show that verapamil reduces the proportion of cells with low rhodamine 123 fluorescence intensity. (B) Purified AMs and LDCs at 1.0×10⁵ cells/ml were loaded with 5 μg/ml of the MRP1 substrate Fluo-3 followed by incubation in the presence or absence of 75 μM of the MRP1-specific inhibitor MK-571. The region of cells with low Fluo-3 fluorescence intensity (left side region: $Fluo^{low}$) indicates the cells that efflux the dye via MRP1. The representative dot plots show that MK-571 reduces the proportion of cells with low Fluo-3 fluorescence intensity.

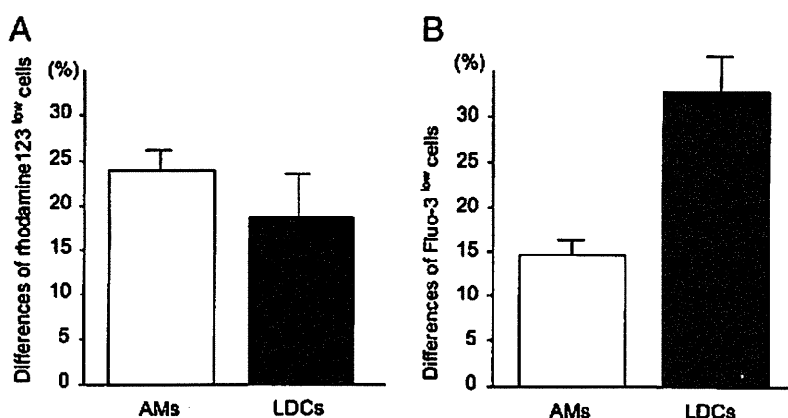

Fig. 3. Reduced percentages of cells with low fluorescence dye contents in the presence of specific transporter inhibitors. (A) Purified alveolar macrophages (AMs) and lung dendritic cells (LDCs) were loaded with 1 μg/ml of the MDR1 substrate rhodamine 123 followed by incubation in the presence or absence of 50 μM of the MDR1-specific inhibitor verapamil. The region of cells with low rhodamine 123 fluorescence intensity (rhodamine 123^{low} cells) indicates the cells that efflux the dye via MDR1. The difference in the proportions of rhodamine 123^{low} cells was calculated by subtracting the percentage of rhodamine 123^{low} cells with verapamil from that of cells without verapamil. (B) Purified AMs and LDCs were loaded with 5 μg/ml of the MRP1 substrate Fluo-3 followed by incubation in the presence or absence of 75 μM of the MRP1-specific inhibitor MK-571. The region of cells with low Fluo-3 fluorescence intensity indicates the cells that efflux the dye via MRP1. The proportion of cells with low fluorescence Fluo-3 intensity in the presence or absence of MK-571 reflects the transporter activity. There are no significant differences in the MK-571-inhibited Fluo-3 efflux among the AMs and LDCs. The data are expressed as the means ± SEM of three independent experiments.

After verapamil or MK-571 treatment, significant downregulation of CD80 was found in LDCs. Laupeze et al. (Laupeze et al., 2001) showed that addition of an MRP1 antagonist significantly induces elevated CD86 and reduced CD40 expression levels on monocyte-derived DCs. In another study, treatment with the MDR1 antagonist PSC833 was found to suppress CD80 and CD1a expressions and elevate CD86 expression in human monocyte-derived DCs (Pendse et al., 2006). The relationship between reduced efflux functional activity after inhibitor treatment and the maturation status of DCs remains undetermined.

What is the functional significance of MDR1 and MRP1 in LDCs? Originally, transporters were considered to have obvious protective functions by eliminating xenobiotics and toxins. The directional transport experiments in the present study confirmed that LDCs had elimination activities. Laupeze et al. (Laupeze et al., 2001) suggested that efflux pumps are involved in protection against oxidative stress and maintenance of the intracellular redox potential, and serve to protect DCs from xenobiotics. MDR1 and MRP1 in LDCs are assumed to be involved in protective functions through their efflux activities. Although other physiological transporter functions, including migration, cytokine secretion and differentiation, have been suggested in other types of DCs (Laupeze et al., 2001; Randolph et al., 1998; Robbiani et al., 2000; Schroeijers et al., 2002; van de Ven et al., 2006), we could not investigate these functions using LDCs. We also need to consider the effects of the transport substrates on the functions of DCs. Exogenous cysteinyl leukotrienes, which are substrates for MRP1, can restore reduced DC migration in MRP1-deficient mice (Robbiani et al., 2000). In a mouse asthma model, the cysteinyl leukotriene C4 was found to enhance antigen presentation and cytokine production by LDCs (Okunishi et al., 2004), although the involvement of the transporters on LDCs is undetermined.

Table 1
Effects of verapamil and MK-571 on immunophenotypic markers of lung dendritic cells.

	Mean fluorescence intensity		
	Control	+ Verapamil	+ MK-571
Isotype control	74.3 ± 3.9	77.7 ± 4.2	80.3 ± 4.3
CD40	399.3 ± 35.5	288.0 ± 11.5	245.0 ± 12.1
CD80	9535.3 ± 4519.6	4585.6 ± 1319.1*	2295.3 ± 245.7*
CD86	738.6 ± 241.0	773.0 ± 251.7	747.0 ± 261.3

Values are expressed as the means ± SEM of three independent experiments.
* $p < 0.05$, significant difference vs. control cells without the specific inhibitors.

Conclusion

LDCs express the ABC transporters MDR1 and MRP1. The efflux functions through MDR1 and MRP1 in LDCs were similar to those in AMs. In addition to antigen uptake and processing, the transporter molecules in LDCs may have protective functions to control the intracellular accumulation of inhaled toxic compounds. Further research, including investigations of the transporters on DCs of different types and origins, may provide important information toward a better understanding of transporters.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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References

Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392(6673):245–52.
Cordon-Cardo C, O'Brien JP, Boccia J, Casals D, Bertino JR, Melamed MR. Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues. J Histochem Cytochem 1990;38(9):1277–87.
Cumberbatch M, Illingworth I, Kimber I. Antigen-bearing dendritic cells in the draining lymph nodes of contact sensitized mice: cluster formation with lymphocytes. Immunology 1991;74(1):139–45.
Donnenberg VS, Burckart GJ, Griffith BP, Jain AB, Zeevi A, Berg AD. P-glycoprotein (P-gp) is upregulated in peripheral T-cell subsets from solid organ transplant recipients. J Clin Pharmacol 2001;41(12):1271–9.
Endter S, Becker U, Daum N, Huwer H, Lehr CM, Gumbleton M, et al. P-glycoprotein (MDR1) functional activity in human alveolar epithelial cell monolayers. Cell Tissue Res 2007;328(1):77–84.
Holt PG, Schon-Hegrad MA. Localization of T cells, macrophages and dendritic cells in rat respiratory tract tissue: implications for immune function studies. Immunology 1987;62(3):349–56.
Jungblut M, Oeltze K, Zehnter I, Hasselmann D, Bosio A. Standardized preparation of single-cell suspensions from mouse lung tissue using the gentleMACS Dissociator. J Vis Exp 2009;29.
Kyle-Cezar F, Echevarria-Lima J, Rumjanek VM. Independent regulation of ABCB1 and ABCC activities in thymocytes and bone marrow mononuclear cells during aging. Scand J Immunol 2007;66(2–3):238–48.
Laupeze B, Amiot L, Bertho N, Grosset JM, Lehne G, Fauchet R, et al. Differential expression of the efflux pumps P-glycoprotein and multidrug resistance-associated

