Methods

Instrumentation

We have previously reported the details of the instrumentation procedure.[13] In brief, hybrid dogs (HBD) mated with the beagle, American fox hound, and Labrador retriever for laboratory use (weighing 15 to 21 kg; Kitayama Labes, Gifu, Japan) were anesthetized by an intravenous injection of sodium pentobarbital (30 mg/kg), intubated, and ventilated with room air mixed with oxygen (100% O_2 at flow rate of 1.0 to 1.5 L/min). The arterial blood pH, Po_2, and Pco_2 before the protocol was begun were 7.38±0.02, 104±3 mm Hg, and 38.7±1.6 mm Hg, respectively. End-diastolic length (EDL) was determined at the R wave on the ECG, and end-systolic length (ESL) was determined at the minimum pressure differential. Then, fractional shortening (FS) was calculated as [(EDL−ESL)/EDL]×100%. Agents were administered into the left anterior descending coronary artery (LAD) via the bypass tube. To constitute the coronary bypass between the carotid artery and the LAD, <30 seconds interruption of the LAD was necessary, but this brief period of ischemia does not provoke either myocardial injury or protection. This study conformed to the *Position of the American Heart Association on Research Animal Use* adopted by the Association in November 1984.

Experimental Protocols

Protocol 1: Effects of Carvedilol on Adenosine Release and CBF in Nonischemic Myocardium

After hemodynamics became stable, coronary arterial and venous blood samples were obtained for the measurement of adenosine concentrations,[11] and the difference between the adenosine levels in coronary arterial and venous blood [VAD(Ado)] was then calculated.

Five HBD dogs were used in protocol 1. Hemodynamic parameters (ie, systolic and diastolic aortic blood pressure and heart rate) were monitored. Carvedilol was infused at 1.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ at a concentration of 0.09 mg/mL) for 10 minutes, and then coronary perfusion pressure (CPP), CBF, FS, and VAD(Ado) were measured. Carvedilol was dissolved in a small volume of DMSO (final concentration, <0.15%). In a preliminary study, this dose of carvedilol was shown to be the minimum dose that caused maximal coronary vasodilation in ischemic or nonischemic hearts. We also confirmed that this volume of DMSO did not change either coronary hemodynamics or VAD(Ado) in ischemic or nonischemic hearts.

Protocol 2: Effects of Carvedilol or Propranolol on Adenosine Release and CBF in Ischemic Hearts

After hemodynamics became stable, coronary arterial and venous blood samples were obtained for blood gas analysis and for measurement of adenosine[11,13] and lactate[14] levels. Lactate extraction ratio (LER) was calculated as the coronary arteriovenous difference of the lactate concentration multiplied by 100 and divided by the arterial lactate concentration.

Twenty HBD dogs were used in protocol 2. Hemodynamic parameters were monitored. To examine whether administration of carvedilol caused coronary vasodilation and reduced the severity of myocardial ischemia and whether adenosine-dependent mechanisms are involved in these actions, saline (n=5), α,β-methyleneadenosine diphosphate (AMP-CP) at 80 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ at a concentration of 4.8 mg/mL, n=5), or 8-sulfophenyltheophylline (8-SPT) at 30 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ at a concentration of 1.8 mg/mL, n=5) was infused into the bypass tube. AMP-CP is an inhibitor of ecto-5′-nucleotidase, whereas 8-SPT is a nonspecific adenosine receptor antagonist. Both agents were dissolved in saline before administration. After confirming that systemic and coronary hemodynamics were unchanged for 5 minutes after each drug infusion, CPP was reduced so that CBF decreased to 50% of the baseline level for 5 minutes. Then, infusion of carvedilol was started at 1.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ at a concentration of 0.09 mg/mL) and was continued for 10 minutes, while CPP was maintained at the reduced level. A preliminary study showed that the above-mentioned dose of 8-SPT was the minimum dose that prevented coronary vasodilation induced by adenosine at 2 $\mu g \cdot kg^{-1} \cdot min^{-1}$, whereas the dose of carvedilol (1.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$) was the minimum level that caused maximal coronary vasodilation.

In addition, propranolol was infused at 30 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ at a concentration of 1.8 mg/mL) to investigate whether it had effects identical to those of carvedilol (n=5). This dose of propranolol corresponds to 15 μg/mL, and the effective dose of propranolol is >10 μg/mL, indicating that the dose of propranolol in the present study is sufficient to antagonize β-receptors of the hearts.

Protocol 3: Influence of the Antioxidant Activity of Carvedilol on CBF

To examine whether carvedilol eliminates oxidative stress and causes adenosine-dependent coronary vasodilation in ischemic hearts, either saline (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ at a concentration of 1.5 mg/mL, n=5) or human recombinant superoxide dismutase (SOD) (5340 IU/mg, >99% purity) at 25 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ at a concentration of 1.5 mg/mL, n=5) was infused into the bypass tube. CPP was then reduced so that CBF decreased to 50% of the baseline level for 5 minutes. Subsequently, infusion of carvedilol at 1.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ at a concentration of 0.09 mg/mL) was initiated and continued for 10 minutes, while CPP was maintained at the reduced value. As a marker of oxidative stress, the 8-iso-prostaglandin $F_{2\alpha}$ level was measured in coronary arterial and venous blood, and the arteriovenous difference of 8-iso-prostaglandin $F_{2\alpha}$ [VAD(8-Iso-$F_{2\alpha}$)] was calculated. We confirmed that this dose of SOD had no effect on either systemic or coronary hemodynamic parameters.[11]

Protocol 4: Effects of Carvedilol on Infarct Size After 90 Minutes of Ischemia

In HBD dogs, the bypass tube to the LAD was occluded for 90 minutes, followed by reperfusion for 6 hours, together with administration of either saline (n=7, control) or DMSO (0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$, n=5) from 10 minutes before occlusion until 1 hour of reperfusion, except at the time of coronary occlusion. Hemodynamic parameters were monitored during myocardial ischemia and after the start of reperfusion. In the carvedilol group (n=5), carvedilol at 1.5 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (an infusion rate of 0.0167 mL $\cdot kg^{-1} \cdot min^{-1}$ at a concentration of 0.09 mg/mL) was infused from 10 minutes before coronary occlusion until 60 minutes after the start of reperfusion, except during occlusion. In the carvedilol+8-SPT group (n=6) and the carvedilol+AMP-CP group (n=6), the effect of carvedilol was tested during concomitant administration of either 8-SPT at 30 $\mu g \cdot kg^{-1} \cdot min^{-1}$ or AMP-CP at 80 $\mu g \cdot kg^{-1} \cdot min^{-1}$. In the 8-SPT group (n=6) and the AMP-CP group (n=7), 90 minutes of ischemia and 6 hours of reperfusion were performed during treatments with 8-SPT and AMP-CP, respectively. Either 8-SPT or AMP-CP was infused from 10 minutes before coronary occlusion until 60 minutes after the start of reperfusion, except during occlusion. In all groups, infarct size was assessed after 6 hours of reperfusion.

Protocol 5: Effects of Carvedilol on 5′-Nucleotidase Activity

In human umbilical vein endothelial cells (HUVECs) cultured with or without xanthine (1×10^{-4} mol/L) and xanthine oxidase (1.6×10^{-3} U/mL), 5′-nucleotidase activity was measured by an enzyme assay after exposure to carvedilol (0, 1×10^{-8} to 1×10^{-5} mol/L) for 15 minutes.[15]

Analyses

The methods of measuring plasma adenosine levels,[11] myocardial ecto-5′-nucleotidase activity,[11,16] and plasma lactate levels[13] have been reported previously.

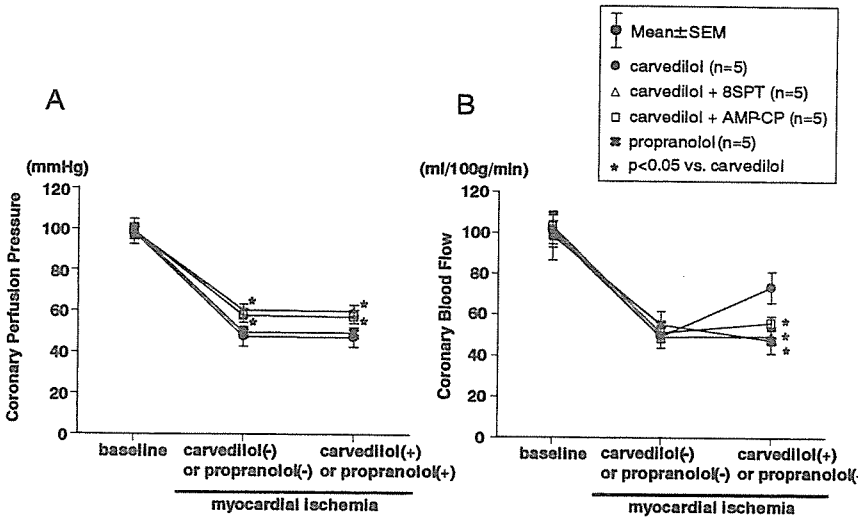

Figure 1. Effects of carvedilol on coronary hemodynamics in ischemic myocardium. A and B show CPP and CBF, respectively. Statistical analysis was performed by ANOVA followed by Bonferroni's test.

Measurement of Infarct Size and Collateral Blood Flow

In protocol 4, the area of myocardial necrosis and the area at risk[16] were measured in all of the dogs upon completion of the protocol by an operator who had no knowledge of the treatment given to each animal. Infarct size was expressed as a percentage of the area at risk.

Regional myocardial blood flow was determined as described previously.[17] Nonradioactive microspheres (Sekisui Plastic Co) made of inert plastic were labeled with bromine. Microspheres were administered at 80 minutes after the start of coronary occlusion. The radio fluorescence of the stable heavy elements was measured with a wavelength dispersive spectrometer (PW 1480, Phillips Co). Because the level of energy emitted is characteristic of specific elements, it was possible to quantify the radio fluorescence of the heavy element with which the microspheres were labeled. Myocardial blood flow was calculated according to the following formula: time flow=(tissue count)×(reference flow)/(reference count), and was expressed in milliliters per minute per gram wet weight. Endomyocardial blood flow was measured at the inner half of the left ventricular wall.

Exclusion Criteria

To ensure that all of the animals used for analysis of infarct size in protocol 4 were healthy and were exposed to a similar extent of ischemia, the following standards were used for exclusion of unsatisfactory dogs: (1) subendocardial collateral blood flow >15 mL · 100 g^{-1} · min^{-1}, (2) a heart rate >170 bpm, and (3) >2 consecutive attempts required to terminate ventricular fibrillation using low-energy DC pulses applied directly to the heart.

Statistical Analysis

Statistical analysis was performed by use of ANOVA[18,19] to compare data among the groups. When ANOVA indicated a significant difference, paired data were compared by use of the Bonferroni test. Changes of the hemodynamic and metabolic parameters over time were assessed by ANOVA with repeated measures. Results were expressed as the mean±SEM, with a value of $P<0.05$ being considered significant.

Results

Effects of Carvedilol on VAD(Ado) in Nonischemic Myocardium

Neither systemic hemodynamic parameters (mean blood pressure, 101.0±2.1 versus 98.6±3.2 mm Hg and heart rate, 130.2±3.7 versus 128.0±3.3 bpm) nor FS (20.1±1.0% versus 21.5±1.0%) changed during the infusion of carvedilol. In contrast, CBF was increased (98.4±8.5 versus 112.6±9.6 mL · 100 g^{-1} · min^{-1}, $P<0.05$), as was VAD(Ado) (40.9±4.0 versus 68.6±5.5 nmol/L, $P<0.05$).

Effects of Either Carvedilol or Propranolol on VAD(Ado) During Coronary Hypoperfusion

Administration of either 8-SPT or AMP-CP did not alter the systemic hemodynamics (mean blood pressure, 98.8±6.1 versus 101.8±5.8 mm Hg before and after 8-SPT and 99.0±3.0 versus 102.0±3.2 mm Hg before and after AMP-CP; heart rate, 132.2±6.9 versus 132.4±6.1 min^{-1} before and after 8-SPT and 131.8±4.6 versus 132.8±3.4 min^{-1} before and after AMP-CP) or the coronary hemodynamic and metabolic parameters (Figures 1 through 3). Before both CBF and CPP were reduced, there were no significant differences in hemodynamic and metabolic parameters among the 3 groups. In untreated dogs, administration of saline did not affect CPP, LER, or FS. However, addition of carvedilol increased VAD(Ado), CBF, LER, and FS, even in the

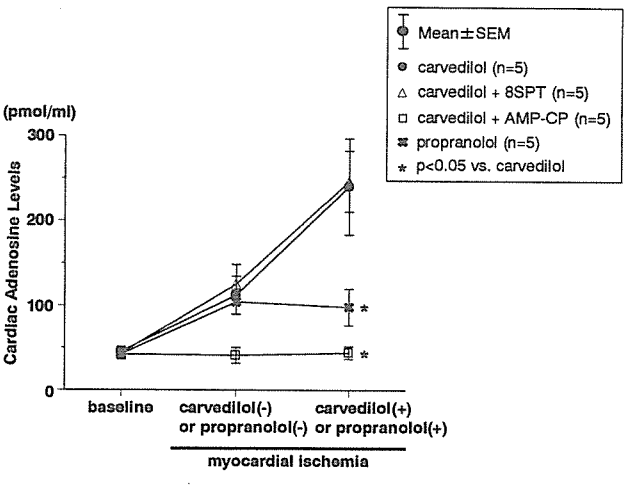

Figure 2. Changes of difference in adenosine levels between coronary venous and arterial blood [VAD(Ado)] in ischemic myocardium. Carvedilol increased VAD(Ado), which was attenuated by an ecto-5'-nucleotidase inhibitor. Statistical analysis was performed by ANOVA followed by Bonferroni's test.

