Phenotyping of CYP1A2 in Japanese Population by Analysis of Caffeine Urinary Metabolites: Absence of Mutation Prescribing the Phenotype in the CYP1A2 Gene

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Abstract
Caffeine has been used as a metabolic probe to determine the relative levels of CYP1A2 activity in different individuals, since this compound is specifically 3-demethylated by CYP1A2. Urine specimen obtained at a 4-5-h interval after caffeine ingestion from 205 Japanese were analyzed using the [1,7-dimethyluric acid + 1,7-dimethylxanthine]/caffeine (1,3,7-trimethylxanthine) ratio, which better correlated with the rate constant for caffeine 3-demethylation than other ratios. The probit analyses of nonsmokers (n = 147) and smokers (n = 58) suggested that the CYP1A2 activity was not normally distributed and appeared bimodal. The breakpoints were at 5.0 and 6.0 of (1,7-dimethyluric acid + 1,7-dimethylxanthine)/1,3,7-trimethylxanthine ratio in nonsmokers and smokers, respectively. The bimodal probit plot suggested the existence of poor and extensive phenotypes. The percentage of individuals with the poor phenotype in Japanese was 14.1%. Induction of CYP1A2 by cigarette smoking was confirmed by the higher molar ratio observed in smokers (P < 0.0001). The CYP1A2 ratio was also higher in males than in females (P = 0.04). A reproducibility study of 12 subjects in an 11 month interval period showed that intraindividual variability did not alter this CYP1A2 phenotypic classification. Family study in eight pedigrees suggested that the poor phenotype of CYP1A2 inherited as an autosomal recessive trait. The sequences of CYP1A2 gene from poor and extensive metabolizers were analyzed. Although no differences of nucleotide sequence were observed in exons, exon-intron junctions and 5'-flanking regions (up to ~2.6 kilobases) of CYP1A2 gene between each phenotype, there were some sequences which differed from the previous reported data. This is the first report in which the

CYP1A2 phenotype and a genetic polymorphism in the CYP1A2 gene were comparably investigated.

Introduction
CYP1A2 is responsible for the metabolic activation of various carcinogens such as aromatic amines (2-amino-3-methylimidazo[4,5-f]quinoxaline), heterocyclic amines [Trp-P-2 (3-amino-1-methyl-5H-pyrido[4,3-b]indole), and PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine)] through N-oxidation (1-4). In humans, large intraindividual differences in metabolism of such carcinogens and in the content of CYP1A2 were suggested from the results of several previous studies (3, 5-7). Such intraindividual differences of CYP1A2 activities have long been regarded as determinants of individual cancer susceptibility.

CYP1A2 was shown to be responsible for the 3-demethylation of caffeine (8), the initial major step in the biotransformation of caffeine in humans. Since caffeine is a commonly ingested and is a relatively innocuous compound, determination of caffeine metabolites in human urine can be useful in the estimation of the CYP1A2 activity of individuals and can serve to assess individual differences in susceptibility to aromatic amine-inducible cancers. Methods to analyze caffeine metabolites and several urinary metabolite ratios reflecting CYP1A2 activity have been reported in different populations (9-12).

In the present study, we analyzed caffeine metabolites in urine obtained from 205 Japanese at a 4-5-h interval after coffee consumption.

A genetic polymorphism for CYP1A2 has been suggested by differences in the in vivo metabolism of phenacetin (13) and theophylline (14, 15). To investigate whether the phenotypic polymorphism of CYP1A2 detected by caffeine metabolism can be ascribed to genetic factors, the caffeine test were performed in eight pedigrees.

The genetic polymorphisms responsible for intraindividual variability of human CYP1A2 have not yet been determined. Therefore, to examine the genetic polymorphism of CYP1A2, we analyzed sequences of CYP1A2 gene from PMs1 and EMs using a PCR method.

