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Original Article

The *CYP3A4* intron 6 C>T polymorphism (*CYP3A4*22*) is associated with reduced CYP3A4 protein level and function in human liver microsomes

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ABSTRACT — Effects of the *CYP3A4* intron 6 C>T (*CYP3A4*22*) polymorphism, which has recently been reported to have a critical role *in vivo*, were investigated by measuring CYP3A4 protein expression levels and CYP3A4-dependent drug oxidation activities in individual human liver microsomes *in vitro*. Prior to protein analysis, analysis of DNA samples indicated that 36 Caucasian subjects were genotyped as *CYP3A4*1/*1* and five subjects were *CYP3A4*1/*22,* with a *CYP3A4*22* allelic frequency of 6.1%. No *CYP3A4*22* alleles were found in the Japanese samples (106 alleles). Individual differences in CYP2D6-dependent dextromethorphan *O*-demethylation activities in liver microsomes from Caucasians were not affected by either the *CYP3A4*1/*22* or *CYP3A5*1/*3* genotype. Liver microsomes genotyped as *CYP3A4*1/*22* (n = 4) showed significantly lower CYP3A-dependent dextromethorphan *N*-demethylation, midazolam 1′-hydroxylation, and testosterone 6β-hydroxylation activities, as well as lower expression levels of CYP3A protein (28% of control), compared with those of the *CYP3A4*1/*1* group (n = 19). The other polymorphism, *CYP3A5*1/*3,* did not show these differences (n = 4). The *CYP3A4*22* polymorphism was associated with reduced CYP3A4 protein expression levels and resulted in decreased CYP3A4-dependent activities in human livers. The present results suggest an important role of low expression of CYP3A4 protein associated with the *CYP3A4*22* allele in the individual differences in drug clearance.

Key words: P450 3A4, P450 3A5, Protein expression, Ethnic difference, Impaired polymorphism

INTRODUCTION

Cytochrome P450 (P450 or CYP) comprises a superfamily of enzymes and plays an important role in the oxidative metabolism of a large number of endogenous and exogenous compounds (Guengerich, 2008). CYP3A4 is the most abundant P450 enzyme in human livers and is involved in metabolism of > 50% of marketed drugs (Rendic, 2002; Shimada *et al.,* 1994). CYP3A activity has been shown to display 10- to 100-fold variation among individuals (Lamba *et al.,* 2002; Shimada *et al.,* 1994; Westlind *et al.,* 1999; Westlind-Johnsson *et al.,* 2003), which may influence drug response and toxicity. Low drug clearances mediated by impaired CYP3A4 would invoke unexpected side effects or drug interactions with co-administered food or medicines. It has been suggested that ~85% of the inter-individual variability in hepatic CYP3A4 expression and activity is attributable to genetic factors (Ozdemir *et al.,* 2000). However, most of the *CYP3A4* polymorphisms examined to date have low frequencies (http://www.cypalleles.ki.se/cyp3a4.htm). The most common *CYP3A4* variant has been a promoter variant (*CYP3A4*1B*) leading to modified CYP3A4 activity (García-Martin *et al.,* 2002), but subsequent work on this has not been clear regarding its significance (Ball *et al.,* 1999; García-Martin *et al.,* 2002).

A recent study identified a functional SNP in *CYP3A4* intron 6 (rs35599367C>T; *CYP3A4*22*) (Wang *et al.,*

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2011). The *CYP3A4*22* genotype is associated with a good lipid-lowering response to the drug simvastatin (Elens *et al.,* 2011a). In addition, renal transplant patients with the *CYP3A4*22* allele show reduced clearance of a calcineurin inhibitor and therefore might be associated with increased risk of drug overexposure (Elens *et al.,* 2011b, 2011c and 2012). However, there are no clear *in vitro* reports regarding any effects of the *CYP3A4*22* allele on protein levels. On the other hand, CYP3A5, demonstrating 84% amino acid sequence identity with CYP3A4, has been associated with the metabolism of a variety of CYP3A4-probe drugs (Wrighton and Stevens, 1992). The most frequent variant of functional importance in the *CYP3A5* gene has been *CYP3A5*3,* leading to alternative splicing with impaired protein expression (Kuehl *et al.,* 2001; van Schaik *et al.,* 2002). Therefore, *CYP3A5* polymorphisms should also have potential effects on CYP3A-dependent activities in human livers. However, there are no available studies to clarify the *CYP3A4*22* and *CYP3A5*3* polymorphisms, in combination, on the catalytic function in human liver microsomes.