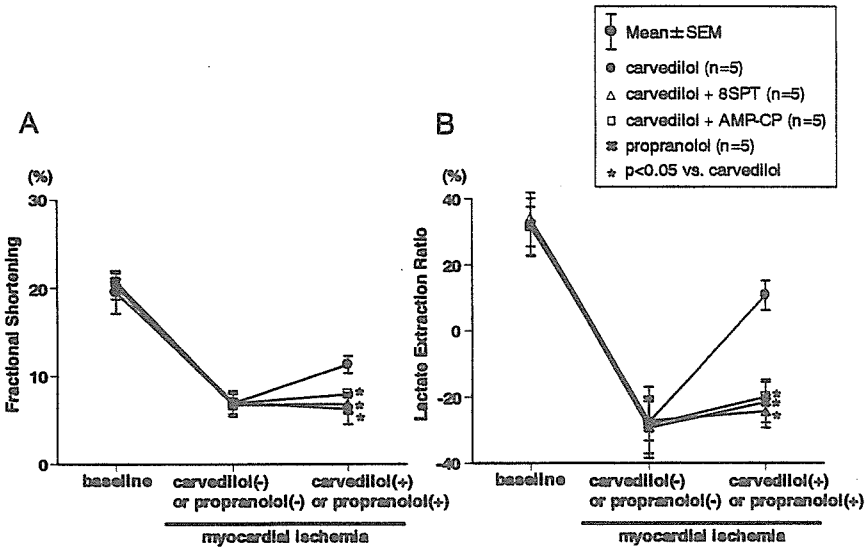

Figure 3. Changes of FS (A) and LER (B) in ischemic myocardium. Statistical analysis was performed by ANOVA followed by Bonferroni's test.

constant low-CPP state, suggesting that myocardial ischemia was improved by carvedilol. These effects of carvedilol were blunted by administration of either 8-SPT or AMP-CP. Unlike carvedilol, an infusion of propranolol did not alter VAD(Ado), CBF, LER, or FS (Figures 1 through 3).

Reduction of Oxidative Stress and Beneficial Effect of Carvedilol in Ischemic Myocardium

In 5 dogs, reduction of CBF caused an increase of VAD(8-Iso-$F_{2\alpha}$), which was reduced by carvedilol (Figure 4A through 4C). Under these conditions, VAD(Ado) was increased by infusion of carvedilol (Figure 4D). In another 5 dogs, an infusion of SOD did not change either hemodynamic parameters or VAD(8-Iso-$F_{2\alpha}$) at nonischemic baseline conditions (Figure 4A through 4C). After the reduction of CBF to 50%, VAD(Ado) increased to the level seen in the presence of carvedilol without SOD (Figure 4D), whereas VAD(8-Iso-$F_{2\alpha}$) did not increase (Figure 4C). Addition of carvedilol did not further attenuate VAD(8-Iso-$F_{2\alpha}$) or increase VAD(Ado) (Figure 4C and 4D).

Effects of Carvedilol on Infarct Size

Seven of 64 dogs were excluded from analysis because their subendocardial collateral flow was >15 mL · 100 g^{-1} · min^{-1}, so 57 dogs completed the protocol satisfactorily. Among these 57 dogs, 18 dogs developed ventricular fibrillation at least once, and ventricular fibrillation that matched the exclusion criteria occurred in 15 dogs, so these animals were also excluded from analysis. The numbers of the dogs that met the exclusion criteria of ventricular fibrillation were 2, 2, 0, 2, 3, 3, and 3 in the saline, the DMSO, the carvedilol, the carvedilol+8-SPT, the carvedilol+AMP-CP, the 8-SPT, and the AMP-CP groups, respectively.

Neither aortic blood pressure (≈104 mm Hg) nor heart rate (≈136 min^{-1}) showed any differences among the 7 groups throughout the protocol. The Table shows the area at risk and the endocardial collateral blood flow in the LAD region during myocardial ischemia. There were no significant differences in the area at risk and collateral flow among the 7 groups during myocardial ischemia (Table). Figure 5 shows

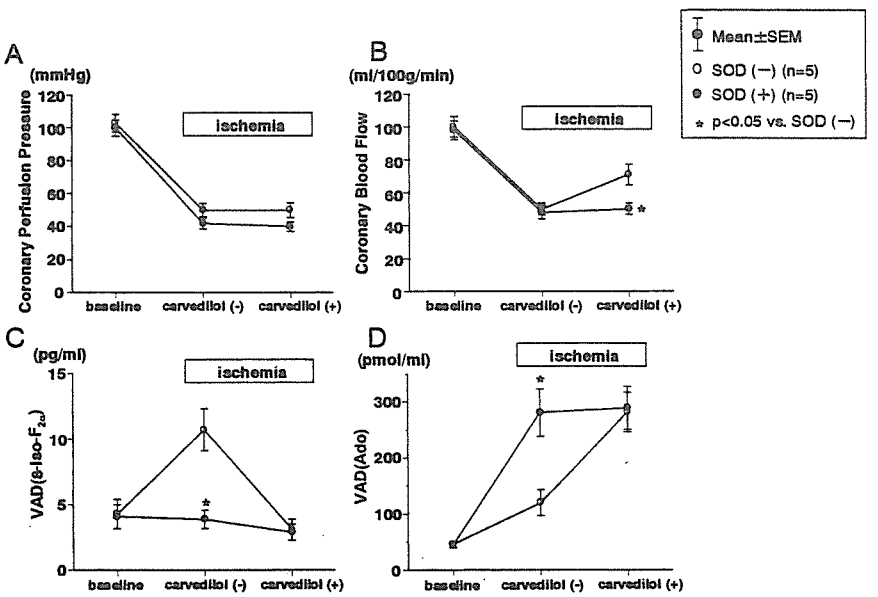

Figure 4. Changes of CPP (A), CBF (B), VAD(8-Iso-$F_{2\alpha}$) (C), and VAD(Ado) (D) in ischemic myocardium. Statistical significance was tested by ANOVA followed by Bonferroni's test.

Area at Risk and Collateral Blood Flow During Myocardial Ischemia in Each Group

Groups	Risk Area, %	CBF During Myocardial Ischemia, mL·100 g^{-1}·min^{-1}
1. Control (saline) group	38.9±1.2	7.8±1.3
2. DMSO group	40.2±1.5	8.1±2.3
3. Carvedilol group	41.5±2.2	8.0±2.6
4. Carvedilol group+8-SPT group	39.3±3.3	9.0±2.0
5. Carvedilol group+AMP-CP group	40.9±3.3	8.7±1.9
6. 8-SPT group	43.1±1.9	8.3±1.9
7. AMP-CP group	41.2±2.1	8.2±1.5

Values are expressed as mean±SEM. There were no differences in the area at risk and collateral blood flow in all of the groups. Statistical significance was tested by ANOVA, followed by Bonferroni's test.

that carvedilol decreased infarct size compared with the control groups. This protective effect was completely blocked by either 8-SPT or AMP-CP, suggesting that the reduction of infarct size by carvedilol was attributable to an adenosine-dependent mechanism.

Effect of Carvedilol on Ecto-5′-Nucleotidase Activity in HUVECs

In HUVECs, carvedilol increased ecto-5′-nucleotidase activity by 35.4±8.4% ($P<0.01$) (Figure 6A). Exposure to xanthine and xanthine oxidase decreased ecto-5′-nucleotidase activity, whereas concomitant addition of carvedilol restored ecto-5′-nucleotidase activity to 104.9±8.7% of the baseline levels ($P<0.01$) (Figure 6B). Neither carvedilol nor xanthine and xanthine oxidase had any effect on cytosolic 5′-nucleotidase.

Discussion

In the present study, we demonstrated that carvedilol increases both adenosine release and CBF in ischemic and nonischemic hearts via reduction of oxidative stress and restoration of ecto-5′-nucleotidase activity. We also showed that carvedilol could limit infarct size and that this effect was attributable to the reduction of oxidative stress and an adenosine- or ecto-5′-nucleotidase–dependent mechanism. These findings suggested that the cardioprotective effect of carvedilol was attributable to an increase of adenosine in ischemic myocardium in addition to its β-blocking action, because propranolol did not mimic this effect.

Influence of Carvedilol on Adenosine Release in Ischemic Hearts

The β-adrenoreceptors in coronary smooth muscle are involved in coronary vasodilation, and their stimulation is thought to increase CBF via the relaxation of vascular smooth muscle and increased myocardial oxygen demand. Therefore, it may seem unusual that a β-blocker like carvedilol would cause coronary vasodilation. There are several possible explanations for the present findings. First, carvedilol itself may cause vasodilation separately from its β-blocking activity. Indeed, although carvedilol does not have a nitroxy moiety, its chemical structure predicts that the drug could also block α_1-adrenoceptors,[20] which would cause vasodilation. We cannot exclude this possibility, but the role of α_1-adrenoceptor blockade in the vasodilatory effect of carvedilol seems likely to be minor, because we have previously reported that blockade of α_1-adrenoceptors attenuates adenosine release in ischemic myocardium,[21] whereas we found that carvedilol caused an increase of adenosine production. Second, carvedilol may increase vasodilatory substances such as NO or adenosine. We demonstrated that carvedilol could increase cardiac adenosine production independently of its β-blocking effect in the present study, because propranolol did not increase CBF under the same circumstances (Figures 1 through 3). Intriguingly, the carvedilol-induced increases in both adenosine release and coronary vasodilation were greater in ischemic heart than in nonischemic heart. There was a significant difference between the influence of carvedilol on coronary vasodilation under nonischemic and ischemic conditions in the present study, because the percent

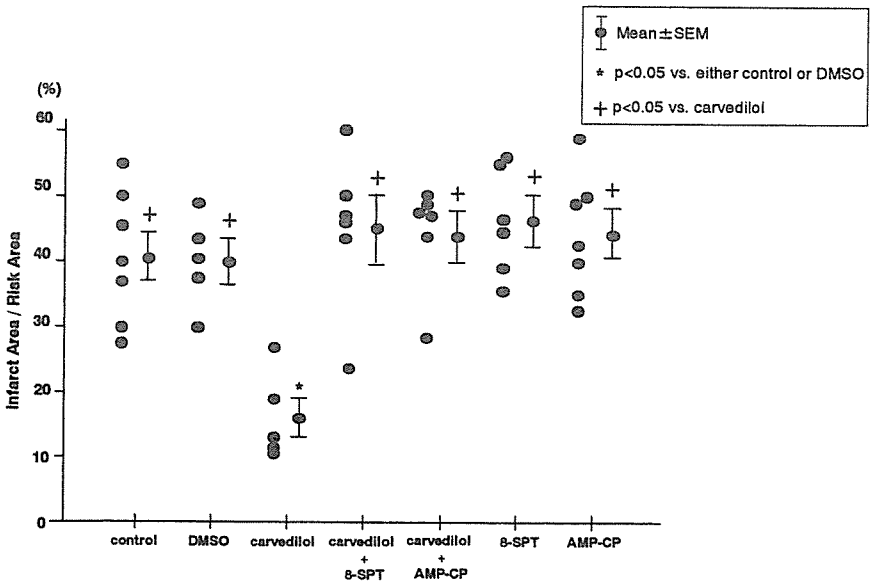

Figure 5. Infarct size as a percentage of area at risk. Infarct size was decreased in carvedilol group compared with control group, and this improvement was blocked by either 8-SPT or AMP-CP. Statistical significance was tested by ANOVA followed by Bonferroni's test.

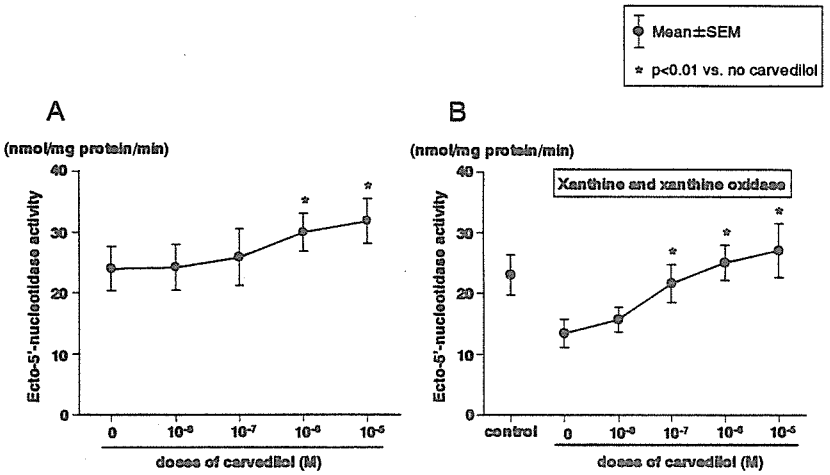

Figure 6. Ecto-5′-nucleotidase activity of HUVECs in presence or absence of carvedilol or xanthine and xanthine oxidase. Statistical significance was tested by ANOVA followed by Bonferroni's test.

increases of CBF in nonischemic and ischemic myocardium were 14.4±1.1% and 50.6±10.1% ($P<0.05$), respectively. One possible explanation is that carvedilol may bind more tightly to β-adrenoreceptors under ischemic conditions than nonischemic conditions, and β-adrenoreceptors are also upregulated in the ischemic heart,[22] which may enhance the adenosine-producing effect of carvedilol. Alternatively, even if carvedilol decreases coronary artery tone in nonischemic heart as well as ischemic heart, the activity of other endogenous vasodilators may decrease to maintain coronary autoregulation. Conversely, the effects of other vasodilators may already be maximal in ischemic hearts, so that carvedilol-induced adenosine release becomes a major determinant of coronary artery tone when adenosine-dependent coronary vasodilation is submaximal. A third possibility is that carvedilol may reduce the levels of substances that attenuate adenosine release and are increased in ischemic myocardium. Because carvedilol is reported to decrease oxidative stress and such stress reduces adenosine production, antioxidant activity of carvedilol may be involved in adenosine-dependent coronary vasodilation and cardioprotection. We showed such evidence in the present study.

In this context, several lines of evidence support the concept that adenosine can markedly attenuate ischemia/reperfusion injury,[12,23] and we suggest that carvedilol-induced adenosine release is important for cardioprotection.

Mechanism of the Carvedilol-Induced Increase of Cardiac Adenosine

In ischemic hearts, carvedilol caused reduction of oxidative stress and increases in both adenosine release and CBF. Also, in HUVECs under oxidative stress, carvedilol restored ecto-5′-nucleotidase activity to the control level. These findings suggest that carvedilol may eliminate the factors that impaired ecto-5′-nucleotidase activity under ischemic conditions. Oxidative stress is one of these factors. Because oxygen-derived free radicals attenuate the ischemia-induced activation of ecto-5′-nucleotidase, elimination of oxidative stress may increase adenosine release in the ischemic myocardium. We observed that carvedilol could reduce oxidative stress, so this action may explain the present findings. Because ecto-5′-nucleotidase is susceptible to impairment by oxygen-derived free radicals, it is likely that the beneficial effect of carvedilol on myocardial ischemia in the present study was attributable to its antioxidant activity.

Clinical Relevance and Limitations

Carvedilol has been shown to be effective for treating heart failure.[5] Its effective clinical dose is about 0.1 to 0.2 μg/mL, and the calculated cardiac concentration of carvedilol in the present study is ≈1 μg/mL. In dogs, carvedilol at 1 and 4 μg/mL decreased blood pressure by 9% and 32%, respectively (data not shown), suggesting that the concentration of 1 μg/mL of carvedilol in canine heats was comparable to a clinical dose of carvedilol. This difference may be also attributable to species differences, the route of administration of carvedilol, or conscious/anesthetic conditions.

The present study hinted that the mechanism by which carvedilol potently ameliorates heart failure, especially ischemic heart failure, may be related to adenosine.[12] Carvedilol may have the ability to both antagonize β-adrenoceptors and increase adenosine release.

Tumor necrosis factor-α is inhibited by both carvedilol and adenosine[24,25] and has been indicated to have a role in the pathology of congestive heart failure. Because the present study hints that the cardioprotection afforded by carvedilol is adenosine-dependent, it follows that the clinical effects of carvedilol may also be adenosine-dependent. If this hypothesis receives further validation, adenosine and potentiators of adenosine production or adenosine receptor agonists may become candidates for the treatment of heart failure.

Acknowledgments

This study was supported by Grants-in-aid for Scientific Research 12470153 and 12877107 from the Japanese Ministry of Education, Culture, Sports, Science, and Technology; a Health and Labor Sciences Research Grant for Human Genome, Tissue Engineering, and Food Biotechnology (H13-Genome-011); and a Health and Labor Sciences Research Grant for Comprehensive Research on Aging and Health (H13-21seiki(seikatsu)-23, H14Tokushitsu-38) from the Japanese Ministry of Health and Labor and Welfare. The authors gratefully acknowledge the technical assistance of Tomi Fukushima and Junko Yamada during the conduct of the experiments.

References

1. Saxenhofer H, Morger D, Weidmann P, et al. Modulation of noradrenergic but not angiotensinergic blood pressure control by beta-blockade with carteolol. *J Hypertens.* 1991;9:1049–1056.
2. Silke B, Verma SP, Hussain M, et al. Haemodynamic dose-response effects of i.v. penbutolol in angina pectoris. *Br J Clin Pharmacol.* 1983; 16:529–535.
3. Packer M, Coats AJ, Fowler MB, et al. Effect of carvedilol on survival in severe chronic heart failure. *N Engl J Med.* 2001;344:1651–1658.
4. Dargie HJ. Effect of carvedilol on outcome after myocardial infarction in patients with left-ventricular dysfunction: the CAPRICORN randomised trial. *Lancet.* 2001;357:1385–1390.
5. Metra M, Nardi M, Giubbini R, et al. Effects of short- and long-term carvedilol administration on rest and exercise hemodynamic variables, exercise capacity and clinical conditions in patients with idiopathic dilated cardiomyopathy. *J Am Coll Cardiol.* 1994;24:1678–1687.
6. Ruffolo RR Jr, Gellai M, Hieble JP, et al. The pharmacology of carvedilol. *Eur J Clin Pharmacol.* 1990;38(suppl 2):S82–S88.
7. Gretzer I, Hjemdahl P. Differences between the effects of metoprolol and prazosin on the forearm vasculature in primary hypertension. *J Hypertens.* 1997;15:1317–1326.
8. Oettl K, Greilberger J, Zangger K, et al. Radical-scavenging and iron-chelating properties of carvedilol, an antihypertensive drug with antioxidative activity. *Biochem Pharmacol.* 2001;62:241–248.
9. Huang WH, Wang Y, Askari A. (Na+/K+)-ATPase: inactivation and degradation induced by oxygen radicals. *Int J Biochem.* 1992;24: 621–626.
10. Guerra L, Cerbai E, Gessi S, et al. The effect of oxygen free radicals on calcium current and dihydropyridine binding sites in guinea pig ventricular myocytes. *Br J Pharmacol.* 1996;118:1278–1284.
11. Kitakaze M, Hori M, Takashima S, et al. Superoxide dismutase enhances ischemia-induced reactive hyperemic flow and adenosine release in dogs: a role of 5′-nucleotidase activity. *Circ Res.* 1992;71:558–566.
12. Kitakaze M, Minamino T, Node K, et al. Adenosine and cardioprotection in the diseased heart. *Jpn Circ J.* 1999;63:231–243.
13. Kitakaze M, Minamino T, Node K, et al. Beneficial effects of inhibition of angiotensin-converting enzyme on ischemic myocardium during coronary hypoperfusion in dogs. *Circulation.* 1995;92:950–961.
14. Bergmeyer HU. *Methods of Enzymatic Analysis.* 1st ed. New York, NY: Academic Press Inc; 1963:266–270.
15. Smith K, Varon HH, Race GJ, et al. Serum 5′-nucleotidase in patients with tumor in the liver. *Cancer.* 1965;19:1281–1285.
16. Kitakaze M, Hori M, Morioka T, et al. Alpha1-adrenoceptor activation mediates the infarct size limiting effect of ischemic preconditioning through augmentation of 5′-nucleotidase activity and adenosine release. *J Clin Invest.* 1994;93:2197–2205.
17. Mori H, Haruyama S, Shinozaki Y, et al. New nonradioactive microspheres and more sensitive X-ray fluorescence to measure regional blood flow. *Am J Physiol.* 1992;263:H1946–H1957.
18. Snedecor GW, Cochran WG. *Statistical Methods.* Ames, Iowa: Iowa State University Press; 1972:258–298.
19. Steel RGD, Torrie JH. *Principles and Procedures of Statistics: A Biomedical Approach.* 2nd ed. New York, NY: McGraw-Hill Publishing Co; 1980:137–238.
20. Feuerstein GZ, Ruffolo RR Jr. Carvedilol, a novel multiple action antihypertensive agent with antioxidant activity and the potential for myocardial and vascular protection. *Eur Heart J.* 1995;16(suppl F):38–42.
21. Kitakaze M, Hori M, Tamai J, et al. α_1-Adrenoceptor activity regulates release of adenosine from the ischemic myocardium in dogs. *Circ Res.* 1987;60:631–639.
22. Brodde OE, Zerkowski HR, Borst HG, et al. Drug- and disease-induced changes of human cardiac beta 1- and beta 2-adrenoceptors. *Eur Heart J.* 1989;10(suppl B):38–44.
23. Babbitt TG, Virmani R, Norton R Jr, et al. Adenosine administration during reperfusion after 3 hours of ischemia: effects on infarct size, ventricular function, and regional myocardial blood flow. *Am Heart J.* 1990;120:808–818.
24. Parmely MJ, Zhou WW, Edwards CK, et al. Adenosine and a related carbocyclic nucleoside analogue selectively inhibit tumor necrosis factor-α production and protect mice against endotoxin challenge. *J Immunol.* 1993;151:389–396.
25. Rossig L, Haendeler J, Mallat Z, et al. Congestive heart failure induces endothelial cell apoptosis: protective role of carvedilol. *J Am Coll Cardiol.* 2000;36:2081–2089.

Drug Metab. Pharmacokin. 19 (3): *SNP8* (239)–*SNP13* (244) (2004).

SNP Communication

Fourteen Novel Single Nucleotide Polymorphisms in the SLC22A2 Gene Encoding Human Organic Cation Transporter (OCT2)

Hiromi FUKUSHIMA-UESAKA[1], Keiko MAEKAWA[1,2], Shogo OZAWA[1,3], Kazuo KOMAMURA[4,5], Kazuyuki UENO[6], Masahiko SHIBAKAWA[6], Shiro KAMAKURA[4], Masafumi KITAKAZE[4], Hitonobu TOMOIKE[4], Yoshiro SAITO[1,2] and Jun-ichi SAWADA[1,2]

[1]*Project team for Pharmacogenetics,* [2]*Division of Biochemistry and Immunochemistry,* [3]*Division of Pharmacology, National Institute of Health Sciences, Tokyo, Japan,* [4]*Department of Cardiology,* [5]*Department of Cardiovascular Dynamics Research Institute,* [6]*Department of Pharmacy, National Cardiovascular Center, Suita, Japan*

Full text of this paper is available at http://www.jssx.org

Summary: Thirty-three genetic variations including fourteen novel ones were found in the *SLC22A2* gene from 116 Japanese individuals. The novel variations were as follows: 596C>T (MPJ6_OC2003), 602C>T (MPJ6_OC2004), IVS5+20A>G (MPJ6_OC2010), IVS5−84_−83insG (MPJ6_OC2013), IVS6+30T>C (MPJ6_OC2014), IVS6+146G>T (MPJ6_OC2016), IVS6+179G>T (MPJ6_OC2017), IVS6−16delT (MPJ6_OC2018), 1920G>A (MPJ6_OC2022), 2153G>A (MPJ6_OC2026), 2157C>T (MPJ6_OC2028), 2306T>C (MPJ6_OC2031), 2342+5T>C (the last nucleotide number of mRNA+the position in the 3′-flanking region; MPJ6_OC2032) and 2342+127T>C (MPJ6_OC2033). Six variations were located in the exons, four of which were in the 3′-untranslated region (3′-UTR) of exon 11; six were in the introns; and two were in the 3′-flanking region. The frequencies were 0.802 for IVS5−84_-83insG, 0.013 for 602C>T, 0.009 for 596C>T, and 0.004 for the other 11 variations. Among them, 596C>T and 602C>T resulted in amino acid substitutions (Thr199Ile and Thr201Met, respectively).

Key words: *SLC22A2* (OCT2); nonsynonymous alteration; intron; novel SNP

Introduction

Human polyspecific organic cation transporter 2 (OCT2) encoded by *SLC22A2* belongs to a superfamily of transporters, solute carrier family 22 (SLC22), which share a highly homologous 12-transmembrane structure.[1)] This family also includes organic cation transporter 1 (OCT1) encoded by *SLC22A1*, which exhibits 70% amino acid similarity to OCT2.[2)] *SLC22A1* and *SLC22A2* are located adjacently on chromosome 6q26 and both consist of 11 exons,[3)] suggesting that they evolved from a common ancestor by gene duplication. Both OCT1 and OCT2 translocate a wide variety of endogenous and exogenous organic cations across the plasma membrane in an electrochemical potential-dependent manner.[4,5)] Although both transporters show an extensive overlap in their substrate specificities, there are distinct differences in tissue distribution. In contrast to OCT1, which is primarily found in the sinusoidal (basolateral) membranes of hepatocytes and, to a lesser extent, in intestinal epithelial cells, OCT2 is mainly found in the basolateral membranes of proximal tubules in the kidney.[2,4-6)] Based on its properties and tissue distribution, OCT2 is thought to mediate the uptake of organic cations from the blood into renal tubular epithelial cells. Indeed, functional studies using isolated human kidney tubules demonstrate their ability to take

On March 5th, 2004, these variations were not found in the Japanese Single Nucleotide Polymorphisms (JSNP) (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB (http://www.pharmgkb.org/do/) databases.

This study was supported by the Program for the Promotion of Fundamental Studies in Health Sciences (MPJ-3 and 6) of the Organization for Pharmaceutical Safety and Research (OPSR) of Japan.

Received; March 9, 2004, Accepted; April 15, 2004

To whom correspondence should be addressed: Keiko MAEKAWA, Ph.D. *Division of Biochemistry and Immunochemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel. +81-3-3700-9453, Fax. +81-3-3707-6950, E-mail: maekawa@nihs.go.jp*

Table 1. Primer sequences used for the analysis of the human *SLC22A2* gene

	Amplified and sequenced region	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	PCR product (bp)
1st PCR	Exons 1 to 9	CCTCTAAGTACCTGATGTCTGGCTT	CATCAACACAGGTGCTCCATTATGC	20556
	Exons 10 to 11	GCATTTGACTGGCTAAAACTTGGTG	GTGGGAGGTCTCTCAGAACAAAAGA	10370
2nd PCR	Exon 1	GTGTGTTTTCTCCATAGGGC	CCAGTTATTCTCCAGACAAA	1060
	Exon 2	GCCACTTAACTATACAGTTC	GGAGATTGTGGTCTGTGTGC	350
	Exons 3 to 4	TGCTGATGAAAAGTGCTACC	GGTCTGGAGAGTGAAAGCAA	1796
	Exon 5	CTGAGGAGGGATTTAGCATT	GGCATAGGAGACTGGTGAAG	586
	Exon 6	GTATGGAAAGTGCTTGCTCT	GTGAAGCCGAGGTTGCCATT	467
	Exon 7	AGCCAGCCACTGAAGTAGAT	GGGCTGTAAAGTCTCTCTTG	618
	Exon 8	CGTGAGAATCTGCTGACATT	ACCCCAGCCTGTAAGTGTGA	449
	Exon 9	GTGATTTCTGAGATTTTGG	GCCACAGACACATCATTACT	413
	Exon 10	GGGTTACAGTCCTCCTTTTC	TTAGAAAGACCTTCTCCACG	652
	Exon 11	CCACCACTCAGAACACATTG	AGAGGTGAAATAGGGCAAGG	1049
Sequencing	Exon 1[a]	GTGTGTTTTCTCCATAGGGC	GGCACCGTGTAGTTCAGTTC	
		ACTCCCCTCTTTGACTTCTG	CCAGTTATTCTCCAGACAAA	
	Exon 2	GCCACTTAACTATACAGTTC	GGAGATTGTGGTCTGTGTGC	
	Exon 3	TCCCTTTGTGGCTATCAGTC	GAGGAATGCTGAATGAGTTG	
	Exon 4	ATTCAGAGAGTTGCGTAGAA	GGTCTGGAGAGTGAAAGCAA	
	Exon 5	CTGAGGAGGGATTTAGCATT	GGCATAGGAGACTGGTGAAG	
	Exon 6	TTGTTATTCCCTATGTGACC	GTGAAGCCGAGGTTGCCATT	
	Exon 7	AGCCAGCCACTGAAGTAGAT	GGGCTGTAAAGTCTCTCTTG	
	Exon 8	CGTGAGAATCTGCTGACATT	ACCCCAGCCTGTAAGTGTGA	
	Exon 9	GTGATTTCTGAGATTTTGG	GCCACAGACACATCATTACT	
	Exon 10	GGGTTACAGTCCTCCTTTTC	TTAGAAAGACCTTCTCCACG	
	Exon 11[a]	TTTGGTTTTCAGCCTCCTCA	TCACCTGTGTTACTGAAAGG	
		TAACCAGAATACATAGGGGA	AGGTGAAATAGGGCAAGGAA	

[a]Two sets of overlapping primers were used for sequencing of exon 1 and exon 11.

up a number of organic cations with a similar specificity to OCT2.[7] A wide array of clinically used drugs (*e.g.*, cimetidine, procainamide and metformin), endogenous bioactive amines such as dopamine and norepinephrine, as well as toxic substances [*e.g.*, MPP^+ (1-methyl-4-phenyl-pyridinium)] were reported to be transported by OCT2.[2,4,5,8,9] Thus, OCT2 is thought to have a critical role in the clearance of these compounds.

Recently, several single nucleotide polymorphisms (SNPs) in *SLC22A2* have been identified both in Japanese populations[10] and in ethnically diverse populations,[11] and these lists have been published in the JSNP database and the PharmGKB database,[12] respectively. Furthermore, several SNPs in OCT2 have been reported to affect the transport function of OCT2 *in vitro*.[11] These variations may influence renal drug elimination and toxicities *in vivo*. Therefore, studies on genetic variations in *SLC22A2* would be useful for investigating possible correlations between genotypes and phenotypes, such as responsiveness to drug therapy and sensitivity to xenobiotics. In this study, we searched for novel SNPs by sequencing all the exons and the surrounding introns of *SLC22A2* from 116 Japanese individuals. We identified 14 novel variations, including two novel nonsynonymous SNPs located in exon 3.

Materials and Methods

Human genomic DNA samples: All of the 116 arrhythmic patients participating in this study were administered cationic antiarrhythmic drugs at the National Cardiovascular Center. Genomic DNA was extracted from blood leukocytes and was used as a template in the polymerase chain reaction (PCR). The ethical review boards of the National Cardiovascular Center and National Institute of Health Sciences approved this study. Written informed consent was obtained from all participating subjects.

PCR conditions for DNA sequencing: First, the entire *SLC22A2* gene was divided into two regions (from exon 1 to exon 9 and from exon 10 to 11), and each region was amplified from 100 ng of genomic DNA using 1.25 units of *Z*-Taq (Takara Shuzo, Tokyo, Japan) with 0.2 μM primers listed in **Table 1** (1st PCR). The first PCR conditions were 30 cycles of 98°C for 5 sec, 55°C for 5 sec, and 72°C for 190 sec. Next, each exon was amplified by *Ex*-Taq (0.625 units) (Takara Shuzo) with an appropriate set of *SLC22A2*-specific primers (2nd PCR; 0.2 μM) designed in the introns, as listed in **Table 1**. The second round PCR conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min,

Table 2. Summary of variations in the *SLC22A2* gene detected in Japanese arrhythmic patients

SNP name			Location	position		Nucleotide change and flanking sequence (5′ to 3′)	Amino acid change	Frequency
SNP ID	NCBI[b]	JSNPs[c]		Accession number NT_007422.12	From the translational initiation site or from the nearest exon			
MPJ6_OC2001	rs624249	ssj0008486	Exon 1	2966741	390[d]	GGTGTACGAGACG/TCCTGGCTCGTCC	Thr130Thr	0.138
MPJ6_OC2002	rs2774230	ssj0008488	Intron 2	2964955	IVS2+32	GTGGAATTTAAAG/CAATCCCAAAGGT		0.177
MPJ6_OC2003[a]			Exon 3	2958998	596[d]	CCATTTCCCCAAC/TCTATACGTGGAT	Thr199Ile	0.009
MPJ6_OC2004[a]			Exon 3	2958992	602[d]	CCCCAACCTATAC/TGTGGATGTTAAT	Thr201Met	0.013
MPJ6_OC2005	rs316016	IMS-JST027183, ssj0008491	Intron 3	2958841	IVS3+80	GGGCGGCATGCAG/AGAAAAGGGACCC		0.246
MPJ6_OC2006	rs316017	IMS-JST027184, ssj0008492	Intron 3	2957874	IVS3−117	TCAGAGAGTTGCA/GTAGAATATTCTA		0.246
MPJ6_OC2007	rs316018	IMS-JST027185, ssj0008493	Intron 3	2957835	IVS3−78	TTTTGAGGTTGGC/TGTTCTAGTTTCC		0.246
MPJ6_OC2008	rs316019	IMS-JST027186	Exon 4	2957623	808[d]	CAGTTCACAGTTG/TCTCTGCCCAACT	Ala270Ser	0.168
MPJ6_OC2009	rs2279463	IMS-JST027187, ssj0008494	Intron 4	2955730	IVS4−59	ATTATAAAAAAAT/CGGGGGATGGGGT		0.078
MPJ6_OC2010[a]			Intron 5	2955537	IVS5+20	AGGGCCTTAAGTA/GTCAAATCAGGGG		0.004
MPJ6_OC2011	rs3219195		Intron 5	2955529_2955528	IVS5+28_+29	AAGTATCAAATCAG/-GGGATGGAGAA		0.078
MPJ6_OC2012	rs316021	IMS-JST027188, ssj0008495	Intron 5	2955382	IVS5+175	ATTTGTGGTTCCA/GTGGGAGAATATT		0.259
MPJ6_OC2013[a]			Intron 5	2954002_2954001	IVS5−84_−83	CGCTCAGGACGG-/GTAAGAAATTATT		0.802
MPJ6_OC2014[a]			Intron 6	2953782	IVS6+30	CTTTGAAATGCCT/CCCAAATTGTTTT		0.004
MPJ6_OC2015	rs617217	ssj0008496	Intron 6	2953697	IVS6+115	TGACGGTGGGCAC/GCAATGTGGGTTG		0.198
MPJ6_OC2016[a]			Intron 6	2953666	IVS6+146	GTAGGTTTTCCTG/TATCGTGTTTTTC		0.004
MPJ6_OC2017[a]			Intron 6	2953633	IVS6+179	ATATTAAGATCGG/TTGTAGCCCCTAA		0.004
MPJ6_OC2018[a]			Intron 6	2952175	IVS6−16	CTTGACCTGAACT/-CTCCTCTTTGCT		0.004
MPJ6_OC2019	rs8177518		Intron 7	2950810	IVS7−35	TGAGGAATCATCT/CGTGTACGGATAA		0.065
MPJ6_OC2020	rs3839344	IMS-JST156304, ssj0005336	Intron 9	2933263_2933262	IVS9−86_−85	TGCTAAAAAAAA-/AGTTTTAAACAAA		0.802
MPJ6_OC2021	rs316003	ssj0008502	Exon 10	2933173	1506[d]	GCTTTCAGGCGTA/GCTTGGCTTGGTT	Val502Val	0.198
MPJ6_OC2022[a]			Exon 11 (3′-UTR)	2925553	1920[d]	GGTTTTCTGGAGG/AGTTTTTTTTCCA		0.004
MPJ6_OC2023	rs3127594	ssj0005340	Exon 11 (3′-UTR)	2925445	2028[d]	AAAATAGAAAAAA/TGTGTGAAAAACA		0.017
MPJ6_OC2024	rs3103353	ssj0005341	Exon 11 (3′-UTR)	2925417	2056[d]	AAGTTGGGAGAGG/AAGCATCTATTTT		0.017
MPJ6_OC2025	rs3127593	ssj0005342	Exon 11 (3′-UTR)	2925344	2129[d]	TGTCAAGAATTTT/AAGATAGGCCTTT		0.017
MPJ6_OC2026[a]			Exon 11 (3′-UTR)	2925320	2153[d]	TCAGTAACACAGG/ATGAAGAAATTTT		0.004
MPJ6_OC2027	rs2450975	IMS-JST156307, ssj0008505	Exon 11 (3′-UTR)	2925316	2157[d]	TAACACAGGTGAC/AGAAATTTTTAAA		0.168
MPJ6_OC2028[a]			Exon 11 (3′-UTR)	2925316	2157[d]	TAACACAGGTGAC/TGAAATTTTTAAA		0.004
MPJ6_OC2029	rs694812	IMS-JST183810	Exon 11 (3′-UTR)	2925231	2242[d]	TACAACTTAAGTA/GATCATGAGGTCC		0.151
MPJ6_OC2030	rs3127592		Exon 11 (3′-UTR)	2925185	2288[d]	GCATGAATGTGTA/GTTTTCTACAAAA		0.017
MPJ6_OC2031[a]			Exon 11 (3′-UTR)	2925167	2306[d]	TACAAAAGACCTT/CGAGAAGAGTTCA		0.004
MPJ6_OC2032[a]			3′-flanking	2925126	2342+5[e]	TTATAAAACCATT/CGATGATCATTTT		0.004
MPJ6_OC2033[a]			3′-flanking	2925004	2342+127[e]	TCATAAGACTTAT/CAATGAAAACAAA		0.004

[a]Fourteen novel variations detected in our study.
[b]The rs number in NCBI dbSNP.
[c]The ID number in the JSNP database.
[d]A of the translation initiation codon ATG is numbered 1.
[e]The last nucleotide number of mRNA + the position in the 3′-flanking region.

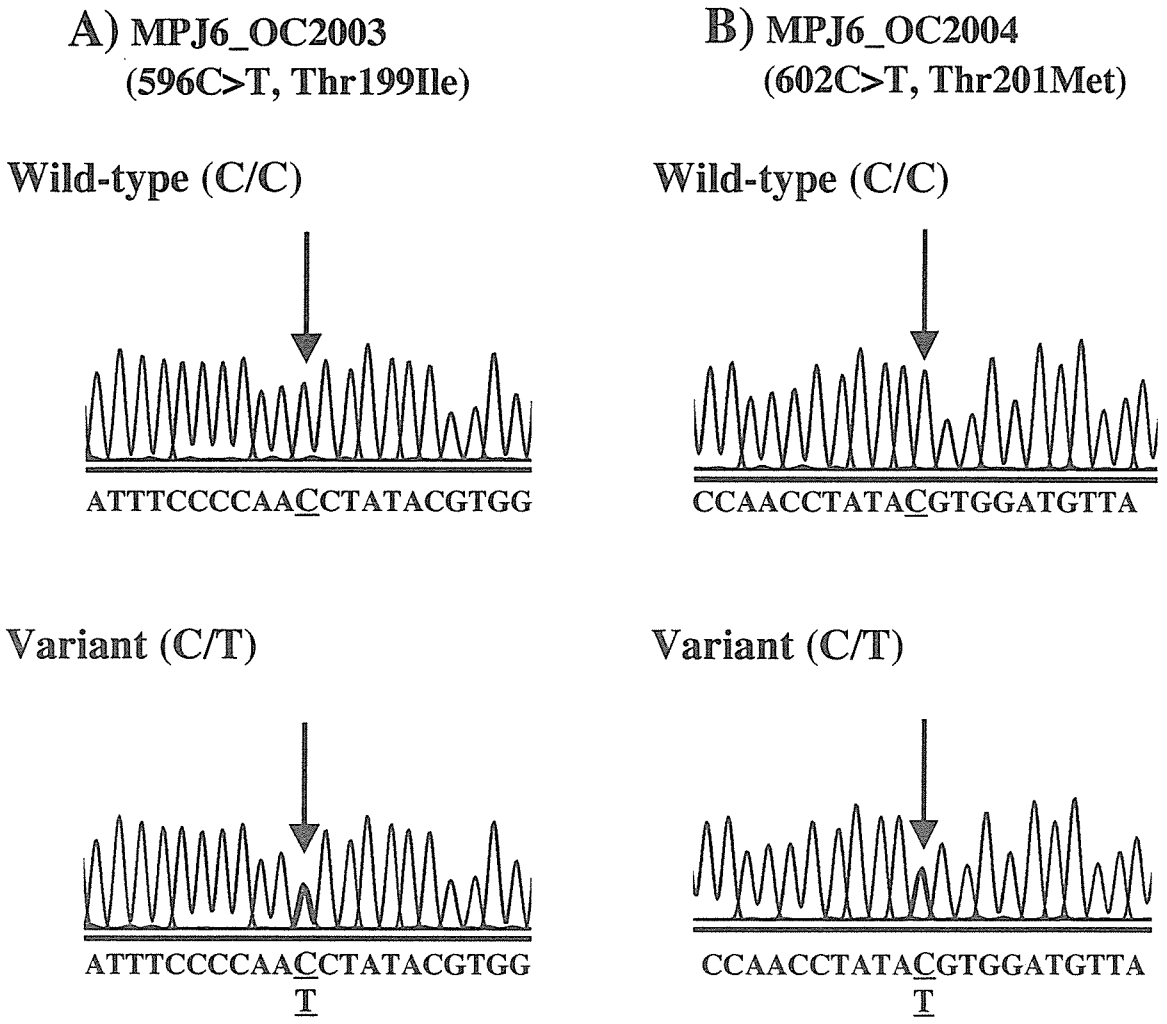

Fig. 1. Electropherograms (sense strands) for the novel nonsynonymous SNPs in *SLC22A2*. (A) MPJ6_OC2003 (wild-type 596C/C; variant 596C/T). (B) MPJ6_OC2004 (wild-type 602C/C; variant 602C/T). The first A of the translation initiation codon ATG is defined as position 1. Arrows indicate the position of the nucleotide changes.

and then a final extension for 7 min at 72°C. The PCR products were then treated with a PCR Product Pre-Sequencing Kit (USB Co., Cleveland, OH, USA) and were directly sequenced on both strands using an ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with the sequencing primers listed in **Table 1** (Sequencing). The excess dye was removed by a DyeEx96 kit (Qiagen, Hilden, Germany). The eluates were analyzed on an ABI Prism 3730 DNA Analyzer (Applied Biosystems). All novel SNPs were confirmed by repeated sequence analyses on PCR products generated by a new genomic DNA amplification.

Results and Discussion

We sequenced all the exons (exons 1～11) and their flanking regions of *SLC22A2* for 116 Japanese arrhythmic patients, and found 33 variations, including 14 novel ones (**Table 2**). In this paper, genomic and cDNA sequences of *SLC22A2* obtained from GenBank (NT_007422.12 and NM_003058.2, respectively) were used as the reference sequences. The novel variations were 596C>T in exon 3 (the A of the translation start codon is numbered +1 in the cDNA sequence; MPJ6_OC2003), 602C>T in exon 3 (MPJ6_OC2004), IVS5+20A>G in intron 5 (20 nucleotides downstream from exon 5; MPJ6_CS2010), IVS5−84_−83insG in intron 5 (from 83 to 84 nucleotides upstream from exon 6; MPJ6_OC2013), IVS6+30T>C in intron 6 (MPJ6_OC2014), IVS6+146G>T in intron 6 (MPJ6_OC2016), IVS6+179G>T in intron 6 (MPJ6_OC2017), IVS6−16delT in intron 6 (MPJ6_OC2018), 1920G>A in exon 11 (MPJ6_OC2022) 2153G>A in exon 11 (MPJ6_OC2026), 2157C>T in exon 11 (MPJ6_OC2028), 2306T>C in exon 11

(MPJ6_OC2031), 2342+5T>C in the 3′-flanking region (5 nucleotides downstream from the last nucleotide of mRNA; MPJ6_OC2032) and 2342+127T>C in the 3′-flanking region (MPJ6_OC2033). The frequencies were 0.802 for IVS5−84_−83insG, 0.013 for 602C>T, 0.009 for 596C>T and 0.004 for the other 11 variations. Nineteen variations we detected were already reported and publicized in the dbSNP database and/or the JSNP database. Their ID numbers in the databases are also given in **Table 2**.

Recently, twenty-eight polymorphisms in *SLC22A2* were identified in several ethnically diverse populations, including 30 Asian-Americans, by Leabman *et al.*[11] and published in the PharmGKB database.[12] Among them, six variations, 390G>T (Thr130Thr) (MPJ6_OC2001), IVS2+32G>C (MPJ6_OC2002), 808G>T (Ala270Ser) (MPJ6_OC2008), IVS5+28_+29delAG (MPJ6_OC2011), IVS7−35T>C (MPJ6_OC2019) and 1506A>G (Val502Val) (MPJ6_OC2021) were also detected in our study. The other SNPs reported in their study were not detected in our study. The 808G>T (Ala270Ser) SNP was the only nonsynonymous SNP found commonly in both their study[11] and ours (**Table 2**) at an allele frequency greater than 10%. The frequency for this SNP was 0.168 in our study, which was comparable to those in Caucasians (0.157), African-Americans (0.110)[11] and the JSNPs database (0.120). However, it was higher than that in 30 Asian-Americans (0.086).[11] This difference in frequency between Asian-Americans and Japanese may be due to the numbers of subjects analyzed. Leabman *et al.*[11] reported that the 808G>T change exhibits a slight increase in the Km value of the prototypical organic cation, MPP^+, and a significant increase in the Ki value of tetrabutylammonium (TBA) when expressed in *Xenopus laevis* oocytes. They suggested that the 808G>T (Ala270Ser) change might influence drug response by altering the renal elimination of xenobiotics *in vivo*.

We identified two novel nonsynonymous SNPs. These SNPs were as follows:

1) SNP, MPJ6_OC2003; GENE NAME, *SLC22A2*; ACCESSION NUMBER, NT_007422.12; LENGTH, 25 bases; 5′-CCATTTCCCCAAC/TCTATACGTGG-AT-3′

2) SNP, MPJ6_OC2004; GENE NAME, *SLC22A2*; ACCESSION NUMBER, NT_007422.12; LENGTH, 25 bases; 5′-CCCCAACCTATAC/TGTGGATGTT-AAT-3′.

The 596C>T (Thr199Ile) (MPJ6_OC2003) change was found in two heterozygous subjects. Another SNP, 602C>T (Thr201Met) (MPJ6_OC2004), was found in 3 different heterozygous subjects. The electropherograms of these two novel nonsynonymous SNPs are shown in **Fig. 1**. Both SNPs were located in exon 3, in a short extracellular loop between the third transmembrane domain (TMD3) and the fourth transmembrane domain (TMD4). These two threonine residues are not conserved among the human OCT family (OCT1, OCT2 and OCT3). Functional significances of these amino acid substitutions on transport activity are currently unknown. Further analyses of these two SNPs are needed. The other 12 novel SNPs were located in introns, the 3′-UTR region or 3′ flanking region. At position 2157, bi-directional changes, C>T (MPJ6_OC2028) and C>A (MPJ6_OC2027), were found. The biological significance of these 12 SNPs also remains to be evaluated.

In conclusion, we identified 33 variations including 14 novel ones in *SLC22A2* of Japanese subjects. Two novel SNPs resulted in amino acid substitutions. The current data may be useful for haplotype analysis and pharmacogenetic studies on OCT2.

Acknowledgments: We thank Ms. Chie Knudsen for her secretarial assistance.

References

1) Marger, M. D. and Saier, M. H., Jr.: A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends Biochem. Sci.*, **18**: 13–20 (1993).
2) Gorboulev, V., Ulzheimer, J. C., Akhoundova, A., Ulzheimer-Teuber, I., Karbach, U., Quester, S., Baumann, C., Lang, F., Busch, A. E. and Koepsell, H.: Cloning and characterization of two human polyspecific organic cation transporters. *DNA Cell Biol.*, **16**: 871–881 (1997).
3) Koehler, M. R., Wissinger, B., Gorboulev, V., Koepsell, H. and Schmid, M.: The two human organic cation transporter genes SLC22A1 and SLC22A2 are located on chromosome 6q26. *Cytogenet. Cell Genet.*, **79**: 198–200 (1997).
4) Burckhardt, G. and Wolff, N. A.: Structure of renal organic anion and cation transporters. *Am. J. Physiol. Renal. Physiol.*, **278**: F853–866 (2000).
5) Dresser, M. J., Leabman, M. K. and Giacomini, K. M.: Transporters involved in the elimination of drugs in the kidney: organic anion transporters and organic cation transporters. *J. Pharm. Sci.*, **90**: 397–421 (2001).
6) Motohashi, H., Sakurai, Y., Saito, H., Masuda, S., Urakami, Y., Goto, M., Fukatsu, A., Ogawa, O. and Inui, K.: Gene expression levels and immunolocalization of organic ion transporters in the human kidney. *J. Am. Soc. Nephrol.*, **13**: 866–874 (2002).
7) Pietig, G., Mehrens, T., Hirsch, J. R., Cetinkaya, I., Piechota, H. and Schlatter, E.: Properties and regulation of organic cation transport in freshly isolated human proximal tubules. *J. Biol. Chem.*, **276**: 33741–33746 (2001).
8) Urakami, Y., Okuda, M., Masuda, S., Saito, H. and Inui, K. I.: Functional characteristics and membrane localization of rat multispecific organic cation transport-

ers, OCT1 and OCT2, mediating tubular secretion of cationic drugs. *J. Pharmacol. Exp. Ther.*, **287**: 800–805 (1998).

9) Okuda, M., Urakami, Y., Saito, H. and Inui, K.: Molecular mechanisms of organic cation transport in OCT2-expressing Xenopus oocytes. *Biochim. Biophys. Acta*, **1417**: 224–231 (1999).

10) Saito, S., Iida, A., Sekine, A., Ogawa, C., Kawauchi, S., Higuchi, S. and Nakamura, Y.: Catalog of 238 variations among six human genes encoding solute carriers (hSLCs) in the Japanese population. *J. Hum. Genet.*, **47**: 576–584 (2002).

11) Leabman, M. K., Huang, C. C., Kawamoto, M., Johns, S. J., Stryke, D., Ferrin, T. E., DeYoung, J., Taylor, T., Clark, A. G., Herskowitz, I. and Giacomini, K. M.: Polymorphisms in a human kidney xenobiotic transporter, OCT2, exhibit altered function. *Pharmacogenetics*, **12**: 395–405 (2002).

12) Leabman, M. K., Huang, C. C., Stryke, D., Johns, S. J., Kawamoto, M., Ferrin, T. E., DeYoung, J., Taylor, T. R., De La Cruz, M., Herskowitz, I. and Giacomini, K. M.: PharmGKB update: I. Genetic variants of the organic cation transporter 2 (OCT2, SLC22A2). *Pharmacol. Rev.*, **55**: 399 (2003).

Drug Metab. Pharmacokin. 19 (4): *SNP19* (313)-*SNP25* (319) (2004).

SNP Communication

Novel Nonsynonymous Single Nucleotide Polymorphisms in the CYP2D6 Gene

Akiko SOYAMA, Takashi KUBO, Atsuko MIYAJIMA[5], Yoshiro SAITO[1], Kisho SHISEKI, Kazuo KOMAMURA[2,3], Kazuyuki UENO[4], Shiro KAMAKURA[2], Masafumi KITAKAZE[2], Hitonobu TOMOIKE[2], Shogo OZAWA[5,*] and Jun-ichi SAWADA[1]

Project team for Pharmacogenetics, [1]Division of Biochemistry and Immunochemistry, [5]Division of Pharmacology, National Institute of Health Sciences, Tokyo, Japan [2]Department of Cardiology, [3]Department of Cardiovascular Dynamics Research Institute, [4]Department of Pharmacy, National Cardiovascular Center, Osaka, Japan

Full text of this paper is available at http://www.jssx.org

Summary: Cytochrome P450 (CYP) 2D6 is an important drug-metabolizing enzyme, and its gene is known to be highly polymorphic. Here, we report five novel nonsynonymous single nucleotide polymorphisms (SNPs), and 65 other sequence variations detected from the gene coding for cytochrome P450 (CYP) 2D6 in 254 Japanese subjects. Two of the novel nonsynonymous SNPs were associated with the **10* key SNP, C100T. Among the 65 variations, 23 were novel, including 12 SNPs in 5′-flanking, 1 in 5′-untranslated, and 10 in intronic regions.

The nonsynonymous SNPs in the *CYP2D6* gene were as follows: 73 C>T (Arg25Trp, exon 1), 972 C>T (Ala90Val, exon 2), 1611 T>A (Phe120Ile, exon 3), 1720 A>C (Glu156Ala, exon 3), 3172 A>C (Glu334Ala, exon 7). The SNPs, 73C>T, 972 C>T, 1611 T>A, 1720 A>C and 3172 A>C were linked with **10*, **1*, **10*, **1* and **2*, respectively.

Key words: CYP2D6; SNP; nonsynonymous SNP

Introduction

Cytochrome P450 (CYP) 2D6 encoded by the *CYP2D6* gene is clinically important since it metabolizes a wide variety of clinical drugs including anti-arrhythmic and psychiatric drugs, as well as endogenous compounds.[1)] The *CYP2D6* gene is highly polymorphic, and nearly 50 different alleles or haplotypes have been described (References 2 and 3 for review and http://www.imm.ki.se/CYPalleles/cyp2d6.htm). These include a deletion of the entire gene, amino acid substitutions, splicing defects, and insertions and deletions of nucleotide(s) resulting in frameshifts in the open reading frame.

During the course of our discovering new *CYP2D6* variants by direct sequencing of leukocyte DNA of Japanese subjects who were administered anti-arrhythmic drugs, we detected 70 sequence variations including 5 novel nonsynonymous single nucleotide polymorphisms (SNPs).

Materials and Methods

Human genomic DNA samples: Total genomic DNA was extracted from blood leukocytes from 254 Japanese individuals, who were administered anti-arrhythmic drugs and/or β-blockers, and was used for DNA sequence analysis. The ethics committees of both the National Cardiovascular Center and the National Institute of Health Sciences approved this study. Written informed consent was obtained from all subjects participating in this study.

