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Regular Article

*Novel Structure of the CYP2D6 Gene that Confuses Genotyping for the CYP2D6*5 Allele*

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Summary: We encountered DNA samples which showed a positive product using a long PCR-based method for the detection of *CYP2D6*5*, indicating deletion of the entire *CYP2D6* gene, but the samples did not show a band related to *CYP2D6*5* in either *Xba*I- or *Eco*RI-RFLP analysis. To achieve genotyping with accuracy, we performed a further genetic analysis to clarify the discrepancy. An unknown 1.6-kb insert was identified in a region downstream from the *CYP2D6* stop codon where a specific primer was designed for long-PCR analysis for *CYP2D6*5* genotyping. This finding suggested that the *CYP2D6* gene might not be deleted in the samples even if a positive product was detected by the long-PCR method. Furthermore, the allelic frequency of this type was found to be approximately 0.3% (4 heterozygous/771 samples) in a Japanese population. In conclusion, we found a novel structure of the *CYP2D6* gene, which might lead to incorrect genotyping for *CYP2D6*5*. Although the long PCR-based strategy for the detection of *CYP2D6*5* has been widely used due to its usefulness and convenience, we recommend caution when adopting this method and propose re-evaluating the method for detecting *CYP2D6*5*.

Key words: CYP2D6; genetic polymorphism; *CYP2D6*5*; genotyping

Introduction

CYP2D6 metabolizes many clinically important drugs, including antidepressants, neuroleptics, β-blockers and antiarrhythmics.[1] Individuals with an impaired ability to metabolize various probe drugs for *CYP2D6*, such as debrisoquine and sparteine, are classified as poor metabolizers (PM). There are important interethnic differences in the frequency of PMs. In Caucasian populations, the frequency of PMs is 5–10%, quite high in comparison with the frequencies (0–4%) observed in Chinese and Japanese.[2] The homozygous presence of defective *CYP2D6* alleles, such as *CYP2D6*4* and *CYP2D6*5*, results in a lack of *CYP2D6* enzymatic activity. *CYP2D6*5* is the most frequent of the PM-related alleles in the Japanese population. Therefore, it is necessary to detect the allele in the genotyping of *CYP2D6*. Southern blot analysis with a *CYP2D6cDNA* probe has been performed to detect the *CYP2D6*5* allele because the *CYP2D6* gene does not exist on the allele. However, this method is time-consuming and requires relatively large amounts of genomic DNA from each subject. Therefore, a long-PCR based assay with specific primers was needed and has been used in pharmacogenetic studies on *CYP2D6* substrates. Several sets of primers have been reported.[3–5]

In our laboratory, a long-PCR based assay was adopted with two different sets of primers, primers D1 and D2 (primers D1/D2)[4] and primers 13 and 24 (primers 13/24)[3] for strict analysis (**Fig. 1A**). Two fragments, 6.0 kb and 3.5 kb, indicative of the presence of the *CYP2D6*5* allele, are amplified by long-PCR, both with primers D1/D2 and with primers 13/24, respectively. Recently, we encountered DNA samples that showed different results using the two sets of primers in the above-mentioned method for *CYP2D6*5* screening. That is, a positive product was obtained by long-PCR analysis with primers 13/24, but not with primers D1/D2. We have identified four samples out of

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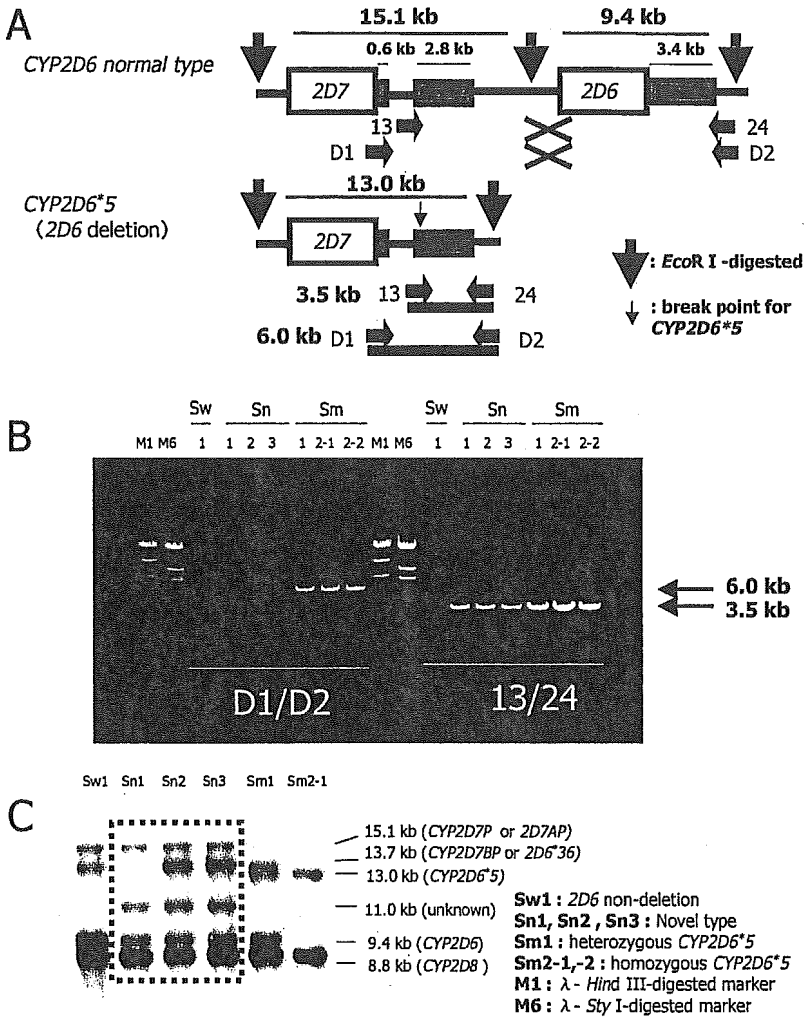

Fig. 1. Methods for detecting *CYP2D6*5* and identifying unknown fragments
A) Two methods of detecting *CYP2D6*5* using long-PCR: Two sets of primers are usually used for *CYP2D6*5* in our laboratory. Arrows show specific primers (primers 13/24 and primers D1/D2). The *CYP2D6*5* allele could be screened rapidly. A gray box indicates the 3.4-kb region which is almost completely identical downstream of both *CYP2D7* and *CYP2D6*. The 3.4-kb homologous region downstream of *CYP2D7* is divided into 0.6-kb and 2.8-kb regions by an insertion of about 1.6 kb. B) Different results using two sets of primers: We identified a sample whose results from the two sets of primers were not consistent. The 3.5-kb product was detected despite a negative finding for the 6.0-kb product. We have observed this in four out of 771 samples so far. C) Southern blot analysis of genomic DNA with *Eco*RI using the *CYP2D6cDNA* probe: The 13.0-kb fragment, indicating deletion of the *CYP2D6* gene, was not detected from the three samples positive for the 3.5-kb PCR product. However, an unknown 11-kb fragment was detected. From the three samples, two novel alleles were detected by *Eco*RI-RFLP analysis. Sn1 showed unknown 11.0-kb band in addition to 15.1-kb, 9.4-kb, and 8.8-kb bands. The other two samples, Sn2 and Sn3, showed a 13.7-kb band suggestive of *CYP2D6*36*(**10C*) or *CYP2D7BP* located between *CYP2D7* and *CYP2D6*.

over 700 samples. To achieve accurate genotyping, we attempted to resolve the discrepancy by using a step by step genetic analysis.

Methods

Samples: All subjects in the study were healthy males who had enrolled for some clinical trials. The protocol for studies of metabolizing enzymes was approved by the local ethics committee, and the study was performed according to public ethics guidelines for human genome/gene analysis research in Japan. Written informed consent for genetic analysis was obtained from each subject. Genomic DNA was isolated from the peripheral lymphocytes of subjects using the QIAGEN Blood kit (QIAGEN, Tokyo, Japan).

Detection of *CYP2D6*5*: Two different sets of primers, primers D1/D2[4] and primers 13/24[3] were adopted for the detection of *CYP2D6*5* (**Table 1**) (**Fig. 1A**). The reaction mixture contained 1.1 mM Mg $(OAc)_2$, 1*XL buffer II (Applied Biosystems, Tokyo, Japan), 0.2 mM of each dNTP, 3 pmol of each primer, 75 ng of genomic DNA and 1 U of rTth DNA polymerase (Applied Biosystems, Tokyo, Japan), with a total volume of 25 μL. The reaction profile was 35 cycles

Table 1. Primers used in the current investigation

Primer name	Sequence
For the detection of *CYP2D6*5*	
2D6 Dl	5′-GCCACTCTCGTGTCGTCAGCTTT-3′
2D6 D2	5′-GGCATGAGCTAAGGCACC-3′
2D6 13	5′-ACCGGGCACCTGTACTCCTCA-3′
2D6 24	5′-GCATGAGCTAAGGCACCCAGAC-3′
For new structure	
2D6 15	5′-CGTCTAGTGGGGAGACAAAC-3′
2D6 24	5′-GCATGAGCTAAGGCACCCAGAC-3′
For sequencing	
Forward primer	
13f	pCR2.1-TOPO vector primer
2D6 15-2	5′-CTCATCACCAACCTGTCATC-3′
2D6 15-3	5′-TCTAATGTACAATAAAGCAA-3′
2D6 15-4	5′-TGTCAGTCAGCCCTGGATGT-3′
2D6 13	5′-ACCGGGCACCTGTACTCCTCA-3′
2D6 13-2	5′-AGGTGCTAAGCCCCTCACTG-3′
2D6 13-3	5′-CCCCTGACCAGTGACGAGTT-3′
2D6 13-4	5′-TCCTTGCTCTGCCATTACCC-3′
Reverse primer	
13r	pCR2.1-TOPO vector primer
2D6 24-2	5′-TTAGGTTTAGGTGTTGACAG-3′
2D6 24-3	5′-GCCCAGTCTGCCCTCTTATT-3′
2D6 24-4	5′-AAGTTGCTGCGTATCCATCT-3′
2D6 24-5	5′-GCTGCCTGTTTCTACTTTGC-3′
2D6 b-657	5′-GAGCCAAACAAGTGTCCT-3′
2D6 13RA	5′-TGAGGAGTACAGGTGCCCGGT-3′
2D6 13RA-2	5′-AAATACACCAATCAGCACTC-3′
2D6 13RA-3	5′-AATCAGCAGGATGTGGGTGG-3′

of 30 sec at 94°C, 30 sec at 66°C and 5 min at 68°C. PCR products were separated by electrophoresis in 1.0% agarose.

Southern blot analysis: *Eco*R I-RFLP and *Xba* I-RFLP analysis was carried out with *CYP2D6cDNA* as a probe using the DIG system (Roche, Tokyo, Japan).[6] In addition, *Bam*H I was also used for Southern blot analysis to obtain additional information on the *CYP2D6* gene structure.

Approach using long-PCR for a novel gene structure: Primers 15 and 24 were used to amplify a region downstream of *CYP2D6* containing a putative inserted sequence (**Table 1**). The reaction mixture contained 1.1 mM Mg $(OAc)_2$, 1*XL buffer II (Applied Biosystems, Tokyo, Japan), 0.2 mM of each dNTP, 7.5 pmol of each primer, 75 ng of genomic DNA and 1 U of rTth DNA polymerase (Applied Biosystems, Tokyo, Japan), with a total volume of 25 μL. The reaction profile was 35 cycles of 30 sec at 94°C, 30 sec at 60°C and 5 min at 68°C. PCR products were separated by electrophoresis in 1% agarose.

Sequencing analysis: The PCR product obtained using the primers 15/24, which contains the unknown sequence, was purified with a QIAquick Gel Extraction Kit (QIAGEN, Tokyo, Japan) and was cloned using a TOPO TA Cloning Kit (Invitrogen, Tokyo, Japan). Sequencing was performed for the plasmid using a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Tokyo, Japan), with the primers listed in **Table 1**. An ABI PRISM310 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) was used for sequencing.

Results and Discussion

Southern blot analysis of genomic DNA was performed with *Xba* I or *Eco*R I using the *CYP2D6cDNA* probe to confirm the *CYP2D6* gene structure. The two *Xba* I fragments we have detected most frequently are 29 kb and 44 kb. The *Xba* I 29-kb fragment contains the two pseudogenes *CYP2D8* and *CYP2D7* in addition to the *CYP2D6* gene. DNA samples carrying the *Xba* I 29-kb band show three bands of 15.1 kb, 9.4 kb, and 8.8 kb, indicating *CYP2D7P/AP*, *CYP2D6*, and *CYP2D8*, respectively, when digested by *Eco*R I.[7] In the present study, we encountered an unknown 11.0-kb band in addition to the 15.1-kb, 9.4-kb, and 8.8-kb bands obtained by *Eco*R I-RFLP analysis, although a 13-kb band, suggestive of *CYP2D6*5*, was not detected.

Firstly, we analyzed three DNA samples from which we had obtained positive products by long-PCR with primers 13/14, but not with primers D1/D2. The three samples were named 'Sn1', 'Sn2' and 'Sn3', respectively in this paper (**Figs. 1B and C**). From the three samples, we found unknown 11.0-kb band in addition to 15.1-kb, 9.4-kb, and 8.8-kb bands by *Eco*R I-RFLP analysis, without the 13-kb band suggestive of *CYP2D6*5*. In addition, two samples, Sn2 and Sn3, showed a 13.7-kb band suggestive of *CYP2D6*36*(**10C*) or *CYP2D7BP* located between *CYP2D7* and *CYP2D6*. Consistent with the *Eco*R I 13.7-kb band, the *Xba* I 44-kb band was detected in the samples.[8,9]

Furthermore, *Bam*H I was used for Southern blot analysis to elucidate the cause of the 11.0-kb band. As a result of *Bam*H I-RFLP, one sample, Sn1, showed 4.9-kb, 1.7-kb, 2.2-kb and 1.9-kb bands in addition to 6.6-kb and 4.1-kb bands. Panserat *et al.* reported that 6.6-kb and 4.1-kb bands, suggestive of *CYP2D7* and *CYP2D6*, respectively, were usually detected by *Bam*H I-RFLP.[10] When a nucleotide substitution exists at the *Bam*H I recognition site in the *CYP2D7* or *CYP2D6* gene, either 4.9-kb and 1.7-kb bands or 2.2-kb and 1.9-kb bands are detected instead of the 6.6-kb or 4.1-kb band, respectively. This result suggests that at least two *CYP2D7* genes and two *CYP2D6* genes exist. Sn1 appeared to have only the 29-kb band as detected by *Xba* I-RFLP probably due to the limited sensitivity although a 30.6-kb band was expected to be detected for the novel allele. *Bam*H I-RFLP and *Xba* I-RFLP analysis suggested that the gene structure of the sample had two *CYP2D7-CYP2D6* alleles. In other words, the sample did not have the *CYP2D6*5* allele, although a

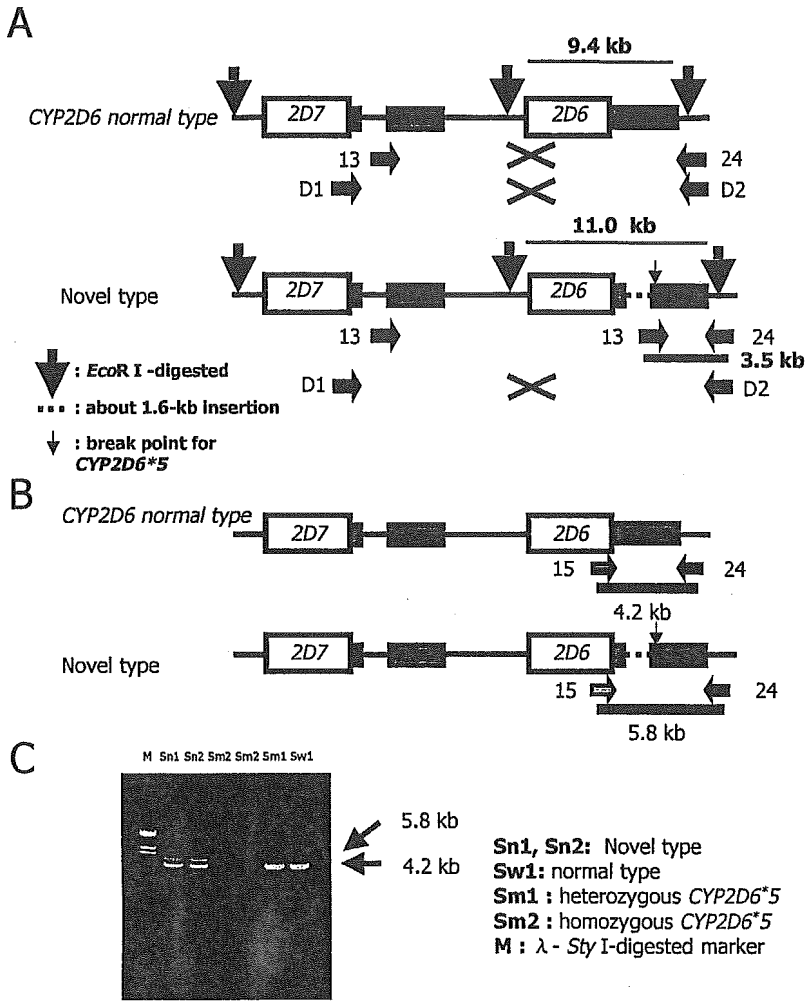

Fig. 2. Analysis for a novel gene structure
A) Hypothesis of a novel gene structure: From the novel type of allele, an 11-kb fragment was detected by *Eco*RI analysis and a positive product was amplified by long-PCR with primers 13/24 but not primers D1/D2. B) Detection of the insertion in the novel allele: Primer 15 is a specific primer located in intron 8 of CYP2D6. If an inserted sequence exists, a 5.8-kb PCR product will appear. C) Confirmation of the PCR product related to the novel allele by electrophoresis: Both the 4.2-kb and 5.8-kb products were amplified from the samples. Neither products were amplified from the homozygote for *CYP2D6*5*. In addition, only a 4.2-kb product was amplified from the heterozygote for *CYP2D6*5*. The 5.8-kb product was directly sequenced by use of the primers listed in **Table 1**.

positive product was detected by the long-PCR with primers 13/24.