In the present study, effects of the *CYP3A4*22* and *CYP3A5*3* polymorphisms were investigated by measuring CYP3A protein expression levels and CYP3A-dependent drug oxidation in individual human livers. Ethnic differences of the frequency of the *CYP3A4*22* allele were seen between Caucasian and Japanese subjects. We report, for the first time, a clear association between the *CYP3A4*22* polymorphism, CYP3A4 protein levels, and catalytic function in human liver microsomes.

MATERIALS AND METHODS

Chemicals

Dextromethorphan was obtained from Sigma-Aldrich (St. Louis, MO, USA). Midazolam and testosterone were obtained from Wako Pure Chemicals (Osaka, Japan). Other chemicals and reagents used in this study were obtained from the sources described previously and were of the highest quality commercially available (Yamazaki *et al.,* 2002, 2006).

Enzyme preparations

The use of the human livers for this study was approved by the Ethics Committees of Vanderbilt University School of Medicine and Showa Pharmaceutical University. The human livers (5- to 74-year-old males and females) were obtained from patients after pathological examination of specimens isolated during hepatic surgery or after death (Inoue *et al.,* 1997; Shimada *et al.,* 2001; Yamaori *et al.,* 2004, 2005; Yamazaki *et al.,* 2003). Human liver microsomes were prepared in 10 mM Tris-HCl buffer (pH 7.4) containing 0.10 mM EDTA and 20% (v/v) glycerol as described previously (Yamaori *et al.,* 2005).

Genotyping

Genomic DNA was isolated from human livers as previously described (Inoue *et al.,* 1997; Shimada *et al.,* 2001; Yamaori *et al.,* 2004, 2005; Yamazaki *et al.,* 2003). The *CYP3A4*22* genotype was determined with 10 ng genomic of DNA in an allelic discrimination reaction performed with TaqMan® (Applied Biosystems, Foster City, CA, USA) genotyping assays (C_59013445_10) using a 7300 Real Time PCR System® (Applied Biosystems). The *CYP3A5*3* genotype was determined according to a previously published method (Adler *et al.,* 2009).

Enzyme assays

Microsomal protein concentrations were estimated using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). Activities for *O*- and *N*-demethylation of dextromethorphan, 1′-hydroxylation of midazolam, and 6β-hydroxylation of testosterone were assayed according to described methods (Kronbach *et al.,* 1989; Uno *et al.,* 2010; Yamazaki and Shimada, 1997). Briefly, the standard incubation mixtures consisted of 100 mM potassium phosphate buffer (pH 7.4), an NADPH-generating system (0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, and 0.25 unit/ml glucose 6-phosphate dehydrogenase), a substrate (400 μM dextromethorphan, 100 μM midazolam, or 50 μM testosterone), and liver microsomes (0.20-0.50 mg protein/ml) in a final volume of 0.25 ml. Dextromethorphan and midazolam were incubated with microsomes at 37°C for 15 min and terminated by the addition of 10 μl of 60% perchloric acid (w/v) or 0.25 ml of ice-cold methanol. Testosterone oxidation reactions were incubated at 37°C for 10 min and terminated by the addition of 1.5 ml of ethyl acetate and 25 μl of 3 M sodium chloride. After extraction, the organic phase of each sample was evaporated under a nitrogen stream. Product formation was determined by high-performance liquid chromatography with an analytical octadecylsilane (C_{18}) column (4.6 mm × 150 mm, 5 μm) according to described methods (Kronbach *et al.,* 1989; Uno *et al.,* 2010; Yamazaki and Shimada, 1997).