Polymerase chain reaction (PCR) conditions for sequencing: 5′-Flanking and exonic regions of the *CYP2D6* gene were separately analyzed by a PCR-based

On April 30, 2004, these SNPs were not found in "dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/)", or "A database for CYP Allele Nomenclature (http://www.imm.ki.se/CYPalleles/)".

This study was supported in part by the Programs for Promotion of Fundamental Studies in Health Sciences (MPJ-6 and MPJ-3) of the Pharmaceuticals and Medical Devices Agency (PMDA) of Japan.

Received; May 13, 2004, Accepted; June 16, 2004

To whom all correspondence should be addressed: Shogo OZAWA, *Division of Pharmacology, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel. +81-3-3700-9737, Fax. +81-3-3707-6950, E-mail: sozawa@nihs.go.jp*

Table 1. Primers used for the amplification and sequencing of 5′-flanking region and all exons of *CYP2D6* gene

5′-Flanking region: 1st PCR			
2D6-CYP505F	CACTGGCTCCAAGCATGGCAG		
2D6-5′2722R	GCTCGGACTACGGTCATCAC		
5′-Flanking region: 2nd PCR and sequencing primers			
2nd PCR primer		Sequencing primer	
2D65′F1s	AGGTCACTCTGGAGTGGGC	2D65′F1s	AGGTCACTCTGGAGTGGGC
2D65′R1s	AGTCCACATGCAGCAGGTTGC	2D65′R1s	AGTCCACATGCAGCAGGTTGC
2D6-CYP505F	CACTGGCTCCAAGCATGGCAG	2D6-CYP505F	CACTGGCTCCAAGCATGGCAG
2D65′R2s	TGCAATGATGCAATCTCGGCTC	2D65′R2s	TGCAATGATGCAATCTCGGCTC
2D65′F3	ATGTTGTCACAGGCTGGGGC	2D65′F3	ATGTTGTCACAGGCTGGGGC
2D65′R3	GGCTCATACACCCTAATGTAGTC	2D65′R3	GGCTCATACACCCTAATGTAGTC
2D65′F7	CTGAAGTCAGGAGTTCAAGAC	2D65′F4	AGCTACTTAGGAGGCTGAGGC
2D65′R4	CGGGCTATGGCTTCTTGTTTGC	2D65′R4s2	GGAGACGGAGATTTCCTCTTGTTG
		2C65′F6s	AAGAATTAGGCTGGGTGGTGC
		2C65′R5s	CTTTAGACAGGGTCTCACTC
		2D65′F4s3	AAAGCAACATATCCTGAACAAAGG
		2D65′R4	CGGGCTATGGCTTCTTGTTTGC
CYP2D6 exons: 1st PCR			
2D6-Dlow	CAGGCATGAGCTAAGGCACCCAGAC		
2D6-Dup	CACACCGGGCACCTGTACTCCTCA		
2D6-DPKup	GTTATCCCAGAAGGCTTTGCAGGCTTCA		
2D6-DPKlow	GCCGACTGAGCCCTGGGAGGTAGGTA		
CYP2D6 exons: 2nd PCR and sequencing primers			
2nd PCR primer		Sequencing primer	
2D6Ex1-2F1	CAGAGGAGCCCATTTGGTAGTGAGGCAGGT	2D6Ex1F3s	GAGGCAGGTATGGGGCTA
2D6Ex1-2R1	GGTCCCACGGAAATCTGTCTCTGT	2D6Ex1R4s	TCAGCAGAAGGGACTTTGTAC
		2D6Ex2F2s	TCATACCTGGGTGACGCATCC
		2D6Ex2R2s	GAAATCTGTCTCTGTCCCCAC
2D6Ex3-4F1	CACGCGCACGTGCCCGTCCCA	2D6Ex3F4s	CACGTGCCCGTCCCAC
2D6Ex3-4R1	CTCTCGCTCCGCACCTCGCGCAGA	2D6Ex3R1s	TTGCTCACGGCTTTGTCC
		2D6Ex4F1s	ACAAAGCGGGAACTGGGA
		2D6Ex4R3s	GCAGAAAGCCCGACTCC
2D6Ex5-6F1	GGACTCTGTACCTCCTATCCACGTCA	2D6Ex5F2s	TTGGTGAGGTCAGTGGTAAGGA
2D6Ex5-6R1	CCCTCGGCCCCTGCACTGTTTCCCAGA	2D6Ex5R1s	CCCCCAAATGACCTCCAAT
		2D6Ex6F1s	AGGAGGAATGAGGGGAGG
		2D6Ex6R3s	GCACTGTTTCCCAGATGG
2D6Ex7F2	TCTGGGAAACAGTGCAGGGGCCGAGGG	2D6Ex7F2s	ATCCTGTAAGCCTGACCTCC
2D6Ex7R2	TCTGGCAGGTCCTGGTTTGTCTCCC	2D6Ex7R2s	CAGTGTGGTGGCATTGAGGA
2D6Ex8-9F1	GGGAGACAAACCAGGACCTGCCAGA	2D6Ex8F2s	GTCCCCCGTGTGTTTGGT
2D6Ex8-9R1	CATCTGCTCAGCCTCAACGTACCCCTGTCT	2D6Ex8R1s	GGGAGGTGAAGAAGAGGAAG
		2D6Ex9F2s	CCTTCCTGCCTTTCTCAGC
		2D8Ex9R2s	GGTAAGCAGGAATGAGGCAG

method. Primer sets used for the amplification and sequencing of the 5′-flanking region and all exons are described in **Table 1.** Two-step PCR reactions were used for the amplification of the 5′-flanking region of the *CYP2D6*.

The first-step PCR amplification of the 5′-flanking region was conducted in a reaction mixture (100 μL) containg 1× Ex Taq buffer, 3.0 mM $MgCl_2$, 5.0 mM dNTPs, 1 unit of Ex Taq polymerase (TaKaRa Shuzo, Kyoto, Japan), and 0.5 μM of each primer. The PCR amplification conditions were as follows: 94°C for 5 min, followed by 30 cycles consisting 94°C for 30 s, 64°C for 1 min, 72°C for 3 min, then 72°C for 7 min, followed by 4°C. For the analysis of each *CYP2D6* exon and surrounding introns, a DNA fragment containing all *CYP2D6* exons was amplified using the Dup, Dlow,

Table 2. SNPs detected in the *CYP2D6* gene in Japanese arrhythmic patients

SNP name			Position				
SNP ID	CYP Allele Nomenclature or NCBI dbSNP or Reference	Location	Position in accession number, M33388	Relative to the translation initiation site[a] or from the nearest exon	SNP and the surrounding sequence (5′ to 3′)	Amino acid change	Frequency
MPJ6_2D6041	Ref. 8	5′-Flanking	NA[b]	−1770	GTGGATGATCCC**G/A**TAGAAGTCCAGA		0.1784
MPJ6_2D6042	Novel	5′-Flanking	13	−1607	GAATTCAAGACC**A/C**GCCTGGACAACT		0.0023
MPJ6_2D6043	*2, Ref. 8	5′-Flanking	36	−1584	CTTGGAAGAACC**C/G**GGTCTCTACAAA		0.1596
MPJ6_2D6044	*10	5′-Flanking	194	−1426	CTACTGAAAATA**C/T**AAAAAGCTAGAC		0.3192
MPJ6_2D6046	*2, *10	5′-Flanking	385	−1235	AAAAAAAAAAAG**A/G**ATTAGGCTGGGT		—[c]
MPJ6_2D6047	*10, Ref. 8	5′-Flanking	620	−1000	GTGGAGGAGGAC**G/A**ACCCTCAGGCAG		0.3099
MPJ6_2D6048	Novel	5′-Flanking	667	−953	CTGGGGCAAGGG**C/T**CTTCCGGCTACC		0.0070
MPJ6_2D6049	Novel	5′-Flanking	672	−948	GCAAGGGCCTTC**C/A**GGCTACCAACTG		0.0023
MPJ6_2D6050	Novel	5′-Flanking	802	−818	CAGGAAACCTCC**G/C**GCATGGCTGGGA		0.0047
MPJ6_2D6051	rs10080991, *2	5′-Flanking	880	−740	GAGAATGTGTGC**C/T**CTAAGTGTCAGT		0.1831
MPJ6_2D6052	Novel	5′-Flanking	902	−718	AGTGTGAGTCTG**T/C**GTATGTGTGAAT		0.0023
MPJ6_2D6053	*2, rs769256	5′-Flanking	942	−678	GTGATTTTCTGC**G/A**TGTGTAATCGTG		0.1831
MPJ6_2D6054	Novel	5′-Flanking	1051	−569	CATGTCAAGAGT**G/A**CAAGGTGAAGTG		0.0023
MPJ6_2D6055	Novel	5′-Flanking	1122	−498	CCCAGGTAAGTG**C/A**CAGTGACAGATA		0.1315
MPJ6_2D6057	Novel	5′-Flanking	1252	−368	GGGAGGTGGATG**G/C**CCGGGTCCACTG		0.0023
MPJ6_2D6058	rs5758604	5′-Flanking	1266	−354	CGGGTCCACTGA**A/G**ACCCTGGTTATC		0.0023
MPJ6_2D6059	Novel	5′-Flanking	1388	−232	AGCAGGAAGCAG**G/C**GGCAAGAACCTC		0.0047
MPJ6_2D6060	Novel	5′-Flanking	1395	−225	AGCAGGGGCAAG**A/G**ACCTCTGGAGCA		0.0047
MPJ6_2D6062	Novel	5′-Flanking	1482	−138	ACAGCAGAGGGC**A/G**AAGGCCATCATC		0.0047
MPJ6_2D6063	Novel	5′-Flanking	1523	−97	GAAGGGTCACGC**G/A**CTCGGTGTGCTG		0.0023
MPJ6_2D6064	Novel	Exon 1 (5′-UTR)	1534	−86	CGCTCGGTGTGC**T/C**GAGAGTGTCCTG		0.0023
MPJ6_2D6065	Novel (*47)	Exon 1	1692	73	GACCTGATGCAC**C/T**GGCGCCAACGCT	R25W	0.0023
MPJ6_2D6066	*22, *44, Ref. 9	Exon 1	1701	82	CACCGGCGCCAA**C/T**GCTGGGCTGCAC	R28C	0.0023
MPJ6_2D6001	*10	Exon 1	1719	100	GCTGCACGCTAC**C/T**CACCAGGCCCCC	P34S	0.3122
MPJ6_2D6067	Novel	Intron 1	1832	213 (IVS1 + 33)	GGGCGGCAGAGG**T/A**GCTGAGGCTCCC		0.0047
MPJ6_2D6002	*2, rs1080995, Ref. 6	Intron 1	1833	214 (IVS1 + 34)	GGCGGCAGAGGT**G/C**CTGAGGCTCCCC		0.1831
MPJ6_2D6003	*2, rs1080996, Ref. 6	Intron 1	1840	221 (IVS1 + 41)	GAGGTGCTGAGG**C/A**TCCCCTACCAGA		0.1831
MPJ6_2D6004	*2, rs1080997, Ref. 6	Intron 1	1842	223 (IVS1 + 43)	GGTGCTGAGGCT**C/G**CCCTACCAGAAG		0.1831
MPJ6_2D6005	*2, rs1080998, Ref. 6	Intron 1	1846	227 (IVS1 + 47)	CTGAGGCTCCCC**T/G**ACCAGAAGCAAA		0.1831
MPJ6_2D6006	*2, rs1080999, Ref. 6	Intron 1	1851	232 (IVS1 + 52)	GCTCCCCTACCA**G/C**AAGCAAACATGG		0.1831
MPJ6_2D6007	*2, rs1080999, Ref. 6	Intron 1	1852	233 (IVS1 + 53)	CTCCCCTACCAG**A/C**AGCAAACATGGA		0.1831
MPJ6_2D6008	*2, rs1081000, Ref. 6	Intron 1	1864	245 (IVS1 + 65)	AAGCAAACATGG**A/G**TGGTGGGTGAAA		0.1831
MPJ6_2D6068	rs1966157	Intron 1	1889	270 (IVS1 + 90)	CCACAGGCTGGA**C/T**CAGAAGCCAGGC		0.0047
MPJ6_2D6009	rs1081002, rs1966156	Intron 1	1929	310 (IVS1 + 130)	GGTTTGGGGGAC**G/T**CCTGGAGAAGGG		0.4977
MPJ6_2D6069	rs1966155	Intron 1	1936	317 (IVS1 + 137)	GGGACGTCCTGG**A/G**GAAGGGCATTTA		0.0047
MPJ6_2D6070	rs1966154	Intron 1	1942	323 (IVS1 + 143)	TCCTGGAGAAGG**G/A**CATTTATACATG		0.0047
MPJ6_2D6071	Novel	Intron 1	2270	651 (IVS1 − 233)	ACTGGGGCCTCT**C/T**GGCAATTTTGGT		0.0023
MPJ6_2D6010	Ref. 7	Intron 1	2365	746 (IVS1 − 138)	ACCCCCGCCCCA**C/G**ATCAGGAGGCT		0.1878

SNP name			Position				
SNP ID	CYP Allele Nomenclature or NCBI dbSNP or Reference	Location	Position in accession number, M33388	Relative to the translation initiation site[a] or from the nearest exon	SNP and the surrounding sequence (5′ to 3′)	Amino acid change	Frequency
MPJ6_2D6012	rs769261, Ref. 7	Intron 1	2462	843 (IVS1 − 41)	TGTAGTCTGGGGT/GGATCCTGGCTTG		0.5070
MPJ6_2D6073	Novel (**48*)	Exon 2	2591	972	CCGTGCGCGAGGC/TGCTGGTGACCCA	A90V	0.0023
MPJ6_2D6013	**10*, rs5030864	Exon 2	2658	1039	GATCCTGGGTTTC/TGGGCCGCGTTCC		0.3028
MPJ6_2D6074	Novel (**49*)	Exon 3	3230	1611	CCCCCAGGGGTGT/ATCCTGGCGCGCT	F120I	0.0047
MPJ6_2D6014	**2*, **10* rs1058164	Exon 3	3280	1661	GCGCTTCTCCGTG/CTCCACCTTGCGC		0.4930
MPJ6_2D6075	Novel (**50*)	Exon 3	3339	1720	GGGTGACCGAGGA/CGGCCGCCTGCCT	E156A	0.0023
MPJ6_2D6015	**14*	Exon 3	3377	1758	GCCAACCACTCCG/AGTGGGTGATGGG	G169R	0.0070
MPJ6_2D6076	Novel	Intron 3	3383	1746 (IVS3 + 6)	CACTCCGGTGGGT/AGATGGGCAGAAG		0.0023
MPJ6_2D6016	Ref. 7	Intron 3	3409	1790 (IVS3 + 32)	GCACAAAGCGGGA/GACTGGGAAGGCG		0.0117
MPJ6_2D6078	Novel	Intron 4	3922	2303 (IVS4 − 138)	GGTGGAGGCTGGC/TACTTGGGGAGGG		0.0141
MPJ6_2D6079	Novel	Intron 4	3981–3992 12bp deletion	2361–2373 (IVS4 − 79_ − 68)	CTGGGTCTACCTGGAGATGGCTGG/− GGCCTGAGACTT		0.0023
MPJ6_2D6017	rs2267444, Ref. 7	Intron 4	4043	2424 (IVS4 − 17)	GGATTGAGACCCC/GGTTCTGTCTGGT		0.0188
MPJ6_2D6018	Ref. 7	Intron 4	4043	2424 (IVS4 − 17)	GGATTGAGACCCC/TGTTCTGTCTGGT		0.0117
MPJ6_2D6088	rs1058169	Exon 5	4089	2470	CGTCCTCCTGCAT/CATCCCAGCGCTG		0.0023
MPJ6_2D6019	**21*	Exon 5	4192	2573	GACCCAGCCCAG−/CCCCCCCCGAGACC	Frameshift	0.0070
MPJ6_2D6020	Ref. 7	Intron 5	4282	2663 (IVS5 + 46)	GGTGGGTTGAGCG/ATCCCAGGAGGAA		0.0469
MPJ6_2D6080	Novel	Intron 5	4339	2720 (IVS5 − 88)	GCATCACCCGGCG/AAGCCGCATCTGG		0.0023
MPJ6_2D6021	Ref. 7	Intron 5	4378	2759 (IVS5 − 49)	AATTGGAGGTCAT/CTTGGGGGCTACC		0.0141
MPJ6_2D6022	**2*, rs4994476	Exon 6	4557	2850	GATGAGAACCTGC/TGCATAGTGGTGG	R296C	0.1831
MPJ6_2D6081	**44*, Ref. 9	Intron 6	4569	2950 (IVS6 + 1)	CGGATGTGCAGCG/CTGAGCCCATCTG	Splicing defect	0.0023
MPJ6_2D6082	Novel (**51*)	Exon 7	4791	3172	GTGTCCAACAGGA/CGATCGACGACGT	E334A	0.0023
MPJ6_2D6023	rs9611741, Ref. 7	Intron 7	4857	3384 (IVS7 + 40)	CTCAGCACCAGCA/CCCTGGTGATAGC		0.5047
MPJ6_2D6024	Ref. 7	Intron 7	5027	3408 (IVS7 + 64)	ATGCCACCACACT/CGACTGTCCCCAC		0.0023
MPJ6_2D6083	Novel	Intron 7	5094	3475 (IVS7 + 131)	ACACTGACTGTCC/TCCACTTGGGTGG		0.0023
MPJ6_2D6084	Novel	Intron 7	5201	3582 (IVS7 + 238)	TTGGAGGACCCAA/GCGCCTGCAGGGA		0.3146
MPJ6_2D6085	**2*	Intron 7	5203	3584 (IVS7 + 240)	GGAGGACCCAACG/ACCTGCAGGGAGA		0.1876
MPJ6_2D6086	Novel	Intron 7	5222	3603 (IVS7 − 196)	GGGAGAGGGGGCA/GGTGTGGGTGCCT		0.0023
MPJ6_2D6025	rs4987144	Intron 7	5409	3790 (IVS7 − 9)	CACCCTGCATCTC/TCTGCCCAGGGAA		0.1831
MPJ6_2D6087	Novel	Intron 8	5698	4019 (IVS8 − 20)	CAGGCTCACTGAC/TGCCCCTCCCCTC		0.0023
MPJ6_2D6026	**18*	Exon 9	5744–5752	4125–4133	TTCAGCTTCTCGGTGCCCACT/GTGCCCAC TGTGCCCACTGGACAGCCCCGGC	ins VPT	0.0023
MPJ6_2D6027	**2*, **10*, rs2103556	Exon 9	5799	4180	CTTTCCTGGTGAG/CCCCATCCCCCTA	S486T	0.4977
MPJ6_2D6038	**5*	Whole Deletion					0.0352

[a] A of the translation initiation codon ATG is numbered +1.
[b] This SNP position is at nucleotide 2431 in a reference sequence, AY545216.
[c] Accurate frequency could not be evaluated due to the surrounding repeat sequences.

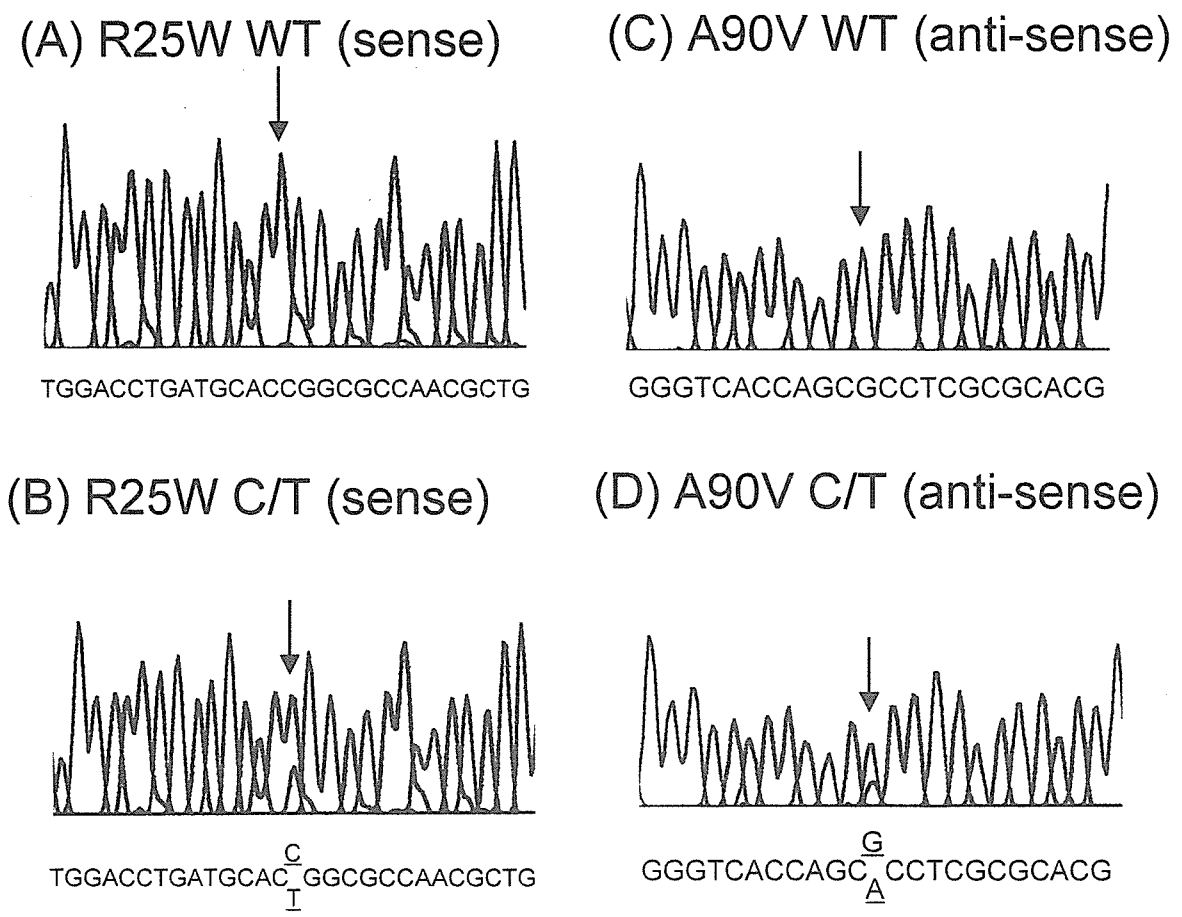

Fig. 1.

DPKup, and DPKlow primers as described by the method of Hersberger *et al.*,[4] which identifies the *CYP2D6*5* allele. The PCR product containing non-*5 5-kb fragment, was purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and subjected to a second round PCR for the analysis of all the *CYP2D6* exons.

The second round PCR for 5′-flanking region and all exons was conducted by the same method as described previously.[5] PCR amplification for the 5′-flanking subregions was done as follows: 94°C for 5 min, followed by 30 cycles consisting of 94°C for 30 sec, 55°C for 1 min and 72°C for 2 min, then, 72°C for 7 min, and kept at 4°C. Cycle sequencing after the incubation with exonuclease I (1 μL) and shrimp alkaline phosphatase (1 μL) was done according to manufacturer's instruction. After cycling, the products were purified and separated on the ABI PRISM 3700 DNA autosequencer (Applied Biosystems, Foster City, CA, USA). Sequencing was done on both strands without any contradiction.

Results and Discussion

We amplified a 5′-flanking region and all exons of *CYP2D6* gene by the two-step PCR and the resultant PCR products were subjected to sequencing as described under Materials and Methods. Sequencing the PCR products resulted in finding 70 sequence variations in the *CYP2D6* gene (accession number, M33388) of the 254 Japanese individuals as described in **Table 2.** Besides the 70 variations, we detected the conversion to *CYP2D7* in exon 9, which was a key variation of *CYP2D6*36*, in 37 samples (All were heterozygotes.). Proposal of Johansson *et al.*[10] that a tandem arrangement of Ch_1 (*CYP2D6*10B*) and Ch_2 (formerly *CYP2D6*10C*) was associated with *CYP2D6*36* suggested that *CYP2D6*36* haplotype contained more than 2 *CYP2D6* gene copies. Since assignment of SNPs found in the *CYP2D6*36*-possessing patients to either of the two copies is difficult, these 37 samples were excluded from the current calculation of SNP frequencies described in **Table 2.** Furthermore, 19 subjects were judged to possess *CYP2D6*5* according to the method by Hersberger *et al.*[4] We found, however, that 4 of the

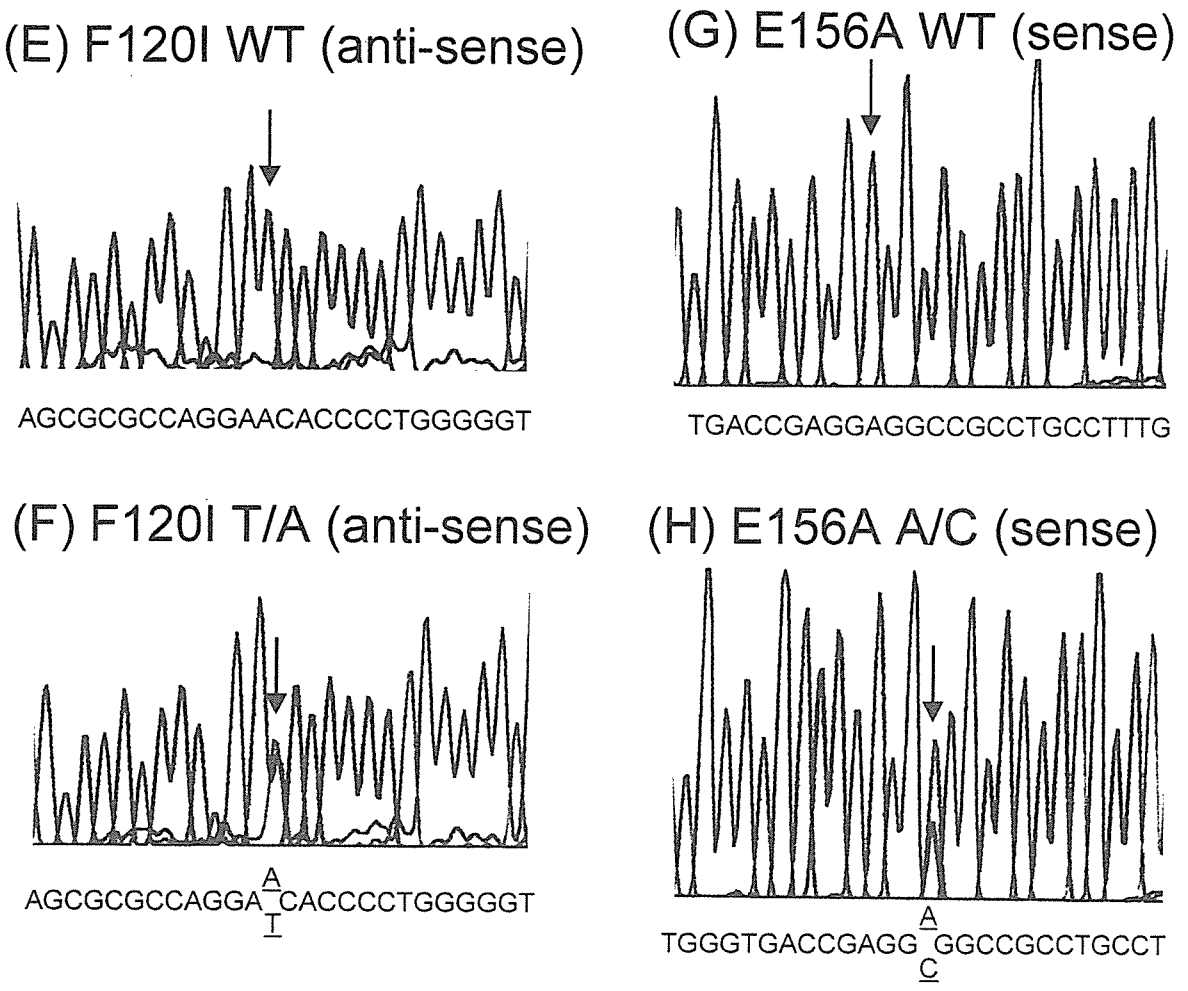

Fig. 1.