protein in human monocyte-derived dendritic cells. Hum Immunol 2001;62(10): 1073-80.
Marzolini C, Paus E, Buclin T, Kim RB. Polymorphisms in human MDR1 (P-glycoprotein): recent advances and clinical relevance. Clin Pharmacol Ther 2004;75(1):13-33.
Naito T, Suda T, Suzuki K, Nakamura Y, Inui N, Sato J, et al. Lung dendritic cells have a potent capability to induce production of immunoglobulin A. Am J Respir Cell Mol Biol 2008;38(2):161-7.
Okunishi K, Dohi M, Nakagome K, Tanaka R, Yamamoto K. A novel role of cysteinyl leukotrienes to promote dendritic cell activation in the antigen-induced immune responses in the lung. J Immunol 2004;173(10):6393-402.
Pendse SS, Behjati S, Schatton T, Izawa A, Sayegh MH, Frank MH. P-glycoprotein functions as a differentiation switch in antigen presenting cell maturation. Am J Transplant 2006;6(12):2884-93.
Randolph GJ, Beaulieu S, Pope M, Sugawara I, Hoffman L, Steinman RM, et al. A physiologic function for p-glycoprotein (MDR-1) during the migration of dendritic cells from skin via afferent lymphatic vessels. Proc Natl Acad Sci USA 1998;95(12):6924-9.
Randolph GJ. Dendritic cell migration to lymph nodes: cytokines, chemokines, and lipid mediators. Semin Immunol 2001;13(5):267-74.
Robbiani DF, Finch RA, Jager D, Muller WA, Sartorelli AC, Randolph GJ. The leukotriene C(4) transporter MRP1 regulates CCL19 (MIP-3beta, ELC)-dependent mobilization of dendritic cells to lymph nodes. Cell 2000;103(5):757-68.
Robey R, Bakke S, Stein W, Meadows B, Litman T, Patil S, et al. Efflux of rhodamine from CD56+ cells as a surrogate marker for reversal of P-glycoprotein-mediated drug efflux by PSC 833. Blood 1999;93(1):306-14.
Scheffer GL, Pijnenborg AC, Smit EF, Muller M, Postma DS, Timens W, et al. Multidrug resistance related molecules in human and murine lung. J Clin Pathol 2002;55(5): 332-9.
Schroeijers AB, Reurs AW, Scheffer GL, Stam AG, de Jong MC, Rustemeyer T, et al. Up-regulation of drug resistance-related vaults during dendritic cell development. J Immunol 2002;168(4):1572-8.
Sertl K, Takemura T, Tschachler E, Ferrans VJ, Kaliner MA, Shevach EM. Dendritic cells with antigen-presenting capability reside in airway epithelium, lung parenchyma, and visceral pleura. J Exp Med 1986;163(2):436-51.
van de Ven R, de Jong MC, Reurs AW, Schoonderwoerd AJ, Jansen G, Hooijberg JH, et al. Dendritic cells require multidrug resistance protein 1 (ABCC1) transporter activity for differentiation. J Immunol 2006;176(9):5191-8.
van de Ven R, Scheffer GL, Scheper RJ, de Gruijl TD. The ABC of dendritic cell development and function. Trends Immunol 2009;30(9):421-9.
van der Deen M, de Vries EG, Timens W, Scheper RJ, Timmer-Bosscha H, Postma DS. ATP-binding cassette (ABC) transporters in normal and pathological lung. Respir Res 2005;6:59.
Vermaelen K, Pauwels R. Pulmonary dendritic cells. Am J Respir Crit Care Med 2005;172 (5):530-51.

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EDITORIAL

Pulmonary Arterial Hypertension Associated With Connective Tissue Disease and Immunosuppressive Therapy

Kazuhiko Takeuchi, MD, PhD; Hiroshi Watanabe, MD, PhD

Connective tissue disease (CTD)-associated pulmonary arterial hypertension (CPAH) is the second most prevalent type of pulmonary arterial hypertension (PAH) after idiopathic PAH (IPAH). CPAH is estimated to account for 30% of all adult cases of PAH. Approximately 15% of mixed CTD (MCTD), 10% of systemic sclerosis (SSc), and several percent of systemic lupus erythematosus (SLE) patients develop PAH.[1–3] In general, patients with CPAH have a poorer prognosis than those with other forms of PAH, except for pulmonary veno-occlusive disease (PVOD).[4] A half of SSc patients may die within 1 year of PAH diagnosis without any treatment.[5]

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The treatment for PAH including CPAH has undergone a remarkable evolution in the recent decade after the advent of the intravenous prostacyclin analog, epoprostenol. New pulmonary vasodilators for PAH, such as the prostacyclin analog, endothelin receptor antagonists and phosphodiesterase type 5 (PDE5) inhibitors, significantly improve patients' symptoms and slow the rate of clinical deterioration. A meta-analysis performed on 23 randomized controlled trials (vs. placebo) in PAH, including CPAH (retrieved from the Medline database from 1990 to 2008), showed a 43% reduction in mortality in patients treated with these new pulmonary vasodilators (RR, 0.57; 95% confidence interval, 0.35–0.92; P=0.023).[6] Despite these positive clinical trial results, PAH remains a chronic disease without a cure.

It is currently accepted that the genesis and progression of PAH are attributable to the immune and/or inflammatory system in CPAH and IPAH patients.[7,8] Macrophages and lymphocytes have been detected in perivascular and plexiform lesions of the pulmonary artery of CPAH patients[9,10] and deposits of antinuclear antibodies, rheumatoid factor, gamma globulins and complement have been identified in the pulmonary vascular wall in lung biopsies of these patients.[11] These findings appear to be supportive of immunosuppressive therapy for CPAH.

Sanchez et al reported that immunosuppressive therapy comprising cyclophosphamide (600 mg/m^2 IV monthly for at least 3 months) and glucocorticosteroids without pulmonary vasodilators (prostacyclin analog, endothelin receptor antagonists or PDE5 inhibitors) could improve the symptoms and prognosis of CPAH patients with SLE or MCTD, but not those of CPAH patients with SSc.[12] A responder in their study was defined as a patient in NYHA functional class I or II with sustained hemodynamic improvement after at least 1 year of immunosuppressive therapy without additional pulmonary vasodilators; 8 of 21 CPAH patients with SLE or MCTD responded to the immunosuppressive therapy and those responders were characterized by a lower NYHA functional class, lower pulmonary vascular resistance and a higher cardiac index at baseline, which indicates that diagnosis and treatment for CPAH in an earlier phase of pathological pulmonary vascular changes may be linked to better outcomes.

Jais et al described the effect of additional pulmonary vasodilators on non-responders to immunosuppressive therapy among CPAH patients with SLE or MCTD,[13] according to their retrospective analysis; 8 non-responders to immunosuppressive therapy were subsequently treated with pulmonary vasodilators 6 months later, and 6 of the 8 non-responders responded to the pulmonary vasodilators.

In this issue of the Journal, Miyamichi-Yamamoto et al[14] report the effectiveness of intensive immunosuppressive therapy (IIT) combined with pulmonary vasodilators (IIT group: mean age, 45±8 years) on pulmonary hemodynamics and prognosis in patients with CPAH. The IIT was a combination therapy with cyclophosphamide (500 mg IV, 10 times in a year) and glucocorticosteroids (1 mg/kg PO daily in the first month, followed by gradual tapering afterward by 5–10 mg/day every 2–4 weeks with a maintenance dose of 5–10 mg/day). The authors compared these patients with a historical control group treated with only pulmonary vasodilators (mean age, 52±18 years) regarding pulmonary hemodynamics and prognosis. Although mean pulmonary arterial pressure (mPAP) remained unchanged in the control group, IIT with pulmonary vasodilators significantly decreased mPAP (40±9 to 29±11 mmHg, P<0.01). Intriguingly, in approximately half of the patients in the IIT group, mPAP was almost normalized (<25 mmHg). Furthermore, the IIT group showed a significantly better prognosis compared with the control group (P<0.01). It should be taken into consideration that there were differences in the baseline characteristics of the 2 groups. For example, no PDE5 inhibitors were given to patients in the historical control group and 6 patients were administered PDE5 inhibitors in the IIT group, there were different proportions of underlying CTD and WHO functional class, and the 6-min walking distance was

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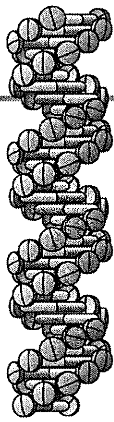
84 m longer in the IIT group than in the control group. However, the data from this study suggest some potential CPAH treatments. The CPAH patients with SSc did not respond to the IIT, which is consistent with previous reports that showed that CPAH patients with SLE or MCTD respond well to immunosuppressive therapy but not patients with SSc.[13] CPAH patients with SSc may be resistant to the present immunosuppressive therapy, and, therefore, an immunosuppressive therapy appropriate to each underlying CTD with CPAH should be examined in future studies. Moreover, in most of the CPAH patients of the present study, IIT with pulmonary vasodilators was initiated within a few months after the diagnosis and indicated good outcomes regardless of the immunological activity of the underlying CTD. Sanchez et al also showed that patients who responded to immunosuppressive therapy had less severe NYHA functional class and pulmonary hemodynamics at baseline than those patients who did not respond.[12] Immunosuppressive therapy for CPAH may be less effective in patients with longstanding PAH because their pulmonary arteries have already sustained irreversible pathological damage. Initiation of treatment in an earlier phase of pathological pulmonary vascular changes could be important for achieving good improvement of pulmonary hemodynamics and long-term prognosis.