1 The abbreviations used are: PM, poor metabolizer; EM, extensive metabolizer; PCR, polymerase chain reaction; 137X, 1,3,7-trimethylxanthine; 13X, 1,3-dimethylxanthine; 17X, 1,7-dimethylxanthine; 37X, 3,7-dimethylxanthine; 1X, 1-methylxanthine; 3X, 3-methylxanthine; 1U, 1-methyluric acid; 137U, 1,3,7-trimethyluric acid; 17U, 1,7-dimethyluric acid; AFMU, 5-acetylamino-6-formylamino-3-methyluracil; HPLC, high performance liquid chromatography; MR, molar ratio; XRE, xenobiotic responsive elements; 7X, 7-methylxanthine; 3U, 3-methyluric acid; 7U, 7-methyluric acid; 13U, 1,3-dimethyluric acid; 37U, 3,7-dimethyluric acid.
Biosystems, Foster City, CA). The sequences of the synthesized primers used for PCR were as follows:

- U1, 5'-TGTCAGATGCTTCTTCAA-3';
- U2, 5'-GTTAGGAGGAGCTTCGCCAT-3';
- U3, 5'-TTGCTCAATTCACATTTGTGA-3';
- U4, 5'-TCACATTCATGCATATGCACA-3';
- U5, 5'-CAGCCATCTCAGGACTG-3';
- U6, 5'-AGCCATCGGGAAGATTATGAT-3';
- U7, 5'-GCTCAGGAAATTACCTTCTAAG-3';
- U8, 5'-CTTGAAGTGCCGAGGTGATA-3';
- P1, 5'-TGATAGGGGGGTGTGCTATTAA-3';
- P2, 5'-TTGACACCACCGATACCGAAG-3';
- P3, 5'-TGTCAGATCCTGGGGGTTCT-3';
- P4, 5'-CAAAGATGCTTACAGGAGTG-3';
- P5, 5'-AGCCAGAACCTCATCCACACA-3';
- P6, 5'-TCTCGAGCTGACAGGACGTAAG-3';
- P7, 5'-CCTTGATCTGCTCTCTCTCTCT-3';
- P8, 5'-AGCTCGAGCTGAGCTGCT-3';
- P9, 5'-CTTCAGTACGATGATGAGG-3';
- P10, 5'-GAACGAGGAGCAGGAGAAG-3';
- P11, 5'-AAACCATGCCCAGCAGTCT-3';
- P12, 5'-AACTCGACATCTCTCTGTCAG-3';
- P13, 5'-AACAGAATCGTCTCTCCCAGC-3';
- P14, 5'-CCCTCAGAAATGTTGCTTCT-3'.

PCR and Sequence Analysis of Amplified DNA Fragments.

DNA samples (1 μg) were added to the PCR mixtures consisting of 20 mM Tris-HCl buffer (pH 8.4), 25 mM KCl, 0.05% Tween-20, 0.1 mg/ml gelatin, 1 mM MgCl₂, 0.4 μM primers, and 250 μM dNTPs. Thirty cycles of amplification were performed using a programmable heat block (Perkin Elmer, Norwalk, CT) under the following conditions: (A) 1.5 min at 94°C for denaturation, 2 min at 56°C for primer annealing and 2 min at 72°C for primer extension; (B) 30 s at 94°C for denaturation, 20 s at 56°C for primer annealing, and 2 min at 72°C for primer extension. The condition (A) was applied in all amplifications. When efficient amplification could not be obtained, the condition (B) was applied. The amplified DNA fragments were blunt-ended by T4 DNA polymerase, phosphorylated by T4 polynucleotide kinase, and subcloned into the blunt-ended M13mp18 vector (18). The nucleotide sequences of the amplified DNA fragments were determined by the dideoxy chain-termination method (19) from selected fragments inserted into M13mp18 vector.

Results

Separation and Identification of Caffeine and Its Metabolites in Urine. A HPLC chromatogram is shown in Fig. 1. 17U, 17X and 177X as well as AFMU, 3X, 1X, 13U, 37X, 13X, and 137X were identified from a urinary extract which was prepared from urinal collected during the 4-5 h interval following ingestion of a 9-ounce cup of coffee prepared from 3.6 g instant coffee. Determination of caffeine and its metabolites was achieved according to the method of Butler et al. (9) with slight modifications in solvent elution and UV detection. Briefly, the modified elution program was selected in order to optimize resolution with the HPLC system used. The spectral information provided by the photo-diode array detection system allowed a comparison of the absorbance spectra of the putative metabolites with known standards and also provided information on peak homogeneity.

CYP1A2 Phenotype of Japanese Subjects. The urinary molar ratio of (17U+17X)/177X was selected for the CYP1A2 activity index, because this ratio was found to reflect caffeine 3-demethylation activity in this caffeine phenotyping procedure better than the other previous proposed ratios of 177X/177X (20), (AFMU+1X+1U)/177X (10) and (AAMU+1X+1U)/177X (11), which was investigated in detail by Butler et al. (9). CYP1A2 phenotyping of Japanese were presented as a frequency distribution in Fig. 2. The MR of (17U+17X)/177X in the 147 nonsmokers (147 unrelated individuals, open column) and smokers (58 unrelated individuals, closed column).
4 1 6

CYP1A2 Phenotypes and No Mutation in CYP1A2 Gene

Fig. 3. Probit analyses of Japanese 147 nonsmokers and 58 smokers for the urinary molar ratio of (17U+17X)/137X. Ordinate, values obtained from analysis of caffeine urinary metabolites in different individuals. Abscissa, the percentage area under the normal probability curve calculated for each data point as described in “Materials and Methods.” Nonsmokers (open circles and open triangles) and smokers (closed circles and closed triangles). Three open and two closed triangles with arrows show the subjects whose genomic DNA were analyzed in the present study.

and EMs exist, sequence analysis of CYP1A2 gene was performed. The location of primers for PCR and PCR-amplified DNA fragments for sequencing are shown in Fig. 6. Genomic DNAs were obtained from two PMs (MR were 2.4 and 3.2, respectively) and one EM (MR, 13.1) in nonsmokers, and from one PM (MR, 1.8) and one EM (MR, 25.1) in smokers indicated by arrows (Fig. 3). These subjects selected for sequence analysis are typical PMs and EMs, respectively. The nucleotide sequences of all exons (whole sequences), intron sequence (at least 30 base pairs) from exon-intron junctions, and 5′-flanking regions (up to −2.6 kilobases) of the CYP1A2 gene were analyzed in nonsmokers, and 5′-flanking region (up to −2.6 kilobases) in smokers was analyzed as described in “Materials and Methods.” The present data were also compared with the previously reported data (21–24).

As shown in Fig. 7, no differences between two PMs and one EM in nonsmokers in all exons and exon-intron junctions were observed; however, the present sequence was different from the data reported by Quattrochi et al. (23) at positions 235, from 1535 to 1536 and 1548, and also different from the data reported by Jaiswal et al. (21) at positions from 1531 to 1533 and 1548. The regions which differed from previous reports were shown in Fig. 7. There were no sequence differences in the other exons and exon-intron junctions as far as we analyzed.

The present sequence of 5′-flanking region (−2578 to −1) was compared with the data reported by Ikeya et al. (22) and Quattrochi and Tukey (24). Although no differences in nucleotide sequence were observed between three PMs and two EMs in Japanese nonsmokers and smokers, there were some sequences which differed from these reported data (Fig. 8). The identity of the present data with the data reported by Ikeya et al. (22) was 98.5%, and with the data reported by Quattrochi and Tukey (24) was 99.5%.

Discussion

The concept of drugs as probes for carcinogen metabolism has been proposed for many years (25). In the present study, we performed a simple and noninvasive assay in which caffeine was administered in coffee, urine was collected
Table 1  Intraindividual differences in the urinary molar ratio of (17U+17X)/137X

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Smoking</th>
<th>MR1</th>
<th>MR2</th>
<th>Coefficient of variation (%)</th>
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<tr>
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<td>1.6</td>
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<td>5.60</td>
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<td>NS</td>
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<td>10.79</td>
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<td>S</td>
<td>19.13</td>
<td>13.08</td>
<td>37.6</td>
</tr>
</tbody>
</table>

*MR1, MR from the first test; MR2, MR from the second test; NS, nonsmoker; S, smoker.*

Fig. 5  Eight pedigrees showing transmission of phenotypes for the urinary molar ratio of (17U+17X)/137X. The values represent the molar ratio of individuals.

between 4–5 h after dosing, and caffeine metabolites were analyzed by HPLC. The data suggested the existence of poor and extensive CYP1A2 phenotypes in Japanese. Induction of CYP1A2 activity by smoking was also confirmed. Bi-linear probit plots of the urinary molar ratio of (17U+17X)/137X were observed in Japanese, although obscure in the nonsmoking population. This may due to the existence of other inducible factors which influence CYP1A2 activities. Caffeine 3-demethylation activity catalyzed exclusively by CYP1A2 is known to be increased by consumption of charcoal-broiled meat (26) and cabbage and brussels sprouts (27). It was reported that the probit plots for Caucasians and Chinese people were resulted in apparent tri-linear distributions (9, 28), whereas that for Caucasians with different calculation formulas for CYP1A2 index were resulted in unimodal distributions (11, 29, 30). The bi-linear probit plots of the urinary molar ratio of (17U+17X)/137X in vivo in the present study is also strongly supported by previous \textit{in vitro} studies with human liver microsomes that show a bimodal distribution for the CYP1A2-dependent N-oxidation of 2-acetylaminofluorene (6) and the O-deethylation of phenacetin, as well as for levels of immunoreactive CYP1A2 (31).

The percentage of individuals with the poor phenotype in Japanese nonsmokers was 14.3% (21/147) and in smokers it was 13.8% (8/58). These values were similar to the result from subjects in Arkansas, Italy, and the People's Republic of China (12–13%) (9). This is also similar to previous findings in England that about 10% of populations have low phenacetin O-deethylase activity (32).

Large interindividual variations in the activity of hepatic CYP1A2 have been demonstrated in the present study, where the urinary molar (17U+17X)/137X ratio that reflects CYP1A2 activity varied 14-fold in the Japanese subjects. This is similar to the observation that phenacetin O-deethylation varied 58-fold (33, 34). The amount of immunoreactive CYP1A2 varied 10-fold in other preparations (3, 5). Furthermore, CYP1A2 mRNA levels also varied 15-fold in different 12 human liver samples (22). Similar interindividual variations in levels of hepatic microsomal CYP1A2 have been shown in several previous \textit{in vitro} studies. For example, rates of N-oxidation of 2-acetylaminofluorene, 2-naphthylamine, and 4-aminobiphenyl varied 67-fold (6), >28-fold (35), and 44-fold (7), respectively, in human liver microsomes.

The mean (17U+17X)/137X ratio in nonsmokers was 7.44, while the values vary from 3.02 to 5.37 to 9.27 in Italian, Chinese, and Arkansas nonsmokers, respectively (9). The racial differences in CYP1A2 activity between these populations may also be due to exposure to dissimilar inducers and/or inhibitors in their diet and environment and may also reflect different genetic background.

The mean (17U+17X)/137X ratio in nonsmokers was significantly higher in male than in female \((P=0.04)\). This was consistent with the previous report in which (AFMU+1U+1X)/177X ratio for CYP1A2 activity was used (38). These results suggest that there is gender difference in CYP1A2 activity. Since it has been reported that oral contraceptives and pregnancy decreased (AFMU+1U+1X)/177U ratio for CYP1A2 activity (29) and decreased the elimina-
CYP1A2 Phenotypes and No Mutation in CYP1A2 Gene

Fig. 6. Structure and sequencing strategy of CYP1A2 gene. Closed boxes, exons. Arrows, the location and direction of primers for PCR. Broken lines with arrows, PCR-amplified DNA fragments. PCR was performed under condition A or B as described in "Materials and Methods." The regions indicated by hatched boxes were completely sequenced.