Western blot analysis

SDS-PAGE was performed using 7.5% (w/v) acrylamide gels. Microsomal protein (5 μg) was separated and transferred onto a nitrocellulose membrane. Immunoblot

quantitation was performed using recombinant CYP3A4 (Supersomes, BD Gentest, Franklin Lakes, NJ, USA) and a mouse antibody to human CYP3A (BD Gentest) as the standard protein and primary antibody for both human CYP3A4 and CYP3A5, respectively. Horseradish peroxidase-conjugated anti-mouse immunoglobulin (BD Gentest) was used as a secondary antibody (Yamaori *et al.,* 2004). A specific band was visualized using LAS-1000UVmini (GE Healthcare, Tokyo, Japan) and analyzed using Multi Gauge software, version 3.0 (GE Healthcare).

Statistical analyses

Statistical analysis was performed by Prism software, version 5.01 (GraphPad, San Diego, CA, USA). The differences in enzymatic activities between two genotypes were evaluated using an unpaired *t*-test with Welch correction. Statistical tests provided two-sided *p* values with a significance level of < 0.05.

RESULTS

DNA samples from 41 Caucasian and 53 Japanese subjects were genotyped for *CYP3A4* using a TaqMan assay method (Table 1). Among the Caucasian samples, 36 subjects were genotyped as *CYP3A4*1/*1* but five subjects were of the *CYP3A4*1/*22* genotype. The *CYP3A4*22* allelic frequency was estimated to be 6.1% in this sample, similar to that reported previously in Caucasians (Elens *et al.,* 2011b). On the other hand, there were no *CYP3A4*22* alleles observed in our Japanese samples (106 alleles). The expected frequency of the *CYP3A4*22* in a Japanese population was calculated to be < 1%; further analysis may be important in this context.

To investigate the effects of genetic polymorphism of the *CYP3A4* and *CYP3A5* genes on the metabolic activities, dextromethorphan *O*- and *N*-demethylation, midazolam 1′-hydroxylation, and testosterone 6β-hydroxylation activities were determined in liver microsomes prepared from 23 Caucasian subjects genotyped for *CYP3A4* (19 *CYP3A4*1/*1* and four *CYP3A4*1/*22*) and *CYP3A5* (four *CYP3A5*1/*3* and 19 *CYP3A5*3/*3*). There were no examples of both *CYP3A4*22* and *CYP3A5*1* among these liver samples. CYP2D6-dependent catalytic activities (dextromethorphan *O*-demethylation) in liver microsomes did not differ between both the *CYP3A4* and *CYP3A5* genotypes (Fig. 1A). On the other hand, the heterozygote group consisted of four liver microsomal preparations derived from subjects genotyped as *CYP3A4*1/*22* (and also *CYP3A5*3/*3*) showed significantly lower CYP3A-dependent activities (*N*-demethylation of dextromethorphan, 1′-hydroxylation of midazolam, and 6β-hydroxylation of testosterone), compared with the wild-type (*CYP3A4*1/*1*) group (Figs. 1B-D, $*p < 0.05$ and $**p < 0.01$). Although these typical substrates of CYP3A are also metabolized by CYP3A5, the *CYP3A5* genotype did not show any significant differences under the present experimental conditions (Fig. 1). CYP3A protein contents in liver microsomes from subjects genotyped as *CYP3A4*1/*22* were significantly lower (28% of control) than from subjects genotyped as *CYP3A4*1/*1*, while there was no significant differences between *CYP3A5* genotypes (Fig. 2). The individuals in the *CYP3A4*1/*22* heterozygote group were all genetically poor expressers of CYP3A5 in this study.

Table 1. *CYP3A4* allele frequencies in 41 Caucasian and 53 Japanese DNA samples

3A4 allele	Number (%)	
	Caucasian n = 82	Japanese n = 106
**1*	77 (94)	106 (100)
**22*	5 (6)	0 (0)

DISCUSSION

A *CYP3A4* intron 6 C>T (*CYP3A4*22*) allele (Elens *et al.,* 2011a, 2011b and 2011c; Wang *et al.,* 2011) is associated with reduced CYP3A4 activity *in vivo*. Wang *et al.* (2011) have recently reported that *CYP3A4*22* is linked to reduced CYP3A4 mRNA production (without any splice variants) and limited testosterone 6β-hydroxylation activity in human livers. However, the finding regarding CYP3A4 mRNA levels has not been strongly supported by another recent study (Klein *et al.,* 2012). Although statistically significant decreases of area-under the curve of drug metabolites per variant allele has been strongly confirmed in the *in vivo* cohort (Klein *et al.,* 2012), we are not aware of clear *in vitro* reports of studies regarding any effects of the *CYP3A4*22* allele on protein levels and multiple enzymatic function.