IIT with pulmonary vasodilators could be a promising therapy for CPAH. However, more reliable data from larger randomized clinical trials are required to establish appropriate therapy for CPAH. CPAH is such a rare disease that cooperation is required in recruiting a sufficient number of patients to reliably estimate the efficacy of therapy for each underlying CTD.

References

1. Mukerjee D, St George D, Coleiro B, Knight C, Denton CP, Davar J, et al. Prevalence and outcome in systemic sclerosis associated pulmonary arterial hypertension: Application of a registry approach. *Ann Rheum Dis* 2003; **62:** 1088–1093.
2. Shen JY, Chen SL, Wu YX, Tao RQ, Gu YY, Bao CD, et al. Pulmonary hypertension in systemic lupus erythematosus. *Rheumatol Int* 1999; **18:** 147–151.
3. Pan TL, Thumboo J, Boey ML. Primary and secondary pulmonary hypertension in systemic lupus erythematosus. *Lupus* 2000; **9:** 338–342.
4. Galie N, Manes A, Branzi A. The endothelin system in pulmonary arterial hypertension. *Cardiovasc Res* 2004; **61:** 227–237.
5. Denton CP, Nihtyanova SI. Therapy of pulmonary arterial hypertension in systemic sclerosis: An update. *Curr Rheumatol Rep* 2007; **9:** 158–164.
6. Galie N, Manes A, Negro L, Palazzini M, Bacchi Reggiani ML, Branzi A. A meta-analysis of randomized controlled trials in pulmonary arterial hypertension. *Eur Heart J* 2009; **30:** 394–403.
7. Dorfmuller P, Perros F, Balabanian K, Humbert M. Inflammation in pulmonary arterial hypertension. *Eur Respir J* 2003; **22:** 358–363.
8. Humbert M, Morrell NW, Archer SL, Stenmark KR, MacLean MR, Lang IM, et al. Cellular and molecular pathobiology of pulmonary arterial hypertension. *J Am Coll Cardiol* 2004; **43:** 13S–24S.
9. Dorfmuller P, Humbert M, Perros F, Sanchez O, Simonneau G, Muller KM, et al. Fibrous remodeling of the pulmonary venous system in pulmonary arterial hypertension associated with connective tissue diseases. *Hum Pathol* 2007; **38:** 893–902.
10. Tuder RM, Groves B, Badesh DB, Voelkel NF. Exuberant endothelial cell growth and elements of inflammation are present in plexiform lesions of pulmonary hypertension. *Am J Pathol* 1994; **144:** 275–285.
11. Yeo PP, Sinniah R. Lupus cor pulmonale with electron microscope and immunofluorescent antibody studies. *Ann Rheum Dis* 1975; **34:** 457–460.
12. Sanchez O, Sitbon O, Jais X, Simonneau G, Humbert M. Immunosuppressive therapy in connective tissue diseases-associated pulmonary arterial hypertension. *Chest* 2006; **130:** 182–189.
13. Jais X, Launay D, Yaici A, Le Pavec J, Tcherakian C, Sitbon O, et al. Immunosuppressive therapy in lupus- and mixed connective tissue disease-associated pulmonary arterial hypertension: A retrospective analysis of twenty-three cases. *Arthritis Rheum* 2008; **58:** 521–531.
14. Miyamichi-Yamamoto S, Fukumoto Y, Sugimura K, Ishii T, Satoh K, Miura Y, et al. Intensive immunosuppressive therapy improves pulmonary hemodynamics and long-term prognosis in patients with pulmonary arterial hypertension associated with connective tissue disease. *Circ J* 2011; **75:** 2668–2674.



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Future perspective on pharmacogenomics of severe hypoglycemia associated with sulfonylureas: reply from the authors

A letter in response to: Holstein JD, Kovacs P, Patzer O, Stumvoll M, Holstein A. The Ser1369Ala variant of *ABCC8* and the risk for severe sulfonylurea-induced hypoglycemia in German patients with Type 2 diabetes. *Pharmacogenomics* 13(1), 5–7 (2012).

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We thank Holstein *et al.* for their valuable comments on our research [1], and would like to respond to the letter with a future perspective.

Holstein *et al.* verified that the *ABCC8* Ser1369Ala variant did not affect severe hypoglycemia due to sulfonylureas in German subjects. Their findings were almost consistent with our results in a Japanese population [2]. It is meaningful that the findings have been validated in a different ethnic group. While the *ABCC8* Ser1369Ala variant was not associated with sulfonylurea-induced severe hypoglycemia in both studies, other unknown genetic factors might influence severe hypoglycemia due to sulfonylureas. Further studies which focus on other genetic factors are now required.

Recently, a new kind of severe hypoglycemia, which is owing to sulfonylurea and the DPP-4 inhibitor, is emerging in Japan [101]. In December 2009, the DPP-4 inhibitor was brought to the market as a brand-new insulin secretagogue. A considerable number of physicians started prescribing DPP-4 inhibitors in addition to traditional sulfonylurea therapy. This comedication improved glycemic control of Type 2 diabetic patients in a relatively short period. However, some of the cases experienced severe hypoglycemia, although their doses of sulfonylureas and DPP-4 inhibitors were within the approved doses in Japan. The addition of DPP-4 inhibitors to sulfonylurea therapy was believed to be a trigger for severe hypoglycemia. Against this urgent problem, the Ministry of Health, Labor and Welfare in Japan issued a proclamation which announced the danger of severe hypoglycemia caused by sulfonylurea and DPP-4 inhibitor treatment in December 2010. Consequently, it is now recommended that the dosage of sulfonylurea is reduced upon addition of DPP-4 inhibitor to sulfonylurea therapy.

The details of pathogenesis for this kind of hypoglycemia remain uncertain. The function of insulin release from pancreatic β-cells by the DPP-4 inhibitor is modest compared to sulfonylurea, and the DPP-4 inhibitor itself seldom causes hypoglycemia in the setting of monotherapy [3]. It is postulated that DPP-4 inhibitor enhances the insulin secretion caused by sulfonylurea as both drugs have a common target in pancreatic β-cells [4]. Sulfonylurea binds to the SUR1 and causes ATP-sensitive K^+ (K_{ATP}) channel closure, triggering the opening of the voltage-dependent Ca^{2+} channel and Ca^{2+} influx, which leads to insulin secretion. A recent study has shown that sulfonylurea binds another target, Epac2 (cAMP-GEFII), which is located inside of pancreatic β-cells [5]. Epac2 activated by sulfonylurea is believed to promote insulin secretion as well as the SUR1 pathway. Epac2 is also activated by DPP-4 inhibitor administration. The DPP-4 inhibitor increases incretin levels (GLP-1, GIP), incretin then binds to the incretin receptor causing insulin secretion through cAMP signaling and Epac2 activation. Epac2 activation by both sulfonylurea and DPP-4 inhibitor causes excessive insulin release and subsequent severe hypoglycemia. These findings should lead to a potential approach to elucidate the pharmacogenomics of hypoglycemia associated with sulfonylurea and DPP-4 inhibitor.

Other approaches may benefit from the findings of genome-wide association studies. Genome-wide association studies have revealed a variety of alleles related to Type 2 diabetes, whose odds ratios are not so high (odds ratio: 1.1–1.4) [6]. Genome-wide association studies are considered to involve numerous, but miscellaneous Type 2 diabetic patients. For the purpose of identifying the genotype highly responsible for sulfonylurea-induced hypoglycemia, as Holstein *et al.* mentioned, it would be favorable to manage

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two conflicting factors: collecting more hypoglycemic cases, and appropriately excluding the hypoglycemia associated with other factors such as renal failure, acute infection and endocrine disorders amongst others.

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References

1 Holstein JD, Kovacs P, Patzer O, Stumvoll M, Holstein A. The Ser1369Ala variant of *ABCC8* and the risk for severe sulfonylurea-induced hypoglycemia in German patients with Type 2 diabetes. *Pharmacogenomics* 13(1), 5–7 (2012).

