Rapid and Reliable Method for Cytochrome P450 2D6 Genotyping

ULRIKE M. STAMER,* BETTINA BAYERER, STEPHANIE WOLF, ANDREAS HOEFFT, AND FRANK STÜBER

Background: Single-nucleotide polymorphisms and single-base deletions within the cytochrome P450 2D6 (CYP2D6) gene have been associated with a poor metabolizer (PM) phenotype and display a frequency of 7–10% in the Caucasian population.

Methods: We developed a reliable and rapid procedure to identify five major PM-associated mutations (CYP2D6*4, *7, and *8) and deletions (CYP2D6*3 and *6) by real-time PCR with subsequent fluorometric melting point analysis of the PCR product. These polymorphisms within the CYP2D6 gene were detected by use of two primer pairs and five different pairs of hybridization probes. DNA extracted from whole blood of 323 individuals was analyzed, and results were compared with genotypes obtained by allele-specific multiplex PCR. In case of uncertain results, additional sequence analysis was performed.

Results: Genotyping results by real-time PCR were 100% reliable, whereas conventional allele-specific multiplex PCR produced uncertain results for 12.1% of samples, as confirmed by sequence analysis. Costs for reagents and consumables were considerably higher for the real-time PCR technology, but labor time was reduced by 2 h compared with allele-specific PCR. The allele frequencies in the population investigated were 0.186 for allele *4, 0.026 for allele *5, 0.009 for allele *3, 0.031 for allele *6, and 0.002 for allele *8. The defective CYP2D6*7 allele was not found. In addition, three additional mutations were detected, one of them displaying a PM genotype.

Conclusion: Genotyping of CYP2D6 by real-time PCR with fluorometric melting point analysis is a rapid and reliable method.

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Materials and Methods

After approval by the local ethics committee and written informed consent was obtained from 323 unrelated

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German Caucasians, EDTA blood was drawn and stored at −20 °C until isolation of genomic DNA (Puregene DNA Isolation Kit; Gentra Systems). A preamplicon was generated as described previously (14) by use of primers specifically designed to avoid amplification from the pseudogenes CYP2D7 and CYP2D8. The PCR product was resolved by gel electrophoresis to confirm correct amplification. This 1654-bp fragment of CYP2D6 was used as template [0.5 μL of the 1:10 diluted preamplicon (5 μL of PCR diluted to 50 μL) per PCR reaction] for allele-specific multiplex PCR and real-time PCR.

**ALLELE-SPECIFIC MULTIPLEX PCR**

Two separate PCR reactions with either wild-type or mutation-specific primers were run for each sample. The detailed methods were adopted from the report by Stüven et al. (14). PCR products were resolved by 2% agarose gel electrophoresis and ethidium bromide staining.

**REAL-TIME PCR AND FLUORESCENCE MELTING CURVE ANALYSIS**

Two real-time PCR reactions using the LightCycler™ (Roche Diagnostics) were performed in parallel. Mutation *4*, *6*, and *8* were investigated in one capillary, mutations *3* and *7* in a second capillary. For both reactions, LightCycler-Red 640- and LightCycler-Red 705-labeled hybridization probes were used in each of the capillaries to perform dual-color hybridization and melting curve analysis after the final amplification cycle.

**ANALYSIS OF ALLELES *4*, *6*, AND *8**

The PCR used the product of the first amplification step as a template and forward and reverse primers (5′-AgAg-gGgCTTCTCCgTