An Investigation of the Catalytic Activity of CYP2A13*4 with Coumarin and Polymorphisms of CYP2A13 in a Chinese Han Population

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List of nonstandard abbreviations:
AFB1, aflatoxin B1; ASA, allele-specific amplification; CYP2A13, cytochrome P450 2A13; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SNP, single nucleotide polymorphism;
ABSTRACT:

Cytochrome P450 2A13 (CYP2A13) has been identified as an efficient catalyst for the metabolisms of coumarin, aflatoxin B₁ (AFB₁), and several tobacco-specific carcinogens. The reported CYP2A13 polymorphisms with missense variations have been studied for their functional consequences, and CYP2A13*4 (R101Q) variant was found to be a null enzyme in metabolizing 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), AFB₁ and 5-methoxypsoralen. In the present study, CYP2A13*4 was expressed in Sf9 cells and evaluated for coumarin 7-hydroxylation activity. Our results demonstrated that CYP2A13*4 showed no activity in coumarin 7-hydroxylation. Furthermore, computer modeling studies were conducted to probe the mechanisms underlying the loss of catalytic activity of CYP2A13*4. The results suggested that the R101Q alteration may result in the absence of several hydrogen bonds involved in heme binding and thus lead to the loss of function in CYP2A13*4. In addition, for the first time, the distribution frequencies of all eight known CYP2A13 missense alleles were examined in a Chinese Han population. The distribution frequencies of CYP2A13*3 allele and CYP2A13*4 allele in the Chinese Han population were statistically significantly different from the reported values in Japanese. Considering that the two variants of CYP2A13 are incapable of metabolic activation of NNK and AFB₁, the susceptibility to NNK or AFB₁ exposure between the Chinese Han population and Japanese can be different.
Introduction

CYP2A13 is a member of the human CYP2A subfamily (Fernandez-Salguero and Gonzalez, 1995) and predominantly expressed in human respiratory tract (Su et al., 2000). Previous studies demonstrated that CYP2A13 plays an important role in the metabolic activation of some toxicants and carcinogens such as hexamethylphosphoramide, N,N-dimethylaniline, and N-nitrosomethyl-phenylamine (Zhang et al., 2002), and is especially highly efficient in AFB1 and NNK activation (He et al., 2006; Jalas et al., 2005). It is expected that CYP2A13 would be involved in the carcinogenicity and toxicity exerted by these carcinogens.

Nine non-synonymous single-nucleotide polymorphisms (SNPs) have been identified for human CYP2A13, including an insertional frame-shift mutation (133_134T insertion, CYP2A13*3) and a substitution of a stop codon (R101X, CYP2A13*7) (Cauffiez et al., 2004, 2005; Cheng et al., 2004; Fujieda et al., 2003; Zhang et al., 2002, 2003). CYP2A13*3 and CYP2A13*7 were predicted to be null in function and the other seven SNPs all result in missense variants (Table 1). The distribution frequencies of some CYP2A13 missense alleles have been investigated in Japanese, French Caucasian and some other populations (Cauffiez et al., 2004, 2005; Cheng et al., 2004; Fujieda et al., 2003; Zhang et al., 2002, 2003). However, up to date, the distribution frequencies of all the CYP2A13 missense alleles in Chinese Han population have not yet been reported.

Among the CYP2A13 missense variants, CYP2A13*4 (R101Q) showed a loss of enzymatic activity in metabolizing NNK, AFB1 and 5-Methoxypsoralen (Wang et al., 2006; Goto et al., 2010). Some characteristics of the CYP2A13*4 enzyme have been clarified in
previous reports, including a loss of P450 spectrum, low electrophoretic mobility and an increased susceptibility to limited protein digestion (Wang et al., 2006; Goto et al., 2010). However, the capacity of CYP2A13*4 to metabolize coumarin has not been characterized yet. Wang et al. (2006) suggested that the instability of CYP2A13*4 variant may be due to the strong interaction of the R101Q substitution with heme. Accordingly, further investigation on the interaction between the R101Q substitution and heme molecule is required.

In the present study, CYP2A13*4 variant was expressed in Sf9 cells to determine the functional consequences of R101Q substitution on coumarin 7-hydroxylation. Computer modeling studies then were conducted to probe the possible mechanisms underlying the loss of enzyme activity of CYP2A13*4. In addition, for the first time, the distribution frequencies of all eight known CYP2A13 missense alleles in a Chinese Han population were investigated.
Materials and Methods

Materials

Polymerase chain reaction (PCR) primers were synthesized by Sangon (Shanghai, China). PCR Enzymes were purchased from Takara (Tokyo, Japan). Mouse anti-his monoclonal antibody was purchased from GE Healthcare Bio-Sciences (Little Chalfont, Buckinghamshire, UK) and peroxidase-conjugated goat anti-mouse antibody was purchased from Zhongshan Biotechnology (Guangdong, China). Bac-to-Bac baculovirus expression system and cell culture media were purchased from Invitrogen (Calsbad, CA). Coumarin, 7-hydroxycoumarin, hemin, human NADPH-P450 reductase, NADPH, NADP+, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO).

Construction of CYP2A13 Variants

The CYP2A13*1 gene with a His-tag at the C-terminus was a generous gift from Dr. Hong (University of Medicine and Dentistry of New Jersey, NJ 08854, USA). Using CYP2A13*1 as template, CYP2A13*4 was generated by amplification using Pyrobest DNA Polymerase. CYP2A13*2/*5/*6/*8/*9 were also produced and served as controls. The PCR primers for site-directed mutagenesis are shown in Supplemental Table 1. All PCR products were sequenced to ensure that no unintended mutation was introduced.

Heterologous Expression of CYP2A13

Expression of CYP2A13 proteins was performed in BAC-to-BAC baculovirus expression system as described previously (He et al., 2004). The microsomes were prepared as described earlier (Patten et al., 1996) and stored at -80 °C prior to use.

Immunoblot Analysis and Determination of P450 content
Expression of each CYP2A13 variant was detected by western blot as described previously (Chen et al., 2006). Microsomal P450 content was determined by reduced CO-difference spectrum as previously described (Omura and Sato, 1964). The CO-difference spectra were recorded using a Jasco V-550 Spectrophotometer (Jasco, Japan).

**Coumarin 7-hydroxylation**

The incubation protocol was modified from a previously reported method (He et al., 2004). The 250 μl incubation system consisted of 20 pmol CYP enzymes, NADPH-P450 reductase (P450:reductase at a molar ratio of 1:5), 10 mM magnesium chloride, an NADPH-generating system, 150 mM potassium chloride, and 0.25-25 μM coumarin in 50 mM Tris buffer (pH 7.4). After incubation at 37 ºC for 10min, the reaction was terminated by addition of ice-cold acetonitrile (Sigma, St. Louis, MO), and then the mixture was centrifuged at 10,000 g for 5 min. 25 μl of the supernatant was analyzed by an Agilent Model 1200 system (Agilent, Palo Alto, CA) as previously described (Von Weymarn et al. 1999). The flow rate was 1.4 ml/min, the column temperature was maintained at 35 ºC and chromatographic separation of 7-hydroxycoumarin was obtained by isocratic elution with 23% acetonitrile and 77% 0.01 M ammonium acetate (Sigma, St. Louis, MO). Each reaction was conducted in triplicate in three independent experiments.

**Molecular Modeling**

Molecular modeling studies were conducted to investigate the effects of mutation on enzymatic activity of CYP2A13*4. The initial structure of CYP2A13 was obtained from the Protein Data Bank (PDB ID: 2P85) (Smith et al., 2007). The mutation model for CYP2A13*4 was constructed using Sybyl 6.9 (Tripos, St. Louis, MO), followed by energy-minimization
using Amber10 (Case et al., 2008). The structure of coumarin was built using Sybyl 6.9 and geometrically optimized at the HF/6-31G* level by Gaussian03 (Frisch et al., 2004). Then, coumarin was docked into CYP2A13*1 and CYP2A13*4 using Autodock4.2 (Morris et al., 2009). Finally, the resulting complexes were subjected to molecular dynamics (MD) simulations by the Amber10 software, using similar parameters as described in previous report (Li et al. 2009).