2 Sato R, Watanabe H, Genma R *et al. ABCC8* polymorphism (Ser1369Ala): influence on severe hypoglycemia due to sulfonylureas. *Pharmacogenomics* 11(12), 1743–1750 (2010).

3 Chia CW, Egan JM. Incretin-based therapies in Type 2 diabetes mellitus. *J. Clin. Endocrinol. Metab.* 93(10), 3703–3716 (2008).

4 Seino S, Zhang CL, Shibasaki T. Sulfonylurea action re-revisited. *J. Diabetes Invest.* 1(1–2), 37–39 (2010).

5 Zhang CL, Katoh M, Shibasaki T *et al.* The cAMP sensor Epac2 is a direct target of antidiabetic sulfonylurea drugs. *Science* 325(5940), 607–610 (2009).

6 Travers ME, McCarthy MI. Type 2 diabetes and obesity: genomics and the clinic. *Hum. Genet.* 130(1), 41–58 (2011).

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101 The Ministry of Health, Labour, and Welfare in Japan. Pharmaceuticals and Medical devices safety information. No. 275, December 2010 (In Japanese). www1.mhlw.go.jp/kinkyu/iyaku_j/iyaku_j/anzenseijyouhou/275.pdf

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Regular Article

Similarities and Differences between US and Japan as to Pharmacogenomic Biomarker Information in Drug Labels

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Summary: Pharmacogenomics (PGx) has been utilized as a tool to improve a drug's benefit/risk ratio and the efficiency of drug developments. In order to examine what factors are involved to determine the level of contexts (contents and descriptions) of drug-PGx biomarker information, we graded sections of Japanese package inserts and US drug labels into six levels according to the importance of cautions in regards to clinical practice and compared similarities and differences of the contexts between the two countries. Out of 54 contexts identified, 33 (61%) were graded differently between Japan and the US. The different contexts were mainly related to metabolizing enzymes used in terms of safety, therapeutic areas other than oncology, outcome before 1993, Japan-based companies having marketing authorization and no PGx data on the Japanese population. We describe the potential reasons that could lead to the differences between the two countries such as genetic differences and quantitative evidence in the Japanese population, and also discuss future perspectives to improve PGx utilization in clinical practices in Japan.

Keywords: pharmacogenomics; genomic biomarker; package insert; drug label; evidence; ethnic difference

Introduction

Various genetic factors affecting inter-individual variability in drug responses such as bioavailability, efficacy and safety of drugs have been reported,[1,2] and pharmacogenomics (PGx) has been utilized to improve a drug's benefit/risk ratio and the efficiency of drug developments.[3] As a result, the number of drug labels (called package inserts (PIs) in Japan) with PGx biomarker information has gradually increased.[4–6] Drug labels are the most fundamental document to provide necessary information on drugs to healthcare professionals. Therefore, to further promote the proper use of drugs based on the concept of personalized medicine, PGx biomarker information should be appropriately included in labels.

In this study, we compared similarities and differences as to the contexts of drug-PGx biomarker information in labels in Japan and the US to examine what factors are involved to determine the level of the contexts of the information. We also discuss future tasks to promote PGx-based medicine in clinical practices.

Methods

In this study, drugs with PGx biomarker information included in their labels were selected based on the "Table of Pharmacogenomic Biomarkers in Drug Labels" publish-

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Table 1. Each section level in the Japanese package inserts and the US labels

Level	Japan	US
1	Warnings (*KEIKOKU*)	Boxed Warning
2	Contraindications (*KINKI*)	Contraindications
3	Indication (*KONO, KOKA*), Dosage and Administration (*YOHO, YORYO*), Precautions for Indications or Dosage and Administration (*KANRENSURUSHIYOJONOCHUI*)	Indications and Usage, Dosage and Administration
4	Careful Administration (*SHINCHOTOYO*), Important Precautions (*JUYONAKIHONTEKICHUI*), Precautions for Combined Use (*HEIYOCHUI*)	Warnings and Precautions, Warnings, Precautions
5	Other Precautions (*SONOTANOCHUI*), Clinical Studies (*RINSHOSEISEKI*), Clinical Pharmacology (*YAKUBUTSUDOTAI*), Pharmacology (*YAKKOYAKURI*)	Adverse Reaction, Drug Interactions, Clinical Studies, Clinical Pharmacology
6	No statement about pharmacogenomic biomarker information	—

ed by the US Food and Drug Administration (FDA) (http://www.fda.gov/Drugs/ScienceResearch/ResearchAreas/Pharmacogenetics/ucm083378.htm, accessed 14 July, 2011). The contexts of drug-PGx biomarker information of the identified labels in the US, such as the generic name of drugs, related biomarkers, therapeutic area and relevant sections of a label, were compared to those of the corresponding drug's PIs in Japan. The information was obtained from the publicly accessible websites of the FDA (http://www.accessdata.fda.gov/scripts/cder/drugsatfda/) and the Pharmaceuticals and Medical Devices Agency (PMDA; http://www.info.pmda.go.jp/psearch/html/menu_tenpu_base.html). In addition, PGx biomarker information found in unlisted sections of US labels was also included for this study. However, the labels of US drugs which did not actually contain any PGx biomarker information were excluded from the study. Cases where selected drugs or PGx biomarker-related indications/usages, such as myelodysplastic/myeloprliferative disease with PDGFR (platelet-derived growth factor receptor) treated with imatinib, had not been approved/marketed in Japan were also excluded.

In order to examine similarities and differences as to the contexts of drug-PGx biomarker information in the labels for Japan or the US, we graded sections of the labels into six levels according to the importance of cautions in clinical practice (as shown in **Table 1**) and then classified the contexts in the labels of the selected drugs according to the grading. If more than one context is included in the labels, the highest graded level was assigned.

We also analyzed associated factors that could cause differences in the level of the contexts between Japan and the US. The chosen factors for analysis were selected based on data availability and were the biomarker type, aim of biomarker use, therapeutic area, company type, year of outcome in Japan and PGx evidence for the Japanese population mentioned in Japanese PIs. Biomarker types were categorized into: metabolizing enzyme, pharmacological target of drugs, and others. Aims of biomarker use were categorized to 2 types: efficacy (including information about pharmacological mechanism and/or efficacy reduction and/or lack of efficacy), and safety (including information about pharmacokinetics (PK) and/or dose adjustment). Therapeutic areas were defined following the classifications in the table by the FDA, *i.e.*, analgesics, antifungals, antimalarials/antiarrhythmics, antivirals, cardiovascular, dermatology-dental, gastroenterology, hematology, metabolic-endocrinology, neurology, oncology, psychiatry, reproductive, reproductive-urologic, and rheumatology. Especially for the oncology area, further improvement in treatment is likely to require introducing novel targeted therapies using biomarkers.[7,8] For analysis, therefore, the areas were grouped as oncology and others, in part to provide a sufficiently large sample size. Company types were categorized according to where the headquarters of a marketing authorization holder (MAH) are presently based (in the EU & US, or Japan). The year of outcome in Japan was categorized according to the periods before 1993, 1994–2003 and after 2004 (when the PMDA was founded). PGx evidence in Japanese PIs was categorized into 3 types according to the evidence in the Japanese population: data on clinical endpoints available (PK data may also be available in some cases), only PK data available, and no data available. For each factor, the distribution of the number of the contexts was compared between with and without grading difference using the chi-square test (SAS software system, ver 9.2; SAS Institute, Cary, NC).

Results

Basic characteristics of the context: A total of 78 contexts of drug-PGx biomarker information were identified in the "Table of Pharmacogenomic Biomarkers in Drug Labels" (see "Methods"). Three contexts (nelfinavir/CYP2C19, timolol/CYP2D6 and tiotropium/CYP2D6) were excluded because we could not find any PGx biomarker information in the US labels. Of the remaining 75 contexts, 21 drugs had not been approved/marketed in Japan. Therefore, 54 contexts were included for analysis in this study.

Table 2 summarizes the basic characteristics of the identified contexts. Of the 54 contexts, the majority (56%) were related to the "metabolizing enzyme." Safety-related biomarkers accounted for a substantial fraction (61%) compared to its counterparts. The most frequent therapeutic area was oncology (37%), followed by psychiatry (11%), cardiovascular (9%) and dermatology and dental (7%). There was little difference in the proportions with regard to the "year of outcome in Japan." The 13 (24%) contexts of Japanese PIs contained data on clinical endpoints in the Japanese population, while 6 (11%) of those only mentioned PK data. There was no data on the Japanese population available in 35 contexts (65%).

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Table 2. Summary of identified drug/biomarker-contexts

	Number of total contexts (%) (n = 54)
Biomarker type	
Metabolizing enzyme	30 (56%)
Pharmacological target	18 (33%)
Others	6 (11%)
Aim of biomarker use	
Efficacy	21 (39%)
Safety	33 (61%)
Therapeutic area	
Analgesics	3 (6%)
Antifungals	2 (4%)
Antimalarials/Antiarrhythmics	1 (2%)
Antivirals	3 (6%)
Cardiovascular	5 (9%)
Dermatology and Dental	4 (7%)
Gastroenterology	2 (4%)
Hematology	3 (6%)
Metabolic and Endocrinology	1 (2%)
Neurology	1 (2%)
Oncology	20 (37%)
Psychiatry	6 (11%)
Reproductive	1 (2%)
Reproductive and Urologic	1 (2%)
Rheumatology	1 (2%)
Year of outcome in Japan	
Before 1993	18 (33%)
1994–2003	11 (20%)
After 2004	25 (46%)
Company type	
EU&US-based company	33 (61%)
Japan-based company	21 (39%)
PGx evidence for the Japanese in Japanese PIs	
Data on clinical endpoints	13 (24%)
Pharmacokinetic data only	6 (11%)
None	35 (65%)

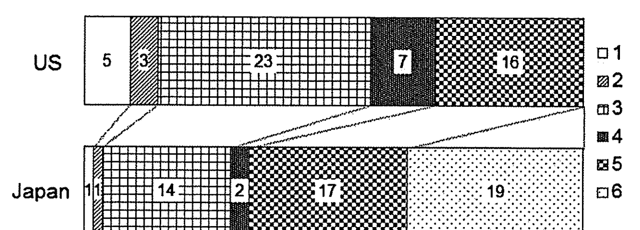

Fig. 1. A pattern of the level of contexts of drug-PGx biomarker information in the Japanese package inserts and the US labels
The contexts of drug-PGx biomarker information in labels were graded into six levels according to importance of cautions in clinical practice as shown in **Table 1**. If more than one context is included in the labels, the highest level of the grading was assigned.

Table 3. Characteristics of drug/biomarker-context without grading difference between Japan and the US ($n = 21$)

Biomarker type	Biomarker name (Number of contexts)	Grading (Number of contexts)	Aim of biomarker use
Metabolizing enzyme (6)	CYP2D6 (1)	Level 3 (1)	Safety
	CYP2C19 (3)	Level 5 (3)	Safety
	G6PD (2)	Level 1 (1)	Safety
		Level 4 (1)	Safety
Pharmacological target (12)	Targets of anticancer drugs* (12)	Level 3 (10)	Efficacy
		Level 5 (2)	Efficacy
Others (3)	CCR 5 (1)	Level 3 (1)	Efficacy
	LDL receptor (1)	Level 3 (1)	Efficacy
	NAGS; CPS; ASS; OTC; ASL; ARG (1)	Level 2 (1)	Safety

*C-Kit, EGFR, Her2/neu, *KRAS*, Philadelphia chromosome, *PML/RARα* translocation.

Grade classification and characteristics of contexts with/without grading difference: Figure 1 shows the graded levels of the contexts in Japan and the US (see **Table 1** for the grading details). In the US, 8 (15%) contexts were graded level 1 or 2, which urged special attention for treatment in clinical practice, while there were only two (4%) similarly graded contexts in Japan. Nineteen (35%) contexts were not mentioned (level 6) in Japan.

Characteristics of drug/biomarker-contexts are separately described in **Tables 3** and **4** according to the presence or absence of grading difference between Japan and the US. **Table 3** aims to describe the characteristics of drug/biomarker-context without grading difference. There was no difference between Japan and the US in the level of 21 (39%) contexts. Most of them were graded level 3 and related to pharmacological targets of anticancer drugs (*e.g.*, C-Kit, epidermal growth factor receptor (EGFR), Her2/neu, *KRAS*, Philadelphia chromosome, *PML/RARα* translocation). The aim of using these biomarkers was mainly for efficacy such as an adequate patient selection in regards to drug administration. Other types of biomarkers of which contexts were graded with a high caution level above level 4 (levels 1, 2, and 3) were mainly for safety, especially risk minimization (glucose-6 phosphate dehydrogenase (G6PD), N-acetylglutamate synthetase (NAGS); carbamyl phosphate synthetase (CPS); argininosuccinate synthetase (ASS); ornithine transcarbamylase (OTC); argininosuccinatelyase (ASL); arginase (ARG), CYP2D6).

Table 4 shows the characteristics of drug/biomarker-context with grading difference between Japan and the US.

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Table 4. Characteristics of drug/biomarker-context with grading difference between Japan and the US ($n = 33$)

Biomarker type	Biomarker name (Number of contexts)	JP grading/US grading (Number of contexts)	Aim of biomarker use
Metabolizing enzyme (24)	CYP2D6 (13)	Level 6/ Level 5 (7)	Safety
		Level 6/ Level 4 (4)	Safety
		Level 6/ Level 3 (1)	Safety
		Level 5/ Level 4 (1)	Safety
	CYP2C9 (2)	Level 5/ Level 3 (2)	Safety
	CYP2C19 (3)	Level 6/ Level 5 (1)	Safety
		Level 6/ Level 4 (1)	Safety
		Level 5/ Level 1 (1)	Efficacy
	DPD (2)	Level 5/ Level 2 (2)	Safety
	TPMT (2)	Level 6/ Level 3 (1)	Safety
		Level 5/ Level 3 (1)	Safety
	UGT1A1 (2)	Level 6/ Level 5 (1)	Safety
		Level 4/ Level 3 (1)	Safety
Pharmacological target (6)	Targets of anticancer drugs* (5)	Level 6/ Level 5 (1)	Efficacy
		Level 5/ Level 3 (3)	Efficacy
		Level 3/ Level 1 (1)	Efficacy
	VKORC1 (1)	Level 6/ Level 3 (1)	Safety
Others (3)	*IL-28b* (1)	Level 6/ Level 5 (1)	Efficacy
	*HLA-B*5701* (1)	Level 5/ Level 1 (1)	Safety
	*HLA-B*1502* (1)	Level 5/ Level 1 (1)	Safety

*Chromosome 5q, EGFR, estrogen receptor, Philadelphia chromosome, *PML/RARα* translocation.

33 contexts were graded differently between Japan and the US, as shown in the table. All these contexts in the US were graded to a higher caution level than in Japan. The most common ($n = 24$) biomarker type was the "metabolizing enzyme" with the likes of CYP, dihydropyrimidine dehydrogenase (DPD), thiopurine methyltransferase (TPMT) and UDP-glucuronosyltransferase (UGT). Out of 18 contexts related to *CYP* mutations, 14 were not mentioned in the Japanese PIs (level 6). The other 4 contexts were only mentioned as a reference without any recommendation or requirement in Japan (level 5), while some of those were described more with caution in the US, such as elevation of the drug concentration and recommended dose adjustment according to the CYP genotype (*i.e.*, celecoxib/CYP2C9 and warfarin/CYP2C9). Marked differences of the levels between Japan and the US were shown in regard to five contexts (clopidogrel/CYP2C19, capecitabine/DPD, fluorouracil/DPD, abacavir/*HLA-B*5701*, and carbamazepine/*HLA-B*1502*). They were graded level 1 (Boxed Warnings) or 2 (Contraindications) in the US, while they were considered level 5 (basically reference information) in Japan.

Table 5. Comparison of drug/biomarker-contexts with ($n = 33$) and without ($n = 21$) grading differences between Japan and the US

	Number of contexts with grading difference (%)	Number of contexts without grading difference (%)	p value
Biomarker type			0.0048
ME[a] ($n = 30$)	24 (80%)	6 (20%)	
PT[b] ($n = 18$)	6 (33%)	12 (67%)	
Others ($n = 6$)	3 (50%)	3 (50%)	
Aim of biomarker use			0.0008
Efficacy ($n = 21$)	7 (33%)	14 (67%)	
Safety ($n = 33$)	26 (79%)	7 (21%)	
Therapeutic area			0.0025
Oncology ($n = 20$)	7 (35%)	13 (65%)	
Others ($n = 34$)	26 (76%)	8 (24%)	
Year of outcome in Japan			0.0104
Before 1993 ($n = 18$)	16 (89%)	2 (11%)	
1994–2003 ($n = 11$)	6 (55%)	5 (45%)	
After 2004 ($n = 25$)	11 (44%)	14 (56%)	
Company type			0.21
EU & US-based company ($n = 33$)	18 (55%)	15 (45%)	
Japan-based company ($n = 21$)	15 (71%)	6 (29%)	
PGx evidence for the Japanese in Japanese PIs			0.0002
Data on clinical endpoints ($n = 13$)	2[d] (15%)	11[e] (85%)	
PK[c] data only ($n = 6$)	3 (50%)	3 (50%)	
None ($n = 35$)	28 (80%)	7 (20%)	

[a]ME: Metabolizing enzyme.
[b]PT: Pharmacological target.
[c]PK: Pharmacokinetic.
[d]Of these, 1 context (panitumumab/EGFR) was related to drug efficacy.
[e]Of these, 10 contexts were related to drug efficacy.

Comparison of contexts with/without grading differences between Japan and the US: Table 5 shows the results of statistical comparisons of the contexts for Japan or the US, with or without grading differences. The different contexts were more likely to be related to metabolizing enzymes, safety, therapeutic areas other than oncology, outcome before 1993, Japan-based companies as MAH, and no data available as PGx clinical evidence for the Japanese. Of 24 biomarkers graded differently and related to metabolizing enzymes, all except one (clopidogrel/CYP2C19) were used in terms of safety. Nearly half ($n = 15$) of contexts relating to the EU & US-based companies as MAH showed no grading difference in contrast to Japan-based companies. Of 14 contexts which came out after 2004 and were graded as identical between the 2 countries, 10 of these were related to the area of oncology. Data on clinical endpoints were available for 11 contexts in Japanese PIs without grading differences, and 10 of these were related to efficacy as the "Aim of biomarker use."

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Discussion

We investigated similarities and differences in regard to the level of the contexts of drug-PGx biomarker information in the labels both in Japan and the US. Moreover, factors associated with the differences were examined. The level of the contexts related to efficacy as the "aim of biomarker use" was mostly identical between the two countries. This would result from the fact that more data on clinical endpoints, specifically for efficacy than for safety, were available in the Japanese population as shown in **Table 5** because efficacy data are critical for regulatory approval and are usually collected from both the Japanese and the US populations in clinical trials before regulatory submission of a new drug application (NDA). Thus, it is indicated that one of the most important factors to determine if the level of the context in the Japanese PIs is identical to the US labels is evidence which is related to clinical endpoints from the Japanese population. This could also explain the fact that the level of the contexts in the area of oncology showed fewer differences because many of the oncology drugs investigated in this study act on target molecules related to drug efficacy, such as HER2/neu and EGFR.

On the other hand, our study also demonstrates a remarkable difference in the level of the contexts between Japan and the US. These differences could be partly due to the differences in the standard format of labels between Japan and the US because the standard volume and length are more limited in the Japanese PIs than those in the US labels, usually resulting in more selected and more limited PGx biomarker information in the Japanese PIs. Other potential reasons to lead these differences in the level of the contexts between the two countries are discussed below.

Genetic differences: One of the reasons for the differences in the level of the contexts is likely related to genetic differences with biomarkers such as different allele frequencies. For example, among "metabolizing enzyme," which was the most popular biomarker type, the difference was prominent in cases with CYP2D6, which is well known for showing polymorphic characteristics.[9] It has been reported that a prevalence of poor metabolizer (PM) allele for *CYP2D6* is approximately 7% in the Caucasian population but very low (<1%) in the Japanese population.[10,11] This different allele frequency may have an impact on PGx biomarker information in different clinical practices in each country and may contribute to resulting in the higher level in the US and the lower level in Japan. Similarly, the very low frequency of the DPD deficiency in the Japanese population may also contribute to the difference.[12,13]

As another example, the *HLA-B*5701* allele has been reported as a useful marker to reduce abacavir-induced hypersensitivity reactions in a Caucasian population.[14] The prevalence of *HLA-B*5701* in Japanese, however, is very low (0.1%), compared with that in Caucasians (5 to 8%).[15,16] The *HLA-B*1502* allele, as a useful marker to reduce the risk of carbamazepine-induced Stevens-Johnson syndrome (SJS)/Toxic Epidermal Necrolysis (TEN), is also known to have ethnic differences in regard to its frequency of occurrence between Han-Chinese (8.6%)[17] and the Japanese population (<0.1%).[18] In these two cases, the low allele frequency in the Japanese population may result in the different level of the contexts in the labels between Japan and the US. Furthermore, in the case of carbamazepine-induced SJS/TEN, a recent genome-wide association study in the Japanese population shows that the risk is associated with a different allele, *HLA-A*3101*, but not with *HLA-B*1502*[18] suggesting that *HLA-A*3101* may be more clinically important than *HLA-B*1502* in the Japanese population. In September 2011, the PI was amended to include this new scientific data on the Japanese population.

Quantitative evidence in Japanese population: Some of the observed differences cannot be illustrated by genetic differences. For example, patients with a variant CYP2C9 or vitamin K epoxide reductase complex subunit 1 (VKORC1) alleles are associated with lower warfarin doses,[19,20] and exist in considerable numbers in both the US and Japan.[21,22] Nevertheless, the starting dose adjustment of warfarin according to a patient's CYP2C9 and VKORC1 genotypes is only recommended in the US label. The case of CYP2C19 is another example; the prevalence of CYP2C19 PM is higher in Japanese (18 to 23%)[23] than in Caucasians (2 to 5%),[11] but some US drug labels (clopidpgrel, diazepam, drospirenone and ethinyl estradiol) include more detailed information about *CYP2C19* polymorphism than the corresponding Japanese PIs. We considered that one of the common factors to cause the difference mentioned above could be a lack of evidence in the Japanese population. As for warfarin and CYP2C9/VKORC1, though some studies showed an association between the polymorphisms and warfarin maintenance dose,[24–26] more evidence, specially an association of testing *CYP2C9* and *VKORC1* polymorphisms with clinical efficacy/safety, will be necessary to recommend the genetic test for selecting appropriate dosage in the Japanese PIs. Similarly, a recommended dose of clopidogrel or alternative drugs to CYP2C19 PM for Japanese patients are still not well established. As mentioned in previous studies in the US,[5,27] there is a gap between published data and evidence such as the clinical relevance of genetic association required to change the contexts in drug labels.

As we described above, the genetic difference and the quantitative evidence would be major factors to determine the level of the contexts of drug-PGx biomarker information in the label. A different level of the context for UGT1A1 between Japan and the US could be a case where both of these factors were associated. *UGT1A1*28* is a popular allele among populations and has been reported to be associated with a risk of irinnotecan-induced neutropenia.[28–31] In addition, the *UGT1A1*6* allele also has been suggested to be a risk factor in the Japanese population,[32,33] though the allele

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has only been detected in Asian populations and not in the US population.[34] Therefore, the Japanese PIs mentioned the *6 allele as well as the *28 allele. In this case, since data from a prospective study to show the higher concentration of SN38 (active substance) and higher frequency of neutropenia in the Japanese subjects homozygous for *6 or *28 or double heterozygous (*6/*28) allele were available,[31] a specific description to warn of the high possibility of a serious adverse event (*e.g.*, neutropenia) in such patients is included in the Japanese PIs.

Others: Some other factors could also be involved to cause a difference in regard to the levels of the contexts. One such factor may be the availability of diagnostic tools for genetic biomarkers. For example, the specific description of UGT1A1 in the Japanese PIs described above was added around the time of the approval of the UGT1A1 diagnostic tool (Invader Assay) in 2008. This suggests that the availability of a diagnostic tool for genetic biomarkers has an impact on the context of drug-PGx biomarker information in labels.

It should also be mentioned that the "year of outcome in Japan," as shown in **Table 5**, may be related to the level of the contexts of drug-PGx biomarker information in the label because the difference of the context was more common for drugs approved before 1993. This suggests that data for PGx biomarker information in the Japanese population for old drugs is more limited. It may result from a low incentive for the pharmaceutical industry to accumulate data on unpatented drugs and less communication focusing on old drugs between the PMDA and the industry. In addition, the higher percentage of the difference in Japan-based companies as a company type shown in **Table 5** could also be related to the year of outcome in Japan because numbers for the outcome before 1993 for Japan-based companies were higher in the context with the differences (67%: 10/15) than that without the differences (33%: 2/6).