Based on these results, we hypothesized that an approximately 1.6-kb fragment was inserted into a region downstream of the *CYP2D6* gene and it created a novel *Eco*R I 11-kb fragment (**Fig 2A**). To confirm this hypothesis, we designed a new specific primer set (primer 15/24) with which the PCR product containing the inserted sequence would be amplified by long-PCR (**Fig. 2B**). As expected, two bands, 4.2 kb and 5.8 kb, were amplified using primers 15 and 24, and the results were confirmed by sequencing analysis (**Fig. 2B and 2C**) (**Table 1**). Finally, we identified the chimeric sequence consisting of exon 9 of *CYP2D6*, the region downstream from *CYP2D7* and the region downstream from *CYP2D6* in that order. We confirmed the sequence where primer 13 could anneal in the region downstream from *CYP2D7* in the chimeric sequence. This was why the positive product was obtained by long-PCR with primers 13/24. On the other hand, primers D1/D2 worked properly in the detection of *CYP2D6*5* because primer D1 was designed based on exon 9 of *CYP2D7*. We also confirmed that false-positive PCR products were detected by long-PCR with primers reported by Hersberger *et al.*[5] because the primer was designed based on the same region as primer 13.

The breakpoint related to *CYP2D6*5* was seen in the 5.8-kb fragment from the chimeric allele. The 3.5-kb PCR product from this chimeric allele was similar to that from *CYP2D6*5*. Therefore, we estimated that a

novel breakpoint is present upstream from the sequence where primer 13 anneals. It was also suspected that this chimeric allele was made from a gene conversion between the *CYP2D6*5* allele and wild-type allele at a region immediately downstream from the *CYP2D6* stop codon. We, however, could not specify the novel breakpoint because of the high homology between *CYP2D6* and *CYP2D7*.

A total of 771 samples have been assessed by long-PCR with the two sets of primers, 13/24 and D1/D2, so far. One sample was detected in addition to the three samples demonstrated above. Therefore, the frequency of the novel allele was estimated to be approximately 0.3% in the Japanese population. After we had demonstrated the estimated structure of this novel allele,[11] Ishiguro *et al.* independently reported the presence of the *Eco*R I-11kb fragment in one Japanese subject.[12] Their observation (n = 1) is probably consistent with our findings. The frequencies in other ethnic groups should be examined as well.

With respect to the activity of *CYP2D6*, the effect of the novel allele could not be evaluated clearly because all four subjects were heterozygous for the allele and no phenotyping test could be conducted. Furthermore, all of them were determined as *CYP2D6*10/*10* by the general genotyping procedure reported previously.[6] It, however, seems to us that this is not the null-allele of *CYP2D6*, such as *CYP2D6*5*, because the coding region of *CYP2D6* exists although this remains to be proven. Thus, it is necessary to characterize the allele with further phenotyping.

Overall, long-PCR analysis is a useful and convenient method to detect *CYP2D6*5*. In fact, this strategy for the detection of *CYP2D6*5* has been widely used as a standard since Steen *et al.* first reported the method.[3] At the moment, the primers provided by Johansson *et al.*[4] can be used to avoid a miscall for *CYP2D6*5* due to its novel structure, although the set of primers is less sensitive than the set provided by Steen *et al.*, and the product related to *CYP2D6*36* could be simultaneously detected by the set of primers as a different product from *CYP2D6*5*.[13] As a new typing strategy, a TaqMan-based assay was used to detect *CYP2D6*5*[14] although the system has not yet been used in a Japanese population.

In conclusion, we strongly recommend that at least two sets of primers, designed at different positions, be used for the detection of *CYP2D6*5*. However, another typing strategy for *CYP2D6*5*, superior to the long-PCR method, will be needed to make accurate decisions.

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ORIGINAL ARTICLE

CYP2A6 polymorphisms are associated with nicotine dependence and influence withdrawal symptoms in smoking cessation

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CYP2A6 is the main enzyme that catalyzes nicotine into cotinine. Interindividual differences in nicotine metabolism result at least partially from polymorphic variation of *CYP2A6* gene. In this study, we evaluated the influence of *CYP2A6* polymorphisms on clinical phenotypes of smoking, such as smoking habit and withdrawal symptoms. Japanese smokers ($n=107$) were genotyped for *CYP2A6*1*, **4* and **9*. Consistent with the previous reports, *CYP2A6* genotypes have a tendency to correlate with the number of cigarettes per day and with daily intake of nicotine. Interestingly, CYP2A6 high-activity group (*CYP2A6*1/*1*, **1/*9*, **1/*4*, **9/*9*) smoked the first cigarette of the day earlier than low-activity group (*CYP2A6*4/*9*, **4/*4*), indicating more remarkable nicotine dependence. Furthermore, nicotine withdrawal symptoms were more serious in smoking cessation in CYP2A6 high-activity group. Collectively, *CYP2A6* genotypes are related with nicotine dependence, influencing smoking habits and withdrawal symptoms in quitting smoking. It is proposed that individualized smoking cessation program could be designed based on *CYP2A6* genotypes.
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Keywords: CYP2A6; polymorphism; smoking; nicotine; individualized medicine

Introduction

Smoking is one of the most important risk factors for serious diseases, including cancers, chronic obstructive pulmonary diseases and cardiovascular diseases. It is strongly recommended that smokers should cease smoking for good health. However, there is an interindividual diversity in the difficulties in quitting smoking, mainly due to nicotine dependence. Therefore, to carry out smoking cessation program effectively, individual status of smoking should be estimated and, more importantly, predicted, based on nicotine dependence.

Nicotine is metabolized to cotinine, an inactive metabolite, principally by CYP2A6.[1] Several *CYP2A6* gene polymorphisms have been identified so far, and three alleles, **1*, **4* and **9*, are shown to be the major polymorphisms in Japanese. *CYP2A6*1* is a wild-type allele with normal enzyme activity. *CYP2A6*4* is a whole deletion type of the *CYP2A6* gene.[2,3] *CYP2A6*9* has a single-nucleotide polymorphism in TATA box, T-48G substitution, which impairs the transcriptional activities[4] and, consequently, its enzymatic activity.[5,6] It has been clearly demonstrated that the pharmacokinetics of nicotine is influenced by *CYP2A6* polymorphisms.

In the present study, as pharmacokinetic changes in plasma nicotine concentration are considered to be related with craving for nicotine,[7] we hypothesized that *CYP2A6* polymorphisms might affect smoking status. And we evaluated the relation between *CYP2A6* genotypes and smoking habits, including nicotine withdrawal symptoms, from the point of view of nicotine dependence. The data presented here provide insights into individualized smoking cessation program based on *CYP2A6* genotypes.

Results

First, we confirmed the relationship between the *CYP2A6* genotypes and the number of cigarettes in the subjects analyzed in the present study (Figure 1a). *In vivo* enzymatic activity of nicotine metabolism decreases in order, *CYP2A6*1/*1*, **1/*9*, **1/*4*, **9/*9*, **4/*9* and **4/*4*.[5] *CYP2A6* genotype, which determines the enzyme activity *in vivo*, had a tendency to be associated with the number of cigarettes smoked per day, as reported previously.[8–10] Next, the amounts of daily nicotine intake were also examined. As shown in Figure 1b, *CYP2A6* genotype is likely to be linked with daily nicotine intake, proposing the possible association between *CYP2A6* genotypes and nicotine dependence.

Association between *CYP2A6* genotypes and smoking habits, such as the number of the cigarettes per day and the daily nicotine uptake, was statistically analyzed. The subjects were divided into the high- and low-activity group, based on their *CYP2A6* genotypes, according to the previous study.[5] The subjects with the **1/*1*, **1/*9*, **1/*4* and **9/*9* genotypes, whose metabolic activities of nicotine are more than 70% of those of the subjects with **1/*1*, were defined as high-activity group, whereas the subjects with the **4/*9* and **4/*4* genotypes with less than 50% of metabolic activities of the subjects with **1/*1* as low-activity group. It was found that the associations of *CYP2A6* genotypes with the number of cigarettes or with the nicotine uptake approached statistical significance ($P=0.09$ or 0.06, respectively).

To evaluate nicotine dependence more directly, we analyzed the relation between *CYP2A6* genotypes and the time to the first cigarette as described in Materials and methods. As shown in Figure 2, proportion of subjects who smoked the first cigarette within 5 min of waking up was significantly higher in CYP2A6 high-activity group than in low-activity group (36.8%, $n=95$ and 8.3%, $n=12$, respectively, $P<0.05$), suggesting that the subjects with high CYP2A6 activity show the severer nicotine dependence than those with low activity.

Fagerstrom Test for Nicotine Dependence (FTND) is commonly performed to estimate nicotine dependence. Thus, the relationship between *CYP2A6* genotypes and nicotine dependence was evaluated according to FTND. Consistent with the results shown in Figure 2, there was significant association between the total score of FTND and CYP2A6 activity (3.95 ± 1.45 in high-activity group, 3.17 ± 0.94 in low-activity group, $P<0.05$) (Figure 3).

Finally, nicotine dependence was diagnosed according to the severity of withdrawal symptoms observed during smoking cessation. In the population of smokers who tried to quit smoking, withdrawal syndrome was categorized into

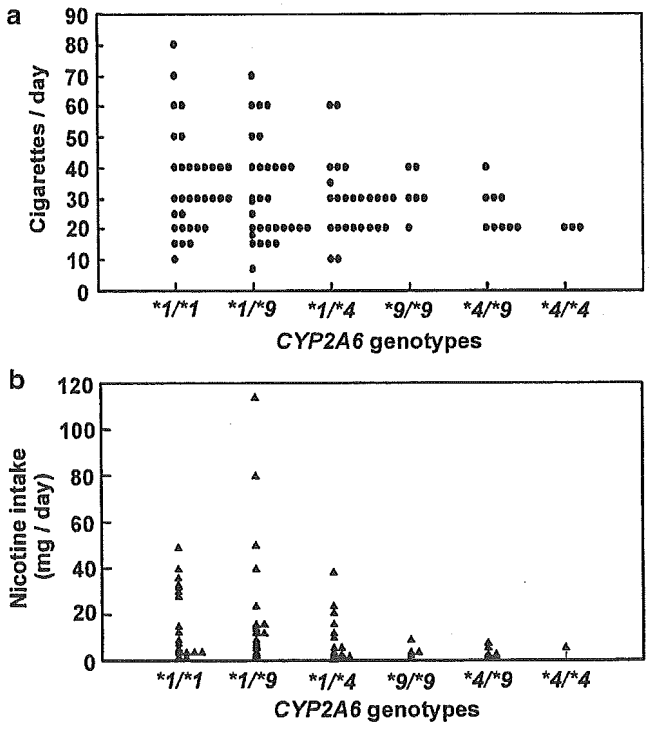

Figure 1 Relationship between the *CYP2A6* genotypes and the number of cigarettes smoked per day (**a**) or the amount of daily nicotine intake (**b**). *CYP2A6* genotypes had a tendency to be associated with the number of cigarettes smoked per day ($n=107$, $P=0.09$) and the amount of daily nicotine intake ($n=70$, $P=0.06$).

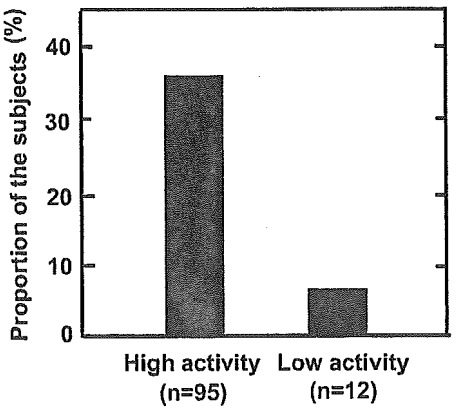

Figure 2 *CYP2A6* genotypes were related to time to the first cigarette of the day. The proportion of subjects who smoked the first cigarette within 5 min of waking up was calculated, as an index for nicotine dependence. The proportion of subjects was significantly higher in CYP2A6 high-activity group than in low-activity group. CYP2A6 high-activity group consists of subjects carrying *CYP2A6*1/*1*, **1/*9*, **1/*4* and *9/*9. Low-activity group consists of subjects carrying *CYP2A6*4/*9* and **4/*4*.

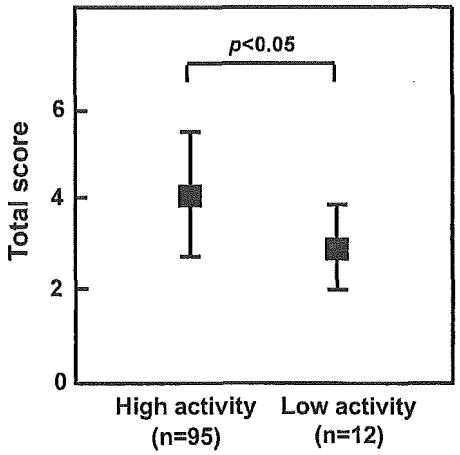

Figure 3 *CYP2A6* genotypes are associated with nicotine dependence, analyzed by Fagerstrom Test for Nicotine Dependence. A total score for nicotine dependence was calculated by total scores on self-reported number of cigarettes smoked per day and time to the first cigarette of the day. The score was significantly high in CYP2A6 high-activity group, compared to low-activity group. Data are shown as mean ± s.d.

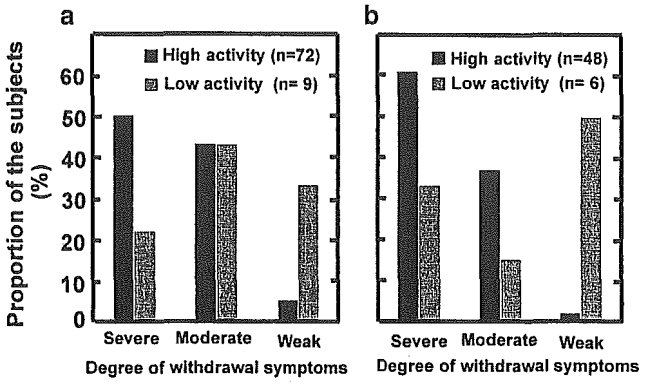

Figure 4 Impacts of *CYP2A6* genotypes on withdrawal symptoms in the subpopulation that tried to quit smoking. Degree of withdrawal symptoms was evaluated using a questionnaire. (a) In CYP2A6 high-activity group, the withdrawal symptoms were significantly more serious than the low-activity group. (b) Degree of withdrawal symptoms and *CYP2A6* genotypes in the population that tried to quit smoking by receiving nicotine replacement therapy. Among the population that tried to quit smoking by receiving nicotine replacement therapy, severe withdrawal symptoms were more remarkable in CYP2A6 high-activity group than in low activity group.

three groups: severe, moderate and weak. The proportion of subjects with severe withdrawal symptoms was higher in CYP2A6 high-activity group than in low-activity group (Figure 4a). The proportion of subjects was 50.0, 44.4 and 5.6% for severe, moderate and weak withdrawal symptoms, respectively, in high-activity group ($n=72$), and 22.2, 44.4 and 33.3%, respectively, in low-activity group ($n=9$) (χ^2 test; $P<0.05$). Furthermore, as nicotine replacement therapy affects the withdrawal symptoms, the degree of withdrawal symptoms was compared in the subpopulation that tried to quit smoking by receiving nicotine replacement therapy. The proportion of subjects was 60.4, 37.5 and 2.1% for severe, moderate and weak symptom, respectively, in high-activity group ($n=48$), and 33.3, 16.7 and 50.0%, respectively, in low-activity group ($n=6$) (χ^2 test; $P<0.01$; Figure 4b). To clarify the association between the CYP2A6 activities and nicotine dependence, the odds ratios (ORs) with 95% confidence intervals (CIs) were estimated relatively to the subjects with the weak withdrawal symptoms. In the total subjects, the ORs (95% CIs) were 6.0 (0.97–37.12, $P=0.128$) for the moderate and 13.5 (1.71–106.56, $P=0.025$) for the severe, respectively. In the subpopulation, they are 54.0 (2.61–1116.96, $P=0.0089$) for the moderate and 43.5 (2.99–633.62, $P=0.00341$) for the severe, respectively. Collectively, the relation between severities of withdrawal symptoms and *CYP2A6* genotypes is more clearly demonstrated in this subpopulation.

Discussion

In the present study, we have demonstrated that *CYP2A6* mutant allele with impaired or null enzyme activity was a negative risk factor for habit of smoking, especially nicotine dependence.

First, the number of cigarettes per day was likely to be associated with the activity of CYP2A6. Relation between the number of cigarettes and *CYP2A6* genotype has been analyzed in several studies, with inconsistent results. Some studies have also shown that subjects who possessed *CYP2A6* mutant allele smoked fewer cigarettes,[8–10] as is the case with the present study, whereas others reported that *CYP2A6* genotypes are not associated with cigarettes consumption in Japanese,[11–13] Chinese[14] and Caucasians.[15] As smoking behavior is also influenced by environmental factors, these conflicting results might be due to interindividual differences in the environmental factors including lifestyles. In this study, a majority of the subjects were working as 'white collar workers', so difference in environmental influence was expected to be minimized.

To our knowledge, this is the first report that evaluated the relationship between the time to the first cigarette of the day and *CYP2A6* genotypes. And it is revealed that the subjects with high-activity alleles of *CYP2A6* smoke the first cigarette earlier than those with low activity. Importantly, as the time to the first cigarette of the day is considered to be influenced by nicotine dependence, it is possible that CYP2A6 activity is related with nicotine dependence. To address this possibility, nicotine dependence was quantified by calculating the score on the number of cigarettes per day and the time to the first cigarette of the day, according to FTND score. These two items are most important factors of FTND score,[16] and are generally used in smoking cessation program. As a result, nicotine dependence was more remarkable in the subjects with CYP2A6 high activity than in those with low activity.

Finally, we investigated the relationship between nicotine withdrawal symptoms and *CYP2A6* genotypes. It was revealed that the subjects in CYP2A6 high-activity group

exhibited manifest withdrawal symptoms, which are clinical phenotypes derived from nicotine dependence in smoking cessation. Moreover, the correlation between *CYP2A6* genotypes and withdrawal symptoms is more remarkable in subjects who received nicotine replacement therapy. Recent studies have provided molecular and cellular aspects of nicotine abuse. From the neuroscientific point of view, withdrawal symptom is considered to be the process of the nicotinic acetylcholine receptor from desensitization/inactivation states to functional states.[17] Importantly, low concentrations of nicotine cause desensitization of its receptors. Therefore, the smokers with high CYP2A6 activity might maintain a low level of nicotine that may inactivate a larger number of nicotinic receptors, compared with those with low activity. As a result, after many hours of abstinence, an excessive number of desensitized/inactivated nicotine receptors may begin to recover to functional states in the smokers with high CYP2A6 activity, resulting in the severe withdrawal symptoms.

In the process of smoking cessation, a number of smokers receive nicotine replacement therapy. High dose of nicotine is administered, for example, with nicotine patch, at the starting point and the subjects gradually weaned themselves from nicotine by reducing the dosage according to the generalized cessation protocol. Considering that the subjects with high CYP2A6 activity are prone to nicotine dependence, it might be beneficial to individualize the protocol for nicotine replacement therapy. Theoretically, by reducing the dosage of nicotine more deliberately in the subjects with high activity than in those with low activity, the success rate in quitting smoking would be improved. At the same time, we have also noticed the limitation of the individualized program for smoking cessation based on *CYP2A6* genotypes alone. It is likely that other gene polymorphisms, in addition to *CYP2A6*, might be involved in nicotine dependence, because interindividual differences were not completely canceled by classifying the subjects based on *CYP2A6* genotypes. Further investigation may be required to understand the genetic background of the susceptibility to nicotine dependence.

In conclusion, we found that *CYP2A6* genotypes affect smoking habit, nicotine dependence, and withdrawal symptoms during smoking cessation. It could be proposed that *CYP2A6* genotyping may be a novel pharmacogenomic strategy for smoking cessation program as an individualized health care.

Materials and methods

Subjects

This study is designed as a multicenter trial. The study subjects consisted of 107 Japanese smokers who attended to a clinic for their health care. The patients with life-threatening diseases, including cancer, heart failure and symptomatic chronic obstructive pulmonary diseases, were excluded. All subjects gave their informed consent to participate in this study.

Table 1 Scoring for the degree of nicotine dependence, analyzed by the Fagerstrom Test for Nicotine Dependence (FTND)

Score	0	1	2	3
Number of cigarettes/day	~10	11–20	21–30	31~
First cigarette of the day (min)	61~	31–60	6–30	~5

~ = from XX to XY.

This study was approved by the institutional review committee of Osaka University.

Estimation of smoking status

All subjects were interviewed about their smoking habits such as the number of cigarettes per day, the nicotine content of the cigarettes, which they usually smoke, and time to the first cigarette of the day, which is generally accepted as a clinical index for nicotine dependence. Daily nicotine intake was calculated by multiplying the number of cigarettes per day by nicotine content of cigarette.

The total score for nicotine dependence was calculated by summing scores on two items that were extracted from FTND: 'the number of cigarettes smoked per day' and 'time to the first cigarette of the day' (Table 1).

In the subpopulation that tried to quit smoking ($n=81$), the degree of withdrawal symptoms was evaluated using a questionnaire. The degree of withdrawal symptoms was categorized into three groups: severe, moderate and weak.

Genotyping

Genomic DNA was extracted from blood using the QIAamp Blood Kit according to the manufacturer's protocol (Qiagen). The genotyping of *CYP2A6*4* was carried out by the PCR-RFLP method, according to the previous report.[18] The primers used for the PCR were as follows: forward – CAC CGA AGT GTT CCC TAT GCT G; reverse – TGT AAA ATG GGC ATG AAC GCC C. Genomic DNA samples (45 ng) were added to the 25-μl PCR mixtures that consisted of 0.2 μM each primer, PCR Gold Buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR was performed with an initial step at 94°C for 5 min, followed by 40 cycles at 95°C for 5 min, at 56°C for 1 min and at 72°C for 2 min, with a final extension at 72°C for 7 min. The PCR product was digested with *Eco*81I. The digestion patterns were analyzed by electrophoresis with 2% agarose gel. Mutation allele was identified from the fragment with 728 bp, whereas the wild-type allele was from that with 789 bp.

*CYP2A6*9* alleles were genotyped by the allele-specific PCR method reported previously,[5] with minor modification. The primers used for the PCR were as follows: forward – GAT TCC TCT CCC CTG GAA C, reverse-wild type: GGC TGG GGT GGT TTG CCT TTA; reverse-mutant type – GGC TGG GGT GGT TTG CCT TTC. The PCR reaction was performed in 25 μl PCR reaction mixtures containing 45 ng genomic DNA, 0.4 μM each primer, PCR Gold Buffer, 2 mM $MgCl_2$, 0.2 mM each dNTP and 1.25 U AmpliTaq Gold DNA

polymerase. PCR was performed with an initial step at 94°C for 5 min, followed by 35 cycles at 94°C for 30 s, at 66°C for 30 s and at 72°C for 25 s, with a final extension at 72°C for 7 min. Under these conditions, amplification of DNA fragments produced single product. The PCR product was detected with ABI PRISM 7700 Sequence detector (Applied Biosystems) using fluorescent dye SYBR Green I (Molecular Probes).

Owing to the low frequency, the alleles, except *CYP2A6*4* and **9*, were defined as *CYP2A6*1*.

Statistical analysis

According to the genotypes, subjects were divided into two groups, high- and low-activity group, as described previously.[5] In brief, subjects with the **4/*9* and **4/*4* genotypes were considered to have less than 50% of the enzyme activity of **1/*1* and, therefore, defined as CYP2A6 low-activity group, whereas those with the **1/*1*, **1/*9*, **1/*4* and **9/*9* genotypes were defined as CYP2A6 high-activity group. All comparisons were carried out between CYP2A6 high-activity group and CYP2A6 low-activity group. Differences in the number of cigarettes, daily nicotine intake and score for nicotine dependence were tested using Mann–Whitney *U*-test. The χ^2 test was used to assess the time to the first cigarette of the day. The frequencies of withdrawal symptoms were also analyzed with χ^2 test. To assess the association of the *CYP2A6* genotypes with the withdrawal symptoms, we calculated ORs and their 95% CIs. An association was reported as statistically significant if the respective null hypothesis of OR = 1 was rejected at $P<0.05$ or when the respective 95% CIs did not include the value 1.

Duality of interest

None declared.

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No positive association between adrenergic receptor variants of α_{2c}Del322–325, β_1Ser49, β_1Arg389 and the risk for heart failure in the Japanese population

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Aims
We investigated the correlation of adrenergic receptor polymorphisms, α_{2c}Del322–325, β_1Ser49Gly and β_1Arg389Gly, with the risk of heart failure in the Japanese population.

Methods
These polymorphisms were analysed by polymerase chain reaction-restriction fragment length polymorphism in patients with chronic heart failure due to idiopathic dilated cardiomyopathy (DCM) and compared with the control group.

Results
There were no differences or any trends in the allele and genotype frequencies of the β_1Ser49Gly and β_1Arg389Gly polymorphisms. The allele frequency of the α_{2c}Del322–325 variant was lower in patients than in controls (0.11 *vs.* 0.04, $P = 0.011 < 0.017$, by Bonferroni correction), while the genotype frequency just failed to reach significance ($P = 0.022 > 0.017$, by Bonferroni correction).

Conclusions
In this population, the variants β_1Ser49, β_1Arg389, and α_{2c}Del322–325 do not appear to be risk factors for chronic heart failure due to DCM. The α_{2c}Del322–325 variant may in fact confer some protection.

Introduction

Neurohumoral factors play important roles in cardiac remodelling, determining the prognosis of heart failure. In particular, the sympathetic nervous system is activated in patients with chronic heart failure (CHF) [1] and sustained stimulation of the adrenergic system exerts direct adverse effects on cardiac function [2]. In spite of the importance of the adrenergic system, the effects of polymorphic mutation of adrenergic receptors on CHF remain to be fully elucidated.

In the present study, we focus on the presynaptic α_{2c} adrenergic receptor (AR) polymorphism with the deletion of four consecutive amino acids, α_{2c}Del322–325, and polymorphic amino acid variants of β_1AR, Ser49Gly and Arg389Gly. These polymorphic changes result in alteration of AR function. The presynaptic

α_2AR negatively regulates the release of norepinephrine from cardiac sympathetic nerves [3] and α_{2c}Del322–325 polymorphism shows a 'loss-of-function' phenotype [4]. The postsynaptic β_1AR polymorphism, β_1Ser49Gly, affects receptor sensitivity and promotes the downregulation of the receptor to agonists *in vitro* [5]. The change of β_1AR from Arg to Gly at the 389 amino acid residue leads to the decrease in G-protein coupling [6]. Considering the importance of the adrenergic system as a modulator of cardiac remodelling, it could be proposed that polymorphisms of adrenergic receptor genes may be closely related to the risk of heart failure.

Recently, Small *et al.* proposed that the polymorphisms of β_1Arg389Gly and α_{2c}Del322–325 are synergistically related to the risk of CHF in a black population [7]. However several concerns, including the aetiology of heart failure and the absence of analysis of β_1Ser49Gly frequency, have been raised against this study [8]. We have investigated the clinical significance of α_{2c}Del322–325, β_1Ser49Gly, and β_1Arg389Gly for the risk of heart failure due to dilated cardiomyopathy (DCM) in the Japanese.

Methods

Subjects

The study subjects consisted of 91 unrelated consecutive patients with CHF due to idiopathic DCM (males 79.5%, age 58.4 ± 13.7 years, ejection fraction 34.6 ± 15.8%) who attended or were admitted to Hokkaido University Hospital, Kyoto Katsura Hospital, Osaka Prefectural Medical Centre for Respiratory and Allergic Diseases, Aizenbashi Hospital or Osaka City University Medical School Hospital. The ratio of the patients, classified as NYHA class I, II, III or IV, was 13.9, 46.8, 15.1, 24.1%, respectively. Patients with ischaemic cardiomyopathy were excluded. One hundred and nineteen subjects (all males, aged from 20 to 40 years) who had no history or symptoms of cardiovascular disease were chosen as controls. This study was approved by the institutional review committee. All subjects gave their informed consent to participate.

Genotyping

Genomic DNA was extracted from samples of peripheral blood leucocytes using the QIAamp DNA Blood Maxi Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's protocol. Genotyping of α_{2c}Del322–325, β_1Ser49Gly and β_1Arg389Gly polymorphisms was performed as described previously [4–6] with minor modifications.

Statistical analysis

Values were expressed as means ± SD. χ^2 test of independence was used to test for associations between heart failure and allele. The 2 × 3 exact probability test was used to evaluate associations between heart failure and genotype. All of the analyses were corrected by Bonferroni correction. $P < 0.017$ was considered to be significant. Statistical analysis was performed with StatView Version 5.0 software (SAS Institute, Cary, NC, USA).

Results

The allele and genotype frequencies of α_{2c}Del322–325, β_1Ser49Gly and β_1Arg389Gly polymorphisms in the patients and controls are shown in Table 1. The allele

Table 1
Distribution of α_{2c} and β_1 adrenergic receptor (AR) variants in controls and patients with heart failure

Alleles and subjects	Allele Frequency	*P*-value	Genotype Frequency			*P*-value
α_{2c}Del322–325			WT/WT	WT/Del	Del/Del	
Controls	0.11	0.011	95/119 (79.8%)	23/119 (19.3%)	1/119 (0.8%)	0.022
Patients with heart failure	0.04		84/91 (92.3%)	7/91 (7.7%)	0/91 (0%)	
β_1Arg389			Gly/Gly	Gly/Arg	Arg/Arg	
Controls	0.81	0.82	5/119 (4.2%)	35/119 (29.4%)	79/ 119 (66.4%)	0.94
Patients with heart failure	0.80		5/91 (5.5%)	26/91 (28.6%)	60/91 (65.9%)	
β_1Ser49			Gly/Gly	Gly/Ser	Ser/ Ser	
Controls	0.84	0.90	3/119 (2.5%)	33/119 (27.7%)	83/119 (69.7%)	0.58
Patients with heart failure	0.84		4/91 (4.4%)	21/91 (23.1%)	66/91 (72.5%)	

P-values for comparisons of allele frequency or genotype frequency between controls and patients with heart failure were determined by 2 × 2 χ^2 or by 2 × 3 exact probability test, respectively. P-value < 0.017 (0.05/3) was considered to be significant.

Table 2
Combined genotypes of β_1AR and the risk for heart failure

β_1Ser49Gly	β_1Arg389Gly	Controls	Patients with heart failure	Odds ratio for heart failure (95%CI)	*P*-value
		No. of subjects			
		119	91		
≧ 1Gly	≧ 1Gly	6	6	1.00	–
≧ 1Gly	Arg/Arg	30	19	0.63 (0.18–2.25)	0.35
Ser/Ser	≧ 1Gly	34	25	0.74 (0.21–2.55)	0.43
Ser/Ser	Arg/Arg	49	41	0.84 (0.25–2.79)	0.50

Subjects with at least one β_1Gly49 allele and at least one β_1Gly389 allele served as the reference group. Odds ratios and P-values between the reference group and each other group were determined by 2 × 2 χ^2 test. P-value < 0.017 (0.05/3) was considered to be significant.

frequency of the α_{2c}Del322–325 variant was lower in patients with CHF than in controls (0.04 *vs.* 0.11, $P = 0.011 < 0.017$, by Bonferroni correction) and the genotype frequency was not significant but showed the *P*-value nearly equal to the borderline of significance ($P = 0.022 > 0.017$, by Bonferroni correction). The allele and genotype frequencies of the β_1Arg389 and β_1Ser49 variants in the patients with heart failure were consistent with those of controls.

Combined genotypes of β_1Arg389 and β_1Ser49 variants were not associated with the risk of heart failure (Table 2).

Discussion

The frequency of β_1Arg389Gly and β_1Ser49Gly polymorphisms did not differ from those in the control group, nor were there any trends, suggesting that these polymorphisms are not associated with susceptibility to CHF. Similarly, the combined genotype of β_1Arg389Gly and β_1Ser49Gly was not associated with the risk of CHF. However, we cannot definitely exclude the possibility that lack of association is derived from a β error problem, although the number of samples in our study was more than that in the previous study [7].

The allele frequency of the α_{2c}Del322–325 variant was statistically lower in CHF than in the controls. However, it is uncertain that the α_{2c}Del322–325 variant is a negative risk factor clinically, because the genotype frequency of this variant was of borderline significance, probably due to the low frequency of the homozygous genotype for α_{2c}Del322–325 variant in the Japanese population. Thus, considering the limitation of low genotype frequency of this polymorphism, a reasonable interpretation of our results is that α_{2c}Del322–325 variant is not a positive risk factor for CHF due to DCM in the Japanese population.

Previously, it was reported that allele frequency of α_{2c}Del322–325 positively correlated with heart failure in both the white and black populations [7]. It remains to be clarified why our data are not consistent with the previous study [7]. One possibility is that the inconsistency might be derived from the difference in the cause of heart failure. The previous study included patients with ischaemic cardiomyopathy, while we excluded ischaemia because some adrenoceptor polymorphisms are related to hypertension, a risk factor for ischaemic heart disease [9, 10]. Another possibility is that the pathophysiological significance of the adrenoceptor polymorphism is closely related to the severity of heart failure. Importantly, the ratio of the patients classified as NYHA III or IV is lower in the present study than that in the previous report [7]. It may be hypothesized that there is a racial difference in the severity of CHF. Indeed, the previous study reported that Japanese patients with congestive heart failure show low mortality [11].