**DNA Isolation**

Two hundred healthy Chinese Han adults were recruited in this study from the clinical laboratory in the Second Affiliated Hospital of Zhejiang University. The research protocol was approved by the Ethics Committee of Zhejiang University. Genomic DNA was isolated from 2 ml blood sample drawn from each volunteer using a blood genomic DNA isolation kit (Axygen, Hangzhou, China). Samples were stored in the TE buffer (pH 8.0) at -20°C before use.

**Distribution of CYP2A13 alleles in a Chinese Han Population**

Two-step allele-specific amplification (ASA) was applied to mutation detection. First, using genomic DNA as template, two long fragments, F1 and F2, were amplified by 25 cycles with two pairs of CTP2A13-specific primers described previously (Supplemental Table 2, Cauffiez et al., 2004). PCR products from five samples were randomly chosen for sequencing and served as positive controls for the second step.

Using the PCR product of the first step as template, the second amplification was performed as described previously (Chen et al., 2006) at an optimized annealing temperature (Supplemental Table 2). Five sequenced samples in step 1 were used as positive controls, and
reaction mixture without template was used as a negative control. The result of each sample was determined by two independent experiments. Chi-square ($\chi^2$) was used to evaluate genotype frequency deviation from Hardy-Weinberg equilibrium and for the statistical comparisons between ethnic groups.
Results and Discussion

To investigate the structural relationship between the R101Q substitution and the capacity of CYP2A13*4 to metabolize coumarin, CYP2A13*4 variant was constructed by site-directed mutagenesis and expressed in a well established Bac-to-Bac insect expression system. The other CYP2A13 missense variants (CYP2A13*2/*5/*6/*8/*9) were also expressed and served as controls. Using anti-His monoclonal antibody, protein bands around 56 KD were detected in the microsomes from all the Sf9 cells transfected with bacmids containing CYP2A13 cDNAs, except the negative control transfected with blank bacmid (Fig. 1A). Consistent with a previous report (Wang et al., 2006), CYP2A13*4 protein showed a reduced expression level in sf9 cells (Fig. 1A) and a loss of P450 spectrum (Fig. 1C) compared with other CYP2A13 variants. Goto et al. (2010) reported that the electrophoretic mobility of the recombinant CYP2A13*4 protein in SDS-PAGE gel was slightly lower than the other recombinant CYP2A13 proteins. However, there was no obvious difference observed in electrophoretic mobility among the CYP2A13 variants in SDS-PAGE analysis in our study. This inconsistency might be partially due to the different expression systems and different purities of the recombinant CYP2A13 proteins and remains to be examined in future work.

Kinetic analysis was conducted for the variant and wild-type proteins using different substrate concentrations (0.5, 2, 5, 10, 15, 20 and 25 μM). Consistent with Schlicht and colleagues’ report (2007), the CYP2A13*2/*5/*6/*8/*9 variants resulted in only modest changes in catalytic efficiency compared with CYP2A13*1. However, the kinetic parameters of CYP2A13*4 can not be determined, as there was no detectable product of coumarin 7-hydroxylation.
Computer modeling studies were then performed to probe the mechanisms underlying the loss of function of CYP2A13*4. The mutation is located within the B-B’ loop and is very close to heme. Previously, Wang et al. (2006) suggested that the effect of R101Q variation on CYP2A13 may be due to its strong interaction with heme. To gain deeper insights into the mechanisms, MD simulations were carried out for both CYP2A13*1 and CYP2A13*4. CYP2A13*4 showed higher RMSD values (Fig. 2) during the simulations, which suggested larger structural deviations and less stability compared with CYP2A13*1 (consistent with the experimental result). In addition, the hydrogen-bonding interactions (Supplementary Table 3, Fig. 3) at the mutation site were analyzed. From Supplementary Table 3, R to Q substitution precluded hydrogen-bonding interaction capability of heme with residue 101 and 372. Besides, the interaction between heme and residue 437 also decreased. As we know, heme is anchored by hydrogen-bonding interactions in the active site and plays an important role in electron transfer during the catalysis. Therefore, the above hydrogen-bonding network changes could greatly affect the appropriate heme binding, and further lead to the loss of the function in CYP2A13*4.