A difference in the timings of regulatory reviews or data packages may also contribute to the levels of the contexts. For example, although the US label for panitumumab states that detection of the EGFR protein expression is necessary for selecting appropriate patients for the drug (level 3), that is not a requirement in the Japanese PIs (level 5); it may be related to the fact that the NDA review of this drug was completed in 2006 by the FDA and in 2010 by the PMDA, and the clinical data package for Japan included 2 new clinical trials which were not originally reviewed by the FDA in 2006.[35,36] In these trials, no significant difference in response rate depending on the state of EGFR expression was observed. Hence, the PMDA concluded that not enough evidence had been elucidated to utilize the diagnostic of EGFR expression as a criterion for patient selection at that time.

The reasons for all cases are not entirely clear; however, complex factors of the reasons mentioned above could contribute to understanding such differences, even in part. It should be noted here that although we selected drug-biomarker contexts based on the list by the FDA in this study, there are several contexts available only in Japanese PIs but not listed in the table by the FDA even if the drug is approved in both countries. Further studies are needed to examine similarities/differences in these cases, but our preliminary study indicates that the results and discussion of this study might not be highly affected.

Future Perspective: In order to improve PGx utilization in clinical practices in Japan, we still have agendas to be taken into consideration. First, an increase in the number of biomarkers qualified by the PMDA is critical. The ICH E16 guideline: "Biomarkers related to drug or biotechnology product development: context, structure and format of qualification submissions" implemented in January 2011 in Japan[37] will facilitate data submission for PGx biomarker information to the PMDA. Especially during developments of a new drug, it is important from an early stage to share the concepts of a biomarker among the pharmaceutical industry, academia and the regulatory agency.

Secondly, it is necessary to collect PGx biomarker data on clinical endpoints as well as PK data on the Japanese population, to provide healthcare professionals with appropriate information in PIs. Therefore, examining the relationships between biomarkers and clinical endpoints during clinical trials is encouraged. In an era with the globalization of drug developments, it would be more practical to accumulate data on clinical endpoints with PGx information in global clinical trials to evaluate similarities and difference of such data among populations. For that purpose, it is necessary to collect enough samples in clinical trials in an internationally harmonized way for genetic analysis, but there are still a wide range of variables and requirements of sample collection in each country.[38,39] To promote sample collection and the accumulation of PGx data efficiently during drug developments, efforts to improve public understanding and perceptions about PGx such as the need to perform a genetic test during clinical trials at a global level are indispensable.

Thirdly, a reliable and convenient diagnostic tool for genetic biomarkers (*i.e.*, companion diagnostics devices) should be co-developed with a drug to practically facilitate the clinical use of biomarkers. Basic research regarding biomarkers and their diagnostic tools should also be conducted taking into account the probable consequences and actual situations in clinical practices. These approaches will facilitate regulatory approval of highly reliable diagnostic tools under the Pharmaceutical Affair Law in Japan. In addition, developing economical genetic tests in terms of cost-effectiveness is also important point for PGx utilization in clinical practices, although this study does not focus on the cost issues.

Finally, in order to accomplish the above-described tasks, all stakeholders including academia, pharmaceutical

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industries and regulatory agencies, should work together. Although the PMDA, the FDA and the European Medicines Agency (EMA) have been conducting qualification meetings for scientific consultation to promote global use of PGx biomarkers,[40–42] further international cooperation/collaborations not only among regulatory agencies but also among ethics committees, clinical trial sites, device industries of genetic tests as well as pharmaceutical industries would assist in promoting biomarker qualification, accumulating PGx information in clinical practices and developing reliable economical genetic tests. These efforts could also be linked to a foundation of scientific consortia among the stakeholders to efficiently provide PGx data.

Through the multiple efforts discussed here, the PGx biomarker information in labels will be improved. Continuous and timely updates of PGx biomarker information in labels are important to promote the proper use of drugs and to realize PGx-based medicine in clinical practices.

References

1) Evans, W. E. and Relling, M. V.: Pharmacogenomics: translating functional genomics into rational therapeutics. *Science*, **286**: 487–491 (1999).
2) Meyer, U. A.: Pharmacogenetics and adverse drug reactions. *Lancet*, **356**: 1667–1671 (2000).
3) Evans, W. E. and Relling, M. V.: Moving towards individualized medicine with pharmacogenomics. *Nature*, **429**: 464–468 (2004).
4) Ishiguro, A., Toyoshima, S. and Uyama, Y.: Current Japanese regulatory situations of pharmacogenomics in drug administration. *Expert Rev. Clin. Pharmacol.*, **1**: 505–514 (2008).
5) Frueh, F. W., Amur, S., Mummaneni, P., Epstein, R. S., Aubert, R. E., DeLuca, T. M., Verbrugge, R. R., Burckart, G. J. and Lesko, L. J.: Pharmacogenomic biomarker information in drug labels approved by the United States food and drug administration: prevalence of related drug use. *Pharmacotherapy*, **28**: 992–998 (2008).
6) Hong, H., Goodsaid, F., Shi, L. and Tong, W.: Molecular biomarkers: a US FDA effort. *Biomark Med.*, **4**: 215–225 (2010).
7) Sawyers, C. L.: The cancer biomarker problem. *Nature*, **452**: 548–552 (2008).
8) Krause, D. S. and Van Etten, R. A.: Tyrosine kinases as targets for cancer therapy. *N. Engl. J. Med.*, **353**: 172–187 (2005).
9) Zhou, S. F.: Polymorphism of human cytochrome P450 2D6 and its clinical significance: Part I. *Clin. Pharmacokinet.*, **48**: 689–723 (2009).
10) Nishida, Y., Fukuda, T., Yamamoto, I. and Azuma, J.: CYP2D6 genotypes in a Japanese population: low frequencies of CYP2D6 gene duplication but high frequency of CYP2D6*10. *Pharmacogenetics*, **10**: 567–570 (2000).
11) Ozawa, S., Soyama, A., Saeki, M., Fukushima-Uesaka, H., Itoda, M., Koyano, S., Sai, K., Ohno, Y., Saito, Y. and Sawada, J.: Ethnic differences in genetic polymorphisms of CYP2D6, CYP2C19, CYP3As and MDR1/ABCB1. *Drug Metab. Pharmacokinet.*, **19**: 83–95 (2004).
12) Hayashi, K., Kidouchi, K., Sumi, S., Mizokami, M., Orito, E., Kumada, K., Ueda, R. and Wada, Y.: Possible prediction of adverse reactions to pyrimidine chemotherapy from urinary pyrimidine levels and a case of asymptomatic adult dihydropyrimidinuria. *Clin. Cancer Res.*, **2**: 1937–1941 (1996).
13) Sumi, S., Imaeda, M., Kidouchi, K., Ohba, S., Hamajima, N., Kodama, K., Togari, H. and Wada, Y.: Population and family studies of dihydropyrimidinuria: prevalence, inheritance mode, and risk of fluorouracil toxicity. *Am. J. Med. Genet.*, **78**: 336–340 (1998).
14) Mallal, S., Phillips, E., Carosi, G., Molina, J. M., Workman, C., Tomazic, J., Jagel-Guedes, E., Rugina, S., Kozyrev, O., Cid, J. F., Hay, P., Nolan, D., Hughes, S., Hughes, A., Ryan, S., Fitch, N., Thorborn, D. and Benbow, A.: HLA-B*5701 screening for hypersensitivity to abacavir. *N. Engl. J. Med.*, **358**: 568–579 (2008).
15) Tanaka, H., Akaza, T. and Juji, T.: Report of the Japanese Central Bone Marrow Data Center. *Clin. Transpl.*, 139–144 (1996).
16) Nolan, D., Gaudieri, S. and Mallal, S.: Pharmacogenetics: a practical role in predicting antiretroviral drug toxicity? *J. HIV Ther.*, **8**: 36–41 (2003).
17) Chung, W. H., Hung, S. I., Hong, H. S., Hsih, M. S., Yang, L. C., Ho, H. C., Wu, J. Y. and Chen, Y. T.: Medical genetics: a marker for Stevens-Johnson syndrome. *Nature*, **428**: 486 (2004).
18) Ozeki, T., Mushiroda, T., Yowang, A., Takahashi, A., Kubo, M., Shirakata, Y., Ikezawa, Z., Iijima, M., Shiohara, T., Hashimoto, K., Kamatani, N. and Nakamura, Y.: Genome-wide association study identifies HLA-A*3101 allele as a genetic risk factor for carbamazepine-induced cutaneous adverse drug reactions in Japanese population. *Hum. Mol. Genet.*, **20**: 1034–1041 (2011).
19) Sanderson, S., Emery, J. and Higgins, J.: CYP2C9 gene variants, drug dose, and bleeding risk in warfarin-treated patients: a HuGEnet systematic review and meta-analysis. *Genet. Med.*, **7**: 97–104 (2005).
20) Wadelius, M., Chen, L. Y., Downes, K., Ghori, J., Hunt, S., Eriksson, N., Wallerman, O., Melhus, H., Wadelius, C., Bentley, D. and Deloukas, P.: Common VKORC1 and GGCX polymorphisms associated with warfarin dose. *Pharmacogenomics J.*, **5**: 262–270 (2005).
21) Takahashi, H., Wilkinson, G. R., Nutescu, E. A., Morita, T., Ritchie, M. D., Scordo, M. G., Pengo, V., Barban, M., Padrini, R., Ieiri, I., Otsubo, K., Kashima, T., Kimura, S., Kijima, S. and Echizen, H.: Different contributions of polymorphisms in VKORC1 and CYP2C9 to intra- and inter-population differences in maintenance dose of warfarin in Japanese, Caucasians and African-Americans. *Pharmacogenet. Genomics*, **16**: 101–110 (2006).
22) Nasu, K., Kubota, T. and Ishizaki, T.: Genetic analysis of CYP2C9 polymorphism in a Japanese population. *Pharmacogenetics*, **7**: 405–409 (1997).
23) Furuta, T., Sugimoto, M., Shirai, N. and Ishizaki, T.: CYP2C19 pharmacogenomics associated with therapy of Helicobacter pylori infection and gastro-esophageal reflux diseases with a proton pump inhibitor. *Pharmacogenomics*, **8**: 1199–1210 (2007).
24) Mushiroda, T., Ohnishi, Y., Saito, S., Takahashi, A., Kikuchi, Y., Shimomura, H., Wanibuchi, Y., Suzuki, T., Kamatani, N. and Nakamura, Y.: Association of VKORC1 and CYP2C9 polymorphisms with warfarin dose requirements in Japanese patients. *J. Hum. Genet.*, **51**: 249–253 (2006).
25) Obayashi, K., Nakamura, K., Kawana, J., Ogata, H., Hanada, K., Kurabayashi, M., Hasegawa, A., Yamamoto, K. and Horiuchi, R.: VKORC1 gene variations are the major contributors of variation in warfarin dose in Japanese patients. *Clin. Pharmacol. Ther.*, **80**: 169–178 (2006).
26) Ohno, M., Yamamoto, A., Ono, A., Miura, G., Funamoto, M., Takemoto, Y., Otsu, K., Kouno, Y., Tanabe, T., Masunaga, Y., Nonen, S., Fujio, Y. and Azuma, J.: Influence of clinical and genetic factors on warfarin dose requirements among Japanese patients. *Eur. J. Clin. Pharmacol.*, **65**: 1097–1103 (2009).
27) Zineh, I., Pebanco, G. D., Aquilante, C. L., Gerhard, T., Beitelshees, A. L., Beasley, B. N. and Hartzema, A. G.: Discordance between availability of pharmacogenetics studies and pharmacogenetics-based prescribing information for the top 200 drugs. *Ann. Pharmacother.*, **40**: 639–644 (2006).
28) Ando, Y., Saka, H., Ando, M., Sawa, T., Muro, K., Ueoka, H.,