In addition to healthy controls, who are all males aged between 20 and 40 years, we analysed 189 diabetes patients (58% male with an age 60.4 ± 9.8 years) who did not suffer from CHF, as an age-matched control. It was found that there were no differences in the allele and genotype frequencies of α_{2c}Del322–325 among healthy controls, male diabetes patients, and female diabetes patients (data not shown). Moreover, the allele frequency of α_{2c}Del322–325 was lower in the patients with CHF than in those with diabetes (0.04 and 0.10, respectively). Thus it is unlikely that α_{2c}Del322–325 polymorphism affected survival through other causes, resulting in the influence on allele frequency of samples.

Recent studies demonstrated that a genetic variability of β_2AR, Thr164Ile, is closely related to heart failure. In patients with congestive cardiac failure, patients with homozygous genotype Ile/Ile show high mortality compared with those with other genotypes [12]. However, we could not statistically confirm the previous findings, probably because of low frequency of the mutation.

In summary, the α_{2c}Del322–325, β_1Ser49 and β_1Arg389 variants do not appear to be risk factors for CHF due to DCM in a Japanese population, and the α_{2c}Del322–325 variant may be protective. Considering the contradiction with the previous report, it is proposed that there may be a racial difference in the clinical importance of this polymorphism. Further efforts should be made to address any possible racial differences in the responsiveness of heart failure from different causes to β-blockers.

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Regular Article

The Frequency of Candidate Alleles for CYP2D6 Genotyping in the Japanese Population with an Additional Respect to the −1584C to G Substitution

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Summary: The −1584C/G single nucleotide polymorphism (SNP) in the promoter region of *CYP2D6* was suggested to have the potential to influence CYP2D6 activity. In this report, we demonstrated the frequencies of −1584C to G substitution-related alleles, such as *CYP2D6*2*, *CYP2D6*21*, *CYP2D6*35* and *CYP2D6*41*, in the Japanese population. The frequencies of *CYP2D6*2*, **41* and **21* were 0.102, 0.026 and 0.005, respectively. We also showed a relationship between the SNP and other common alleles, *CYP2D6*4*, **5*, **10*, **14* and **18*. Interestingly, the SNP was detected in all three subjects carrying *CYP2D6*14*. This finding suggests the −1584G is included in the *CYP2D6*14* allele, which is a null-allele characteristic to the Japanese population. This report presents practical information on *CYP2D6* alleles that should be considered in the pharmacokinetic study of CYP2D6 substrates in the Japanese population.

Key words: CYP2D6; frequency; *CYP2D6*41*; *CYP2D6*35*; *CYP2D6*21*; Japanese

Introduction

CYP2D6 metabolizes many clinically important drugs including antidepressants, neuroleptics, β-blockers and antiarrhythmics.[1] There is a wide interethnic variation in the frequency of the *CYP2D6* genotypes. In a previous study, we reported the frequencies of *CYP2D6* genotypes in a Japanese population.[2] However, some alleles have since been reported which were not included in our study.

In the present study, we focused on the −1584C/G substitution, because the subjects with −1584G were suggested to have higher CYP2D6 enzyme activity than those with −1584C.[3] The locations of the mutations in common *CYP2D6* alleles are displayed in **Fig. 1** according to the CYP nomenclature committee (http://www.imm.ki.se/CYPalleles/cyp2d6.htm), with some modifications. Since the single nucleotide polymorphism (SNP) in −1584 seems to be mainly associated with *CYP2D6*2*, the committee has designated *CYP2D6*2* with −1584G as *CYP2D6*2A* and *CYP2D6*2* with −1584C as *CYP2D6*41*, according to Zanger *et al.*[3] Recent studies have suggested that individuals with *CYP2D6*41* have lower CYP2D6 enzymatic activity *in vivo* than those with *CYP2D6*2A*,[4] possibly as a consequence of lower expression of CYP2D6 protein.[3] The −1584G substitution is also found in the *CYP2D6*35* allele, which has a 31G to A substitution in addition to the SNPs of the *CYP2D6*2* allele, but does not have the gene conversion mutation from *CYP2D7* in intron 1 of *CYP2D6*. Although the allele has been identified in many duplication-negative "Ultra rapid" metabolizers,[5] the activity of recombinant CYP2D6.35 is comparable to that of the wild-type.[6] Furthermore, the −1584G is found in the *CYP2D6*21* allele. However, these effective alleles have been classified as *CYP2D6*2* according to previous detection criteria. Therefore, the consideration of these alleles may result in a better understanding of the phenotype-genotype correlation.

In view of the importance of the −1584C/G substitution-related alleles in the Japanese population, we examined the frequencies of *CYP2D6*41* and **35*, possibly included in *CYP2D6*2*, **21* and other alleles in the present study.

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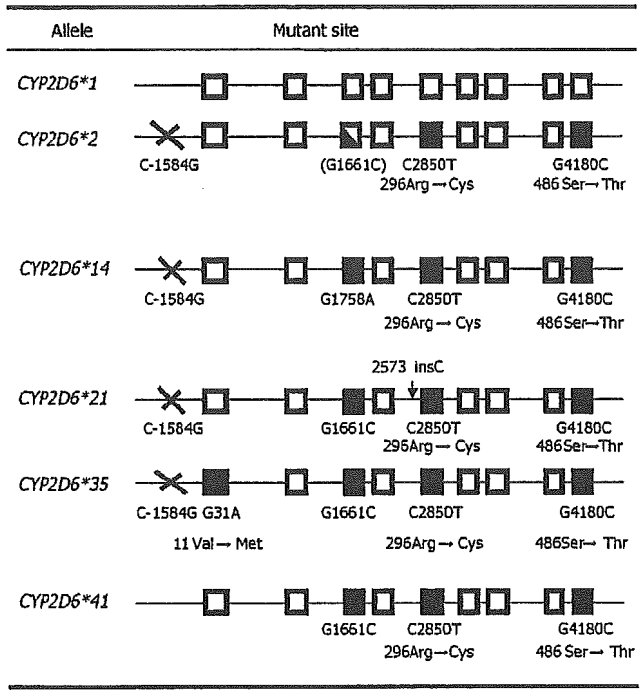

Fig. 1. Schematic representation of the position of mutations on the *CYP2D6* allele

The nature of the mutations and the positions where nucleotide changes occur are indicated. The structures are illustrated with closed boxes (exon containing at least one mutation) and open boxes (exon with wild-type sequence). The position of mutations is in accordance with the numbering used in the CYP2D6 allele nomenclature (http://www.imm.ki.se/CYPalleles/cyp2d6.htm).

Methods

All subjects in the present study were healthy males who had enrolled for several clinical trials. We regenotyped 206 subjects whose data had previously been reported.[2] An additional 79 subjects were newly included, and consequently a total of 285 subjects were examined for the *CYP2D6* genotype. This study was approved by the ethic committees of Osaka University. Written informed consent for genetic analysis was obtained from all subjects. Genomic DNA was isolated from peripheral lymphocytes from each subject.

In our previous study, each *CYP2D6* genotype was defined from the results of PCR-RFLP methods for 100C/T, 1846G/A, 2850C/T and 4180G/C[2] and from the results of *Xba*I and *Eco*RI-RFLP methods or the long-PCR method for the *CYP2D6*5* allele.[7,8]

A PCR-RFLP method for the −1584C to G substitution was developed for it's direct detection. A mismatch PCR-RFLP assay was based on a recognition site for the restriction enzyme *Sma*I by utilizing an oligonucleotide mismatch primer (CYP2D6*41 Mut.R; 5′-TTG TAT TTT TTG TAG AGC CC -3′; the letter with the underline is the mismatch nucleotide). This antisense primer introduces a *Sma*I recognition site by extension when a cytosine is present at the first base. In contrast, in the present of a guanine no recognition site for *Sma*I is introduced. The PCR reaction was carried out in a 25-microl reaction volume containing 1.5 mM $MgCl_2$, 10 mM Tris/HCl (pH 8.3), 0.2 mM of each dNTP, 10 pmol of each primer (CYP2D6*41F; 5′-TTC AAG ACC AGC CTG GAC AAC -3′ and CYP2D6*41Mut.R), 30 ng genomic DNA and 1U AmpliTaqGold™DNA polymerase (Applied Biosystems). An initial denaturation step at 95°C for 10 min was followed by 35 cycles of 95°C for 30 sec, 60°C for 1 min and 72°C for 30 sec, and a final elongation step of 72°C for 5 min. PCR products were then digested with *Sma*I and separated on 4% agarose gel. Product from the −1584C allele was not cut and remained 53 bp in length, while the −1584G allele was cut into 32 and 21 bp.

The 31G/A substitution was detected to find the *CYP2D6*35* allele according to Lovlie *et al.*[5] In brief, a 341-bp fragment covering the SNP was amplified by a set of primers (CYP-511; 5′-AGG TTC ACT CAC AGC AGA GGG-3′ and CYP-518; 5′-CCT GGT CGA AGC AGT ATG GTG-3′), then digested with *Nla*III. The wildtype (31G) was cut into 305 and 36 bp fragments, while the mutant (31A) allele generated 193, 112 and 36 bp fragments. The PCR reaction product was purified and directly sequenced by DNA sequencer ABI 310 using dye-terminater chemistry to confirm the nucleotide substitution and search for other related substitutions. Then, to determine the haplotype, the fragment was cloned using the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Additionally, after genotyping, some clones with insertions were randomly selected and directly sequenced.