19 subjects possessed heterozygous SNPs. This suggested that the 4 subjects possessed aberrant **5* (probably two *CYP2D6* copies), though they were initially judged to have the *CYP2D6*5* haplotype. Thus, we also excluded these 4 subjects from the calculation of SNP frequencies (**Table 2**). Recently, Ishiguro *et al.* have reported similar observation.[11]

Among the 5 nonsynonymous SNPs (**Table 2**), the 1611T>A (F120I, MPJ6_2D6074) was heterozygously detected in an individual who was a homozygous *CYP2D6*10* subject. The 1720A>C (E156A, MPJ6_2D6075) was heterozygously detected in a homozygous **1* individual. The 972C>T, (A90V, MPJ6_2D6073) was found in a subject with **1/*10* genotype. Cloning and sequencing of DNA fragments obtained from the 972C>T subject revealed that 100C and 972T located on the same DNA strand, indicating the association of the MPJ6_2D6073 with the **1* haplotype. Allele-specific PCR analysis revealed that 3172A>C (E334A, MPJ6_2D6082), which was found in a subject with **1/*2* genotype, was associated with *CYP2D6*2* haplotype. Similarly, by allele-specific PCR analysis, 73C>T (R25W, MPJ6_2D6065) was shown to be linked with C100T, a key SNP of **10* allele. For all the nonsynonymous SNPs, electropherograms are shown for each SNP and the SNP and surrounding nucleotide sequences are written in **Fig. 1** (A–J).

Functional characterization of these SNPs is very important especially for those which resulted in the changes of their electronic charges (e.g. E156A and E334A), or for those which are associated with the **10* haplotype (e.g. R25W and F120I).

The known *CYP2D6* alleles, so far observed specifically in Japanese and/or Chinese populations, **14* (1758G>A, G169R), **18* (9 bp insertion, insertion of VPT in exon 9), **21* (2573 ins C, frameshift), and **44* (IVS6+1 G>C, aberrant splicing) were also found, but their frequencies were very low (3/426, 1/426, 3/426 1/426), respectively.

In conclusion, we detected 70 sequence variations in the *CYP2D6* gene including 5 novel nonsynonymous SNPs. These amino acid alterations should further be

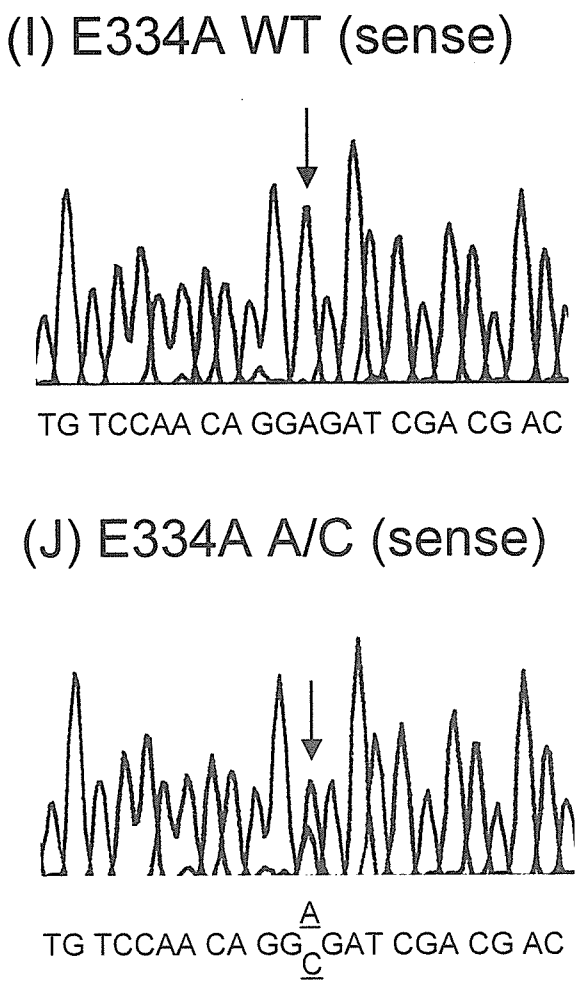

Fig. 1. Electropherogram of 5 nonsynonymous SNPs and their flanking sequences.

The electropherograms are shown in forward or reverse orientation as indicated. Arrows indicate the polymorphic and heterozygous positions and the heterozygous nucleotides are underlined. Bases colored with green, blue, black, and red represent adenine, cytosine, guanine, and thymine, respectively.

evaluated for the influence on catalytic activity, as low activity haplotypes of *CYP2D6* have not been fully elucidated.

Note Added in Proof

The 5 *CYP2D6* haplotypes that possess each of 73C>T (R25W), 972C>T(A90V), 1661T>A (F120I), 1720C>A (E156A), and 3172A>C (E334A) were assigned as **47*, **48*, **49*, **50*, and **51*, respectively, by the CYP Allele Nomenclature Committee (http://www.imm.ki.se/CYPalleles).

References

1) Rendic, S.: Summary of information on human CYP enzymes. *Drug Metab Rev.*, **34**: 83–448 (2002).
2) Daly, A. K., Brockmoller, J., Broly, F., Eichelbaum, M., Evans, W. E., Gonzalez, F. J., Huang, J.-D., Idle, J. R., Ingelman-Sundberg, M., Ishizaki, T., Jacqz-Aigrain, E., Meyer, U. A., Nebert, D. W., Steen, V. M., Wolf, C. R. and Zanger, U. M.: Nomenclature for human *CYP2D6* alleles. *Pharmacogenetics*, **6**: 193–201 (1996).
3) Meyer, U. A. and Zanger, U. M.: Molecular mechanisms of genetic polymorphisms of drug metabolism. *Annu. Rev. Pharmacol. Toxicol.*, **37**: 269–296 (1997).
4) Hersberger, M., Marti-Jaun, J., Rentsch, K. and Hanseler, E.: Rapid detection of the *CYP2D6*3*, *CYP2D6*4*, and *CYP2D6*6* alleles by tetra-primer PCR and of *CYP2D6*5* allele by multiplex long PCR. *Clin. Chem.*, **46**: 1072–1077 (2000).
5) Ishida, S., Soyama, A., Saito, Y., Murayama, N., Saeki, M., Sai, K., Ozawa, S. and Sawada, J.: Determination of CYP2D6 gene alleles by the CYP450 probe array using the Affymetrix GeneChip system: Comparison with sequencing results. *Drug Metabol. Pharmacokinet.*, **17**: 157–160 (2002).
6) Johansson, I., Lundqvist, E., Bertilsson, L., Dahl, M., Sjoqvist, F. and Ingelman-Sundberg, M.: Inherited amplification of an active gene in the cytochrome P450 *CYP2D* locus as a cause of ultrarapid metabolism of debrisoquine. *Proc. Natl. Acad. Sci. USA.*, **90**: 11825–11829 (1993).
7) Soyama, A., Saito, Y., Komamura, K., Ueno, K., Kamakura, S., Ozawa, S. and Sawada, J.: Novel single nucleotide polymorphisms in the *CYP2D6* gene associated with *CYP2D6*2* and/or *CYP2D6*10* alleles. *Drug Metabol. Pharmacokinet.*, **17**: 475–478 (2002).
8) Lovlie, R., Daly, A. K., Matre, G. E., Molven, A. and Steen, V. M.: Polymorphisms in the *CYP2D6* duplication-negative individuals with the ultrarapid metabolizer phenotype: a role for the *CYP2D6*35* allele in ultrarapid metabolism. *Pharmacogenetics*, **11**: 45–55 (2001).
9) Yamazaki, H., Kiyotani, K., Tsubuko, S., Matsunaga, M., Fujieda, M., Saito, T., Miura, J., Kobayashi, S. and Kamataki, T.: Two novel haplotypes of *CYP2D6* gene in a Japanese population. *Drug Metab. Pharmacokinet.*, **18**: 269–271 (2003).
10) Johansson, I., Oscarson, M., Yue, Q.-Y., Bertilsson, L., Sjoqvist, F. and Ingelman-Sundberg, M.: Genetic analysis of the Chinese cytochrome *P4502D* locus: Characterization of variant *CYP2D6* genes present in subjects with diminished capacity for debrisoquine hydroxylation. *Mol. Pharmacol.*, **46**: 452–459 (1994).
11) Ishiguro, A., Kubota, T., Sasaki, H. and Iga, T.: A long PCR assay to distinguish *CYP2D6*5* and a novel *CYP2D6* mutant allele associated with 11 kb *Eco*RI haplotype. *Clin. Chim. Acta*, *in press* (2004).

Drug Metab. Pharmacokin. 19 (4): *SNP14* (308)-*SNP18* (312) (2004).

SNP Communication

Seven Novel Single Nucleotide Polymorphisms in the Human SLC22A1 Gene Encoding Organic Cation Transporter 1 (OCT1)

Masaya ITODA[1], Yoshiro SAITO[1,2], Keiko MAEKAWA[1,2], Hiroyuki HICHIYA[1], Kazuo KOMAMURA[3,4], Shiro KAMAKURA[3], Masafumi KITAKAZE[3], Hitonobu TOMOIKE[3], Kazuyuki UENO[5], Shogo OZAWA[1,6] and Jun-ichi SAWADA[1,2]

[1]*Project team for Pharmacogenetics,* [2]*Division of Biochemistry and Immunochemistry,* [6]*Division of Pharmacology, National Institute of Health Sciences, Tokyo, Japan* [3]*Department of Cardiology,* [4]*Department of Cardiovascular Dynamics Research Institute, National Cardiovascular Center, Suita, Japan* [5]*Department of Pharmaceutical Sciences, Niigata University of Pharmacy and Applied Life Sciences, Niigata, Japan*

Full text of this paper is available at http://www.jssx.org

Summary: Twenty genetic variations, including seven novel ones, were found in the human *SLC22A1* gene, which encodes organic cation transporter 1, from 116 Japanese individuals. The novel variations were as follows: −94C>A in the 5′-untranslated region (A of the translation start codon is numbered +1 in the cDNA sequence; MPJ6_OC1001), 350C>T (MPJ6_OC1004), IVS1−35T>C (MPJ6_OC1006), 561G>A (MPJ6_OC1010), IVS6+75C>G (MPJ6_OC1014), IVS8+108A>G (MPJ6_OC1017), and 1671_1673delATG (MPJ6_OC1020). The frequencies were 0.082 for IVS1-35T>C, 0.022 for IVS6+75C>G, 0.009 for 561G>A, and 0.004 for the other 4 variations. Among them, 350C>T resulted in the amino acid substitution Pro117Leu, which is located in the large extracellular loop between transmembrane domains 1 and 2. Also, we detected the four previously reported nonsynonymous variations, 123C>G (Phe41Leu), 480C>G (Phe160Leu), 1022C>T (Pro341Leu), and 1222A>G (Met408Val) with frequencies of 0.004, 0.086, 0.168, and 0.810, respectively.

Key words: ***SLC22A1*** **(OCT1); nonsynonymous alteration; intron; novel SNP**

Introduction

Human polyspecific organic cation transporter 1 (OCT1), encoded by *SLC22A1*, belongs to the largest superfamily of transporters, the solute carrier family.[1] This transporter is predicted to have 12 transmembrane domains and translocates a wide variety of organic cations including drugs, toxins, and neurotransmitters in an electrochemical potential-dependent manner.[1,2] Since the OCT1 is primarily expressed in the liver (probably in the sinusoidal (basolateral) membrane of hepatocytes), it is expected to play a fundamental role in the uptake of substrates into the liver.[1-4] Indeed, *SLC22A1*-knockout mice exhibited decreased liver accumulation of an anti-tumor drug metaiodobenzylguanidine and an antidiabetic drug metformin.[5,6]

SLC22A1, located on chromosome 6q26, is adjacent to *SLC22A2* (encoding OCT2) and *SLC22A3* (encoding OCT3), and consists of 11 exons spanning approximately 37 kb.[7] Several genetic polymorphisms have been found in *SLC22A1* in different ethnic groups. From Caucasians, Kerb *et al.* found 25 polymorphisms, including 8 nonsynonymous ones, three of which showed reduced activities.[8] Shu *et al.* detected 15 nonsynonymous polymorphisms from 5 different ethnic groups and assessed their functional effects on 1-methyl-

On April 12th, 2004, these variations were not found in the Japanese Single Nucleotide Polymorphisms (JSNP) (http://snp.ims.u-tokyo.ac.jp/), dbSNP in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/SNP/), or PharmGKB (http://www.pharmgkb.org/do/) databases.

This study was supported by the Program for the Promotion of Fundamental Studies in Health Sciences (MPJ-3 and 6) of the Pharmaceuticals and Medical Devices Agency (PMDA) of Japan.

Received; April 15, 2004, Accepted; May 12, 2004

To whom correspondence should be addressed: Yoshiro SAITO, Ph.D., *Division of Biochemistry and Immunochemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel. +81-3-3700-9453, Fax. +81-3-5717-3832, E-mail: yoshiro@nihs.go.jp*