Fig. 7. Nucleotide sequence of the human CYP1A2 gene from two PMs and one EM. Nucleotide sequence of a part of exon 2 from -8 to 292 (A) and a part of exon 7 from 1293 to 1592 (B) are shown. The sequence from 293 to 1292 is omitted, due to no difference between the present data and the data reported by Jaiswal et al. (21) and Quattrochi et al. (23). Only nucleotides that are not identical with the present data are shown. Nucleotide number 1 indicates the initiation site of translation and the number corresponds to nucleotide number of complementary DNA. The initiation codon and stop codon are boxed. Boldface letters, the different sites from the sequence reported by Jaiswal et al. (21) and Quattrochi et al. (23). Dashes, deletions. There were no differences between PMs and EM.

The molecular basis of phenotypic polymorphism can often be ascribed to a mutation or deletion in the gene itself. For example, several CYP2D6 gene mutant which cause phenotypic (debrisoquine-sparteine) polymorphism were reported (42). Since genetically controlled polymorphism of CYP1A2 was suggested by in vivo metabolism of phenacetin (13) and theophylline (14, 15), we initiated this study. To investigate whether or not phenotypic polymorphism of CYP1A2 can be ascribed to differences of sequence between PMs and EMs, the nucleotide sequences of all exons (whole sequences), intron sequence (at least 30 base pairs) from exon-intron junctions, and 5'-flanking region (up to -2.6 kilobases) of CYP1A2 gene were analyzed. It was considered that there was individual variability in the induction of CYP1A2. Therefore, sequences in 5'-flanking region were investigated in addition to exon and exon-intron junction. Although there were some regions which differed from the previous reported data, no differences between PMs and EMs were observed. Thus, the phenotypic polymorphism of CYP1A2 was not due to mutation or deletion in exon, splice junction and 5'-flanking region (up to -2.6 kilobases).

CYP1A1, a closely related isofrom to CYP1A2, is also known to be induced by cigarette smoking in extrahepatic
Fig. 8. Nucleotide sequence of the 5'-flanking region of human CYP1A2 gene from three PMs and two EMs. Nucleotide number indicating the start site of transcription with a 1 was according to the data by Quattrochi and Tukey (24). Nucleotides upstream of the transcription start site are numbered consecutively with negative numbers, whereas nucleotides downstream of the transcription start site are designated by consecutively positive numbers. Only nucleotides that are not identical with the present data are shown for Ikeya (22) and Quattrochi (24). The sequence of the 5'-flanking region to -1907 was reported by Ikeya et al. (22). Dotted area, unreported nucleotide region of Ikeya et al. (22). Dashes, deletions. There were no differences in nucleotide sequence between PMs and EMs.

tissues such as placenta, lung, and peripheral blood cells and in cultured cells (43, 44). Induction mechanism of CYP1A1 has been extensively characterized. The 5'-flanking region of the gene for CYP1A1 contains several short sequence motifs, termed XREs. It is known that polycyclic aromatic hydrocarbons such as benzo[a]pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin bind Ah receptor and ligand-bound Ah receptor interacts with XREs and finally activate the CYP1A1 gene transcriptionally (45). In contrast to CYP1A1, much less information is reported on the regulation mechanism of CYP1A2. Although the CYP1A2 gene lacks apparent XREs in the 5'-flanking region, the latter is required for induction by 3-methylcholanthrene in human liver HepG2 cells but not in human breast MCF-7 cells (24). Similarly, cigarette smoking is known to induce CYP1A2 in human liver (9, 31, 36, 37), but not lung tissue (46, 47), while CYP1A1 is induced in human lung but not in liver by cigarette smoking (46, 47). These data suggest that there are tissue-specific factors that control CYP1A2 expression. Accordingly, the polymorphism could involve further processing of the Ah receptor, the arnt protein (e.g., phosphorylation), or even another receptor and...
could result in individuals who are slow, intermediate, or highly inducible phenotypes.

CYP1A2 is responsible for metabolic activation of numerous procarcinogens, mutagens, and heterocyclic arylamines. Therefore, the polymorphism of CYP1A2 may influence individual susceptibility to chemical-induced cancers. Further investigation is needed to clarify the mechanism of genetic polymorphism of the human CYP1A2 gene.

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References


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