The presence of the *CYP2D6*21* allele was determined by an allele specific PCR method using genotyping kit "-SNP Typing Kit-Cytochrome P450 2D6*21 (2573C insert)" (TOYOBO, Japan) according to the manufacturer's instructions.

Results

The −1584G alleles were detected in the subjects previously determined as having the *CYP2D6*2* genotype. The frequency of −1584G substitution in the Japanese population was estimated to be 0.114 and *CYP2D6*41* allelic frequency 0.026 (**Table 1**).

Interestingly, there were four unusual subjects among all of the subjects with the −1584G allele. One subject was heterozygous for −1584G and had substitutions classified as *CYP2D6*1/*10*. Two subjects were also heterozygous for −1584G although both had *CYP2D6*1/*14*. The last was homozygous for −1584G in spite of *CYP2D6*2/*14* carrier according to the previous classification.

The insertion of cytosine at 2573 was detected in three

Table 1. The allele frequency of *CYP2D6* in Japanese compared with frequencies in other ethnic populations

(Number of subjects)	Allele frequency (95% confidential interval; lower/upper)				
	−1584C	−1584G			
	*CYP2D6*41*	*CYP2D6*2*	*CYP2D6*2×n*	*CYP2D6*21*	*CYP2D6*35*
Japanese (n=285)	0.026 (0.016/0.043)	0.102 (0.082/0.132)	—#	0.005 (0.002/0.015)	0.000 (0.000/0.005)
Caucasian (n=203)[9]	0.094	0.172	—	—	0.074
Caucasian (n=206)[10]	0.102	0.187	0.010	—	—
African-American (n=193)[9]	0.114	0.047	—	—	0.010

Allelic frequency of *CYP2D6*2*×n and *CYP2D6*1*×n was reported to be 0.01 (n=206)[2].

subjects, which indicates that they had the *CYP2D6*21* allele. Consistent with the allelic information on *CYP2D6*21*, the heterozygous for −1584G was detected from all three. Therefore, one subject was genotyped as *CYP2D6*10/*21* and the other two as *CYP2D6*1/*21*. As a result, the frequency of *CYP2D6*21* allele was estimated to be 0.005 in the present study.

One subject was found to be heterozygous for the 31G to A substitution with *CYP2D6*1/*10* according to the previous classification. In the same subject, we also detected an allele carrying 100T and 31A by subcloning analysis. However, the allelic information on *CYP2D6*35* showed no link between the 31A and the 100T. Therefore, we did not consider the subject to be a *CYP2D6*35* carrier, but provisionally as a *CYP2D6*1/*10* carrier, which may be a new type of allele related to *CYP2D6*10* with 31A.

Discussion

In the past, we have classified *CYP2D6*2* as the wild-type enzymic status according to the phenotype test. However, we have, on occasion, encountered subjects with a lower enzyme activity than that expected of the *CYP2D6*2* genotype. In the Caucasian population, subjects with −1584G substitutions including *CYP2D6*2* have been reported to achieve higher CYP2D6 activity *in vivo* than those with −1584C, possibly as a consequence of greater expression of the CYP2D6 protein.[3,4] In addition, irrespective of the genotype, individuals with −1584C/C expressed less CYP2D6 protein than individuals with at least one −1584G allele.[3] There has been no study concerning the −1584C/G substitution in the Japanese population.

In the present study, we confirmed that the −1584G allele was also detected in the Japanese population although its frequency is lower than in other ethnic groups (**Table 1**). Therefore, our results did not disprove the presence of a possible inconsistency between the phenotype and genotype of CYP2D6 found in several previous studies for Japanese, regarding the effect of this SNP on the activity of CYP2D6.

We also examined the 31G to A substitution for presence of *CYP2D6*35* as a related-allele with the −1584C/G substitution. In this study, the heterozygous for 31A was found in only one subject with *CYP2D6*1/*10*. We subcloned the sample and detected the **10* allele with the 31A mutation as a minor novel allele. This was not regarded as *CYP2D6*35*. Although the 31G/A substitution is not likely to influence CYP2D6 enzyme activity and protein expression level *in vitro*,[6] the *CYP2D6*35* allele is found in many ultra rapid metabolizers.[5,9] The present findings suggest that *CYP2D6*35* allele frequency in the Japanese population is very low, compared with some other ethnic populations, which is consistent with the fact that the ultra rapid metabolizers are rare in Japanese[2] and occur in less than about 10% of Caucasians.

The result from four unusual subjects suggests that the −1584G substitution might be associated with *CYP2D6*14* and possibly *CYP2D6*1* or **10* in addition to *CYP2D6*2*, **21* and **35*. Zanger *et al.* also mentioned that one subject with *CYP2D6*1/*1* possessing −1584G was detected in their study,[3] supporting the present result. On the other hand, Gaedigk *et al.* suggested that CYP2D6 poor metabolizers (PMs) can be detected by the selection of −1584G carriers, as −1584G is exclusively linked to functional allelic variants.[9] Their study seems to be of value for the simple clinical use of the SNP information. However, the connection between the **14* allele and the −1584G substitution was found in the present study in addition to **21*. Since *CYP2D6*14* is a null-allele characteristic to the Japanese population, these connections may limit the genotyping strategy to Japanese.

In conclusion, we examined the distribution of −1584C/G substitutions in the Japanese population mainly associated with *CYP2D6*2*, and found the frequency showed interethnic variation. Since the −1584C/G substitution-related alleles potentially effect CYP2D6 enzyme activity, these alleles should be consi-

dered in *CYP2D6* genotyping. The present study found that the −1584C/G substitution has a potential link to other alleles, and further studies on the related alleles in various ethnic populations are necessary.

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Regular Article

Influence of Itraconazole Co-administration and CYP2D6 Genotype on the Pharmacokinetics of the New Antipsychotic ARIPIPRAZOLE

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Summary: The results of *in vitro* studies indicated that ARIPIPRAZOLE, a newly developed antipsychotic, is mainly metabolized by the human cytochrome P450 isozymes CYP3A4 and CYP2D6. The objective of the present study was to investigate the influence of itraconazole (hereafter referred to as ITZ) co-administration (CYP3A4 inhibition) on the pharmacokinetics of ARIPIPRAZOLE administered to 24 healthy adult male volunteers in a fasting condition. The influence of CYP3A4 inhibition was also examined by CYP2D6 genotype.

All subjects were administered a single oral dose of ARIPIPRAZOLE alone in Period I and a single oral dose of ARIPIPRAZOLE following administration of ITZ at 100 mg/day for 7 consecutive days in Period II. The pharmacokinetic parameters of ARIPIPRAZOLE and its main metabolite OPC-14857 were determined.

Co-administration of ITZ increased the C_{max}, $AUC_{336\,hr}$, and $t_{1/2,z}$ of ARIPIPRAZOLE and OPC-14857 by 19.4%, 48.0%, and 18.6% and by 18.6%, 38.8%, and 53.4%, respectively.

By co-administration of ITZ, the CL/F of ARIPIPRAZOLE in extensive metabolizers was decreased by 26.6%, with an even greater decrease (47.3%) in intermediate metabolizers. For the co-administration period, the CL/F of ARIPIPRAZOLE in intermediate metabolizers was about half of that in extensive metabolizers. For C_{max}, there was no significant difference between extensive metabolizers and intermediate metabolizers, and the percent change by co-administration of ITZ was less than 20% in both extensive metabolizers and intermediate metabolizers.

For OPC-14857, the t_{max} in intermediate metabolizers was longer than that in extensive metabolizers, with the difference being amplified by co-administration of ITZ. The $AUC_{336\,hr}$ showed similar increases by co-administration of ITZ in all genotypes. The urinary 6β-hydroxycortisol/cortisol concentration ratio following ITZ administration for 7 consecutive days was about half of that before the start of ITZ administration, indicating that CYP3A4 metabolic activity was inhibited by administration of ITZ. The influence of CYP3A4 inhibition on the pharmacokinetics of ARIPIPRAZOLE was not considered to be clinically significant. On the other hand, definite differences in pharmacokinetics were observed between CYP2D6 genotypes.

Key words: ARIPIPRAZOLE; pharmacokinetics; CYP3A4 inhibition; CYP2D6 genotypes

Introduction

In recent years the investigation of drug interaction has come to be essential for appropriate evaluation of the safety and efficacy of drugs and for appropriate use of drugs after market launch. Especially in the psychiatric field, where it is quite common to provide multiple-drug combination therapy, obtaining adequate information on drug interaction becomes very important.

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