A novel intronic mutation that may affect genotyping result of *CYP2C8* by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) is strongly associated with *CYP2C8**3 in a South American population

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Abstract During the investigation of a *CYP2C8**3 genetic polymorphism in a South American population, we obtained a discrepant result using two different polymerase chain reaction (PCR) protocols. A single nucleotide polymorphism (SNP) (IVS3+43 G>C) was identified in the intron 3 region, which was used as a primer-annealing site of one of the two PCR protocols. A genotyping method was developed to enable discrimination of the *CYP2C8**1A, *CYP2C8**3 (416 G>A), and *CYP2C8**3 (416G>A; IVS3+43 G>C) alleles. In a screen of a South American population, we found that individuals carrying the *CYP2C8**3 (416 G>A) polymorphism also carried the *CYP2C8**3 (416G>A; IVS3+43 G>C). However, we did not find any carriers of *CYP2C8**3 (416G>A; IVS3+43 G>C) in a Japanese population.

Keywords *CYP2C8* genotyping · Allele-specific PCR · South Americans · Japanese · SNP linkage

Introduction

The *CYP2C8* gene is an isoform of the human cytochrome P450 (CYP) 2C subfamily. Nine allele variants

of the *CYP2C8* gene have been recognized to date (See <http://www.imm.ki.se/CYPalleles>). Of these alleles, *CYP2C8**3 has been shown to be markedly defective in the metabolism of important *CYP2C8* substrates, such as arachidonic acid and the anticancer drug paclitaxel. *CYP2C8**3 has additionally been proposed to have important clinical and physiological implications for individuals homozygous for the allele (Dai et al. 2001). The *CYP2C8**3 variant has two separate coding mutations: a nucleotide change 416 G>A in exon 3 (R 139 K); and a 1196 A>G change in exon 8 (K 399 R).

During a genotyping screen using the 416 G>A nucleotide change in exon 3 to identify carriers of *CYP2C8**3, we found a discrepant result when we used two different polymerase chain reaction (PCR) protocols. Protocol A, using primers described by Badahur et al. (2002; Table 1), yielded the expected wild-type pattern of *CYP2C8**1A/*1A after *Bse*RI digestion was applied. However, protocol B, using primers described by Dai et al. (2001; Table 1), produced an unexpected, heterozygous pattern (*CYP2C8**1A/*3) in one of the samples (Fig. 1-I). Because in this initial screen the DNA samples analyzed were from South American and Japanese subjects and the discrepant result was observed in a sample of a Bolivian subject, the cause of the discrepancy and its possible presence in both populations were further investigated, and data are shown in this study.

Materials and methods

Subjects

Participants were unrelated healthy volunteers from South America and Japan. Subjects from South

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Table 1 Polymerase chain reaction (PCR) protocols

Protocol	Target	Primers 5'-3'	Method	Cycle conditions			Product size (bp)	References
				Number of cycles	Denaturation	Annealing	Extension	
A	416 G>A IVS3+43G>C	F aattctccagtttctgccc R cgtcactagtgaagaCaggt	PCR-RFLP	30	94°C 1 min	64°C 1 min	72°C 1 min	(Badahur et al. 2002)
B	416 G>A	F aggaattcccaatctc R actctccacaagcagta	AS-PCR PCR-RFLP	38	94°C 20 s	55°C 10 s	72°C 10 s	(Dai et al. 2001)
C	416 G>A IVS3+43G>C	F ggtaactgggtgagggg R gaggcigacaaccaaggat	Direct sequence	30	94°C 45 s	66°C 45 s	72°C 45 s	(Soyama et al. 2001)
D	IVS3+43G>C	F aattctccagtttctgccc R cgtcactagtgaagaCaggt	AS-PCR	30	94°C 1 min	64°C 1 min	72°C 1 min	This study
E	1196 A>G	F cttecgctcatgatgaag R ctgctgagaaggcatgaag	PCR-RFLP	38	94°C 20 s	55°C 10 s	72°C 10 s	(Dai et al. 2001)

Position of IVS3+43G>C nucleotide change into the primer sequence is shown in upper cases
F forward primer, *R* reverse primer, *RFLP* restriction fragment length polymorphism, *AS* allele-specific

America comprised eight Argentines, 210 Bolivians, 17 Brazilians, 12 Chileans, and 13 Peruvians. Of them, 158 were included in the frequency determination, and the 102 remaining subjects were included for further intron–exon linkage determination. Ages ranged from 15 to 65 years, with a mean of 25.3 years. The ethnicity of each participant could not be determined accurately; however, subjects who to their own knowledge did not have ancestors from Europe, Asia, or other continents other than South America were included in the study. It was estimated that subjects belong to the Mestizo population (Amerindian and Caucasian admixture). The Bolivian samples were in part from a previously described study (Bravo-Villalta et al. 2005). Japanese subjects were mostly students, with a mean age of 23 years. The study was approved by the Ethics Committees of Univalle University, Bolivia, and Gunma University, Japan. All subjects gave their written consent prior to inclusion in the study.

Genotyping

Genomic DNA was extracted from peripheral blood samples from South American and Japanese subjects. A summary of PCR protocols performed in this study is shown in Table 1. All PCR mixtures contained 200 µM of dNTP mix, 2.5 mM of MgCl₂, 1X PCR Gold Buffer (15 mM Tris–HCl, pH 8.0, 50 mM KCl), 0.25 µM of each primer, 0.5 U of AmpliTaq Gold (Perkin-Elmer, Branchburg, NJ, USA), and genomic DNA (60–100 ng). Thermal cycling was performed in GeneAmp PCR System 9700 (Applied Biosystems). The restriction fragment length polymorphism (RFLP) screen for the 416 G>A and 1196 A>G polymorphism was determined by digestion with *Bse*RI and *Xmn*I, respectively.

Statistics

Allele frequencies, 95% confidence intervals (CI) and Fisher's exact test were determined by GraphPad software (GraphPad, San Diego, CA, USA; www.graphpad.com). Fisher's exact test was performed to detect difference in allele frequencies between Bolivian and Japanese populations. Other South American populations included in the study were not considered for the statistical analysis due to the small sample size. A *P* value of less than 0.05 was considered to be statistically significant. Linkage disequilibrium (LD) analysis was performed by SNPalyze software (Dynacom Co., Yokohama, Japan), and pairwise LD between variations was analyzed by rho square (*r*²).

Fig. 1 **I a** Polymerase chain reaction–restriction fragment length polymorphisms (PCR–RFLPs) of individuals 1–5.

The *left panel* shows the pattern after use of PCR protocol A, the *right panel* after use of PCR protocol B. Note that subject 3 in the latter shows a variant doublet band. Individuals 1 and 2 are Japanese subjects. Individuals 3, 4, and 5 are Bolivian subjects of admixture ethnic origin (Amerindian and Caucasian). **b** PCR–RFLPs of Bolivian subjects show examples of results after screening the 416 G>A *CYP2C8**3 variant. *W* homozygous wild type; *M* homozygous mutant; *H* heterozygous. **II** Direct sequence shows exon–intron relation (416G>A–IVS3+43G>C) **a** heterozygous pattern, **b** wild-type, **c** mutant. **III** Allele-specific (AS) PCR for the IVS3+43 G>C variant showing band patterns for the wild-type (*W*), heterozygous (*H*), and homozygous mutant (*M*) genotypes

Results and discussion

We observed a discrepant result during the 416 G>A (*CYP2C8**3) screening when we performed two different PCR protocols (protocols A and B, Table 1). To determine the cause of the discrepant results, we used a third PCR protocol (protocol C, Table 1) to amplify the region containing exon 3 and the two primer sites used by protocol A. The amplification product was then directly sequenced (reference sequence: Gen-

Bank AF136833). For the sample showing the discrepant result as a doublet band (Fig. 1-Ia), a heterozygous signal (G/C) was observed at position 43 of intron 3 (Fig. 1-IIa). This is the site in protocol A where the reverse primer anneals (Table 1).