In addition to coumarin, CYP2A13*4 also showed a decreased metabolic activity on NNK and aflatoxin B1 (Jalas et al., 2005; He et al., 2006). The mechanism of such enzyme activity loss suggested that the same tendency will be also observed in metabolisms of other environmental chemicals. It is reasonable to assume that the ethnic difference in distribution
of CYP2A13*4 allele may lead to the ethnic difference in the human susceptibility to some environmental chemicals. Therefore, the frequency distribution of CYP2A13*4 in a Chinese Han population was investigated, along with the other seven CYP2A13 alleles.

To investigate the distribution frequencies of CYP2A13 alleles in a Chinese Han population, a two-step ASA method was applied to genomic DNA samples of 200 unrelated volunteers. Five DNA samples, analyzed by sequencing, were used as positive controls, and the results of ASA of the five positive controls were consistent with the results of sequencing. The distribution of CYP2A13 polymorphisms conformed to the prediction of Hardy–Weinberg. The frequency of CYP2A13*2 (R257C) allele, CYP2A13*3 (133_134T insertion) allele, CYP2A13*4 (R101Q) allele, CYP2A13*5 (F453Y) allele, CYP2A13*6 (R494C) allele, CYP2A13*7 (R101X) allele, CYP2A13*8 (D158E) allele and CYP2A13*9 (V323L) allele was 6.5%, 1.0%, 3.8%, 1.0%, 1.5%, 4.0%, 1.3% and 0.5%, respectively (Table 1, details in Supplementary Table 4).

Compared with other ethnic groups reported previously, the distribution frequencies of certain CYP2A13 alleles in the Chinese population were significantly different from that in Japanese. CYP2A13*3 allele, reported as an unstable variant (Schlicht et al. 2007), has a much higher frequency in Japanese (4.9%) than that in Chinese Han population (1.0%) (p < 0.01). CYP2A13*4 allele, which results in a null allelic variant of CYP2A13 (Wang et al., 2006), is distributed more widely in the Chinese Han population (3.8%) than in Japanese (0.3%) (p < 0.01). There are no significant distribution differences of CYP2A13*7 allele, CYP2A13*8 allele and CYP2A13*9 allele between the French Caucasian (Cauffiez et al., 2004) and
Chinese Han population. The ethnic differences in these null alleles of CYP2A13 suggested that there be a different susceptibility to NNK or AFB₁ exposure between the Chinese Han population and Japanese, considering that these null allelic variants of CYP2A13 are incapable of metabolic activation of NNK and AFB₁.

In summary, CYP2A13*4 showed no metabolic activity on coumarin. Furthermore, MD simulations indicated that the amino acid substitution in CYP2A13*4 resulted in the absence of several hydrogen bonds involved in heme binding, and thus lead to the loss of function. And for the first time, the frequencies of all eight reported CYP2A13 alleles in Chinese Han subjects were determined. The statistically different distribution frequencies of CYP2A13*3/*4 alleles between Chinese Han population and Japanese indicate that these two populations may have different susceptibility to NNK or AFB₁ exposure.
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Authorship Contributions

Participated in research design: Liu, Y.J. Hong, J.Y. Hong, Zeng, and Chen.

Conducted experiments: Liu, Y.J. Hong, and Li.

Contributed new reagents or analytic tools: Zheng.

Performed data analysis: Liu, Y.J. Hong, and Chen.

Wrote or contributed to the writing of the manuscript: Liu, Y.J. Hong, J.Y. Hong, and Chen.
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Footnotes

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T.L. and Y.H. contributed equally to this work.
Legends for Figures

Figure 1. Heterologous expression of CYP2A13 recombinant enzymes in Sf9 cells. Microsomes were prepared from the Sf9 cells infected for 72 h with recombinant bacmids containing either CYP2A13 wild-type or variant cDNAs. Sf9 cells transfected with the vector alone (without CYP2A13 cDNA) were used as negative control. (A) Western blot of microsomal proteins. 5 μg of each sample were used. From left to right: wild-type CYP2A13, the variants and negative control (B) Representative CO-difference spectrum, which showed the characteristic P450 peak (C) microsomes containing CYP2A13*4 protein only showed an absorption peak at 420 nm.

Figure 2. Backbone RMSD variations of CYP2A13*1 and *4 (red) with respect to the starting structures along the simulations.

Figure 3. Structure of the heme-binding region in CYP2A13*1 (A) and CYP2A13*4 (B). The substitution in CYP2A13*4 precluded hydrogen-bonding interaction (yellow, dash line) capability of heme (gray) with residue 101 (red) and 372 (yellow).
Table 1 Comparison of allele frequencies for the CYP2A13 alleles between Chinese Han and other populations

<table>
<thead>
<tr>
<th>Allele</th>
<th>Missense mutations</th>
<th>Frequency in Chinese</th>
<th>Frequency in other populations</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2A13*2</td>
<td>R257C</td>
<td>6.5%</td>
<td>7.7% (Asian), 5.6% (Chinese), Cheng et al., 2004, 5.8% (Hispanic), Cheng et al., 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0% (French Caucasians), Cauffiez et al., 2005</td>
</tr>
<tr>
<td>CYP2A13*3</td>
<td>133_134T insertion</td>
<td>1.0%</td>
<td>4.9% (Japanese), Fujieda et al., 2003</td>
</tr>
<tr>
<td>CYP2A13*4</td>
<td>R101Q</td>
<td>3.8%</td>
<td>0.3% (Japanese), Fujieda et al., 2003, 3.8% (French Caucasians), Cauffiez et al., 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0% (Gabonese), 1.0% (Tunisians), Cauffiez et al., 2005</td>
</tr>
<tr>
<td>CYP2A13*5</td>
<td>F453Y</td>
<td>1.0%</td>
<td>0.3% (Japanese), Fujieda et al., 2003</td>
</tr>
<tr>
<td>CYP2A13*6</td>
<td>R494C</td>
<td>1.5%</td>
<td>1.0% (Japanese), Fujieda et al., 2003</td>
</tr>
<tr>
<td>CYP2A13*7</td>
<td>R101X</td>
<td>4.0%</td>
<td>5.0% (French Caucasian), Cauffiez et al., 2004, 4.9% (Japanese), Fujieda et al., 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.2% (Chinese), Zhang et al., 2003</td>
</tr>
<tr>
<td>CYP2A13*8</td>
<td>D158E</td>
<td>1.3%</td>
<td>1.0% (French Caucasian), Cauffiez et al., 2004</td>
</tr>
<tr>
<td>CYP2A13*9</td>
<td>V323L</td>
<td>0.5%</td>
<td>1.0% (French Caucasian), Cauffiez et al., 2004</td>
</tr>
</tbody>
</table>
Figure 1

A

2A13*1  2A13*2  2A13*5  2A13*6  2A13*8  2A13*9  2A13*4  vector

B

Abs

0.05

-0.05

400  420  440  460  480  500
Wavelength [nm]

C

Abs

0.008

0.005

0.002

-0.005

-0.006

400  420  440  460  480  500
Wavelength [nm]
Figure 2

![RMSD vs Time Graph]

- **CYP2A13*1**
- **CYP2A13*4**

RMSD (Å) vs Time (ps)
Figure 3