In the present study, decreased CYP3A protein contents (28% of control) in human liver microsomes associated with the *CYP3A4*22* genotype were clearly shown for the first time, using a mouse anti-human CYP3A antibody (Fig. 2). Under the present condition, CYP3A5 could be detected, along with CYP3A4 in liver microsomes; however, most Caucasians are genetically poor expressers of CYP3A5 (*CYP3A5*3/*3*) (Kuehl *et al.,* 2001; van Schaik *et al.,* 2002). In the four Caucasian liver samples that were heterozygous expressers of CYP3A5,

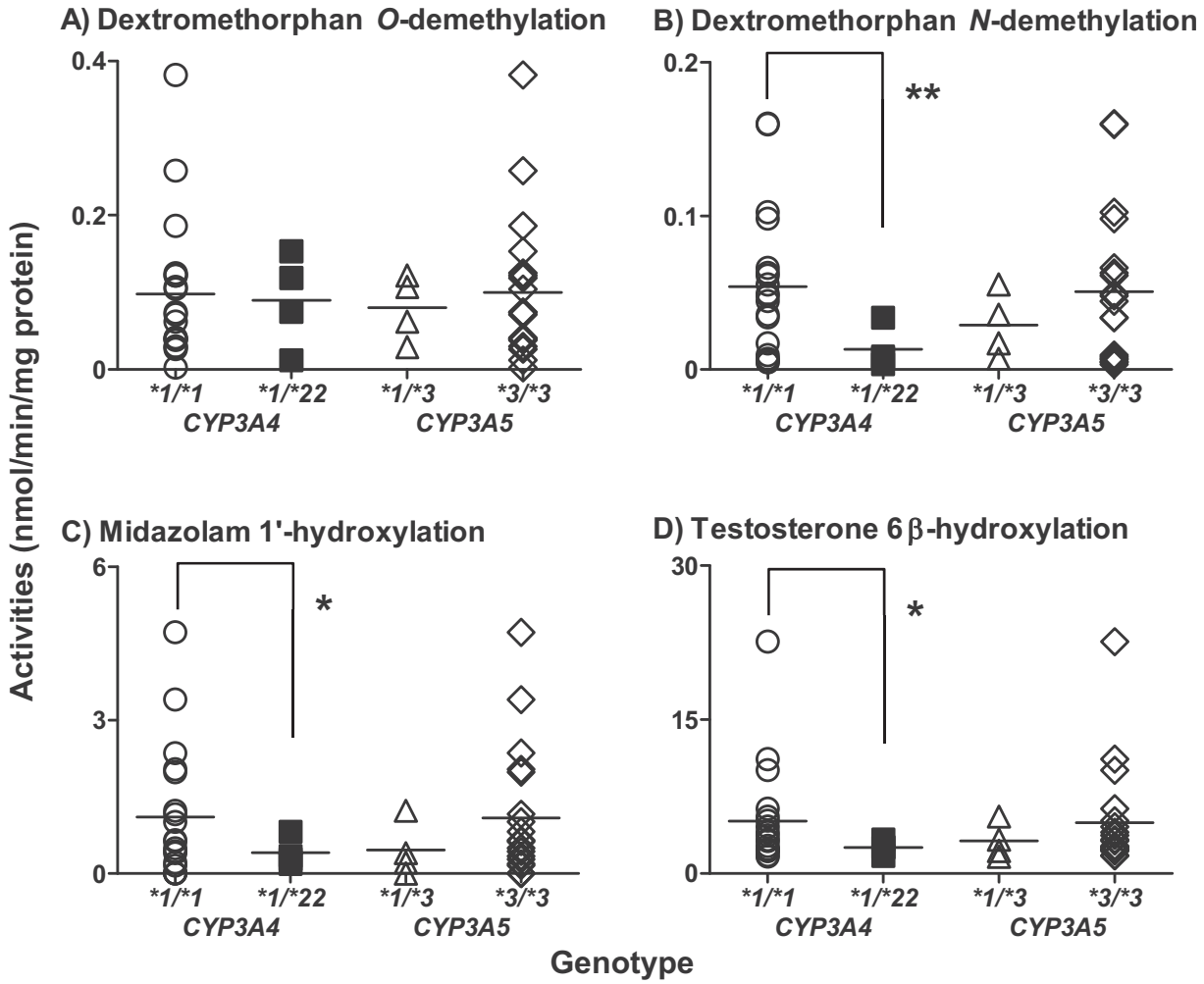

Fig. 1. Association between *CYP3A4* and *CYP3A5* genotypes and P450-dependent drug oxidation activities in human liver microsomes. Dextromethorphan *O*- (A) and *N*-demethylation (B), midazolam 1′-hydroxylation (C), and testosterone 6β-hydroxylation (D) activities were analyzed in liver microsomal samples from 23 Caucasians genotyped for *CYP3A4* and *CYP3A5*. The horizontal lines indicate the mean activities, respectively. *$p < 0.05$; **$p < 0.01$, significantly different by unpaired *t*-test with Welch correction.

these levels were low and < ~5 pmol CYP3A5/mg protein (Yamaori *et al.,* 2005; Yamazaki *et al.,* 1995). Reduced CYP3A4 protein expression levels resulted in decreased CYP3A4-mediated enzymatic activities in liver microsomal samples derived from Caucasians (24-50% of controls, an average of 37%, shown in Fig. 1). Consequently, these results regarding impaired metabolic function in liver microsomes *in vitro* caused by the *CYP3A4*22* genotype could support the recent reported findings of low clearance of clinical CYP3A-related drugs *in vivo* (Elens *et al.,* 2011a*,* 2011b*,* 2011c and 2013; Wang *et al.,* 2011). The reason why this *CYP3A4*22* polymorphism in the intronic part of the gene can lead to lowered the mRNA and/or protein expression should be investigated in the future.

In conclusion, the *CYP3A4*22* allele was associated with decreased CYP3A4 protein expression levels and resulted in decreased CYP3A4-mediated enzymatic activities. These results suggest an important role of low expression of CYP3A4 protein associated with this *CYP3A4*22* allele in the individual differences in drug clearance in Caucasians. Drug therapy for the Caucasian patients harboring the *CYP3A4*22* allele with CYP3A4-dependent drugs should be paid attention. Furthermore, it was also

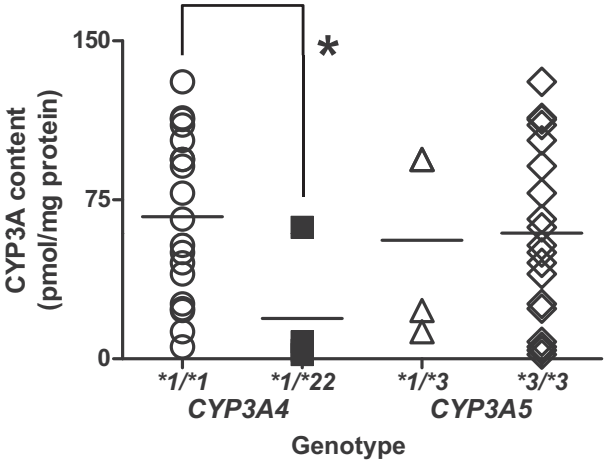

Fig. 2. Association between *CYP3A4* and *CYP3A5* genotypes and expression levels of CYP3A protein in human liver microsomes. CYP3A contents were measured in liver microsomal samples from 23 Caucasians genotyped for *CYP3A4* and *CYP3A5* by immunoblotting. *$p < 0.05$, significantly different by unpaired *t*-test with Welch correction.

demonstrated that the frequency of the *CYP3A4*22* allele shows ethnic differences. Shi *et al*. (2013) have recently reported that they found no CYP3A4*22 alleles in 216 Chinese subjects. Because no *CYP3A4*22* allele carriers were found in a Japanese population in the present study, it may be implied that the effects of *CYP3A4*22* polymorphism might be limited in Japanese subjects, which show a wide variety of inter-individual differences in inductive CYP3A4-dependent drug disposition.

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