To confirm that this mutation (IVS3+43 G>C) was the cause of the discrepancy, allele-specific PCR (AS-PCR) was carried out (protocols A and D, Table 1). When enzyme digestion was applied to the samples judged as heterozygous by PCR protocol B, they

showed wild or mutant patterns after using AS-PCR primers, confirming that the mutant was responsible for the variant band (Fig. 1-III). A sample of 158 individuals from South America was screened for the IVS3+43G>C mutation. This sample comprised three Argentineans, 129 Bolivians, 11 Brazilians, six Chileans, and nine Peruvians. Of them, two subjects from Argentina, ten from Bolivia, and four from Brazil were found to be heterozygous for IVS3+43G>C. The remaining subjects had the wild-type genotype. The homozygous mutant genotype was not found. We additionally genotyped 104 samples from Japanese subjects; none carried the novel SNP. Furthermore, the allele frequency of IVS3+43G>C in the Bolivian population was compared with that observed in the Japanese population. The frequency of 3.9% (95% CI 2.0–7.1%) in the Bolivian population was evidently higher than that of 0% (95% CI 0–2.1%) observed in the Japanese population. Statistical analysis detected a significant difference in the frequencies between both populations, represented by a *P* value of 0.002.

Interestingly, subjects from the South American sample who carried the intron mutation in a heterozygous genotype were also heterozygous for 416 G>A (*CYP2C8**3). To determine whether this linkage was consistently present in South American populations, we investigated a further sample of 102 subjects whose 416 G>A genotype was already known. This sample comprised five Argentineans, 81 Bolivians, six Brazilians, six Chileans, and four Peruvians. Of them, one subject from Argentina, 15 from Bolivia, and one from Brazil were wild-type homozygotes. Four subjects from Argentina, 65 from Bolivia, five from Brazil, six from Chile, and four from Peru were heterozygotes. Only one subject from Bolivia was homozygous for the 416 G>A (*CYP2C8**3) mutation. In all cases, the presence or absence of the IVS3+43G>C intron mutation coincided with the genotype shown for the 416 G>A (*CYP2C8**3) exon mutation (Fig. 1-II), suggesting strong linkage of *CYP2C8**3 (416 G>A) and IVS3+43 G>C in the *CYP2C8* gene. Since the 416 G>A polymorphism has been reported as highly linked to the 1196 A>G polymorphism in exon 8 of the *CYP2C8* gene, we also screened this latter polymorphism (protocol E, Table 1) in all the South American samples. The genotypes observed for the 1196 A>G polymorphism also coincided with both the 416 G>A and the IVS3+43G>C polymorphisms (data not shown). All detected variations were found in Hardy–Weinberg equilibrium (*P* value 0.874), and the LD analysis demonstrated that 416 G>A, IVS3+43G>C, and 1196 A>G were in perfect linkage ($r^2 = 1$).

In the Japanese population, the IVS3+43 G>C mutation was not detected. This outcome is consistent with the report of Nakajima et al. (2003), who did not find the *CYP2C8**3 allele in the Japanese population of their study.

Badahur et al. (2001) detected the 416 G>A mutation by single-strand confirmation polymorphism (SSCP) using the primer of PCR protocol A (Table 1), suggesting that linkage between IVS3+43 G>C and 416 G>A mutation may not be complete in Caucasian populations. The intron mutation (IVS3+43 G>C) was apparently identified in US citizens, as it is reported in data of GenBank (accession number AY514490: variation 4198), but no comment on its relationship to the exon variant is mentioned.

Dai et al. (2001) identified 11 intron mutations by direct sequence of 72 genomic DNA samples from Caucasian (*n* = 24), Asian (*n* = 24), and African American (*n* = 24) subjects. They amplified exon and intron–exon areas by using specific intron primers. Despite that, the intron mutation we identified was absent in their screening. This may due to the small sample size of subjects with whom the sequence was performed. However, it is also possible that the linkage we observed between the intron and exon mutation in the South American population is not complete in these other populations. Evidently, they did not detect the intron polymorphism in the genotyping screen by PCR–RFLP due to the fact that the reverse primer was located in the exon area.

To date, the intron variant does not appear to have been the cause of incorrect genotyping of the *CYP2C8* gene. The genotyping strategy we developed here allows identification of the intron mutation (Figure 1-III); this strategy will be of value when screening populations, such as those in South America, known to carry *CYP2C8* polymorphisms.

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