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Yokoyama, A., Saitoh, S., Shimokata, K. and Hasegawa, Y.: Polymorphisms of UDP-glucuronosyltransferase gene and irinotecan toxicity: a pharmacogenetic analysis. *Cancer Res.*, **60**: 6921–6926 (2000).

29) Iyer, L., Das, S., Janisch, L., Wen, M., Ramirez, J., Karrison, T., Fleming, G. F., Vokes, E. E., Schilsky, R. L. and Ratain, M. J.: UGT1A1*28 polymorphism as a determinant of irinotecan disposition and toxicity. *Pharmacogenomics J.*, **2**: 43–47 (2002).

30) Innocenti, F., Undevia, S. D., Iyer, L., Chen, P. X., Das, S., Kocherginsky, M., Karrison, T., Janisch, L., Ramirez, J., Rudin, C. M., Vokes, E. E. and Ratain, M. J.: Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. *J. Clin. Oncol.*, **22**: 1382–1388 (2004).

31) Minami, H., Sai, K., Saeki, M., Saito, Y., Ozawa, S., Suzuki, K., Kaniwa, N., Sawada, J., Hamaguchi, T., Yamamoto, N., Shirao, K., Yamada, Y., Ohmatsu, H., Kubota, K., Yoshida, T., Ohtsu, A. and Saijo, N.: Irinotecan pharmacokinetics/pharmacodynamics and UGT1A genetic polymorphisms in Japanese: roles of UGT1A1*6 and *28. *Pharmacogenet. Genomics*, **17**: 497–504 (2007).

32) Sai, K., Saeki, M., Saito, Y., Ozawa, S., Katori, N., Jinno, H., Hasegawa, R., Kaniwa, N., Sawada, J., Komamura, K., Ueno, K., Kamakura, S., Kitakaze, M., Kitamura, Y., Kamatani, N., Minami, H., Ohtsu, A., Shirao, K., Yoshida, T. and Saijo, N.: UGT1A1 haplotypes associated with reduced glucuronidation and increased serum bilirubin in irinotecan-administered Japanese patients with cancer. *Clin. Pharmacol. Ther.*, **75**: 501–515 (2004).

33) Onoue, M., Terada, T., Kobayashi, M., Katsura, T., Matsumoto, S., Yanagihara, K., Nishimura, T., Kanai, M., Teramukai, S., Shimizu, A., Fukushima, M. and Inui, K.: UGT1A1*6 polymorphism is most predictive of severe neutropenia induced by irinotecan in Japanese cancer patients. *Int. J. Clin. Oncol.*, **14**: 136–142 (2009).

34) Saito, Y., Maekawa, K., Ozawa, S. and Sawada, J.: Genetic Polymorphisms and Haplotypes of Major Drug Metabolizing Enzymes in East Asians and Their Comparison with Other Ethnic Populations. *Curr Pharmacogenomics*, **5**: 49–78 (2007).

35) PMDA: Panitumumab review report. http://www.info.pmda.go.jp/shinyaku/P201000024/400256000_22200AMX00307_A100_2.pdf (April 2010).

36) FDA: Panitumumab Medical Review. http://www.accessdata.fda.gov/drugsatfda_docs/nda/2006/125147s0000_MedR.pdf (Sep 2006).

37) MHLW: [Biomarkers Related to Drug or Biotechnology Product Development: Context, Structure and Format of Qualification Submissions] (Japanese) (PFSB/ELD Notification No. 0120-1/PFSB/SD Notification No. 0120-1). http://www.pmda.go.jp/ich/e/e16_11_1_20.pdf (January 20, 2011).

38) Ricci, D. S., Broderick, E. D., Tchelet, A., Hong, F., Mayevsky, S., Mohr, D. M., Schaffer, M. E., Warner, A. W., Hakkulinen, P. and Snapir, A.: Global requirements for DNA sample collections: results of a survey of 204 ethics committees in 40 countries. *Clin. Pharmacol. Ther.*, **89**: 554–561 (2011).

39) Warner, A. W., Bhathena, A., Gilardi, S., Mohr, D., Leong, D., Bienfait, K. L., Sarang, J., Duprey, S., Franc, M. A., Nelsen, A. and Snapir, A.: Challenges in obtaining adequate genetic sample sets in clinical trials: the perspective of the industry pharmacogenomics working group. *Clin. Pharmacol. Ther.*, **89**: 529–536 (2011).

40) PMDA: [Consultation on Pharmacogenomics/Biomarkers] (Japanese). http://www.pmda.go.jp/operations/shonin/info/consult/m03_pharma.html

41) EMA: Qualification of novel methodologies for drug development: guidance to applicants (EMEA/CHMP/SAWP/72894/2008). http://www.ema.europa.eu/docs/en_GB/document_library/Regulatory_and_procedural_guideline/2009/10/WC500004201.pdf (January 22, 2009).

42) FDA: Guidance for Industry: Qualification Process for Drug Development Tools. http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM230597.pdf (October 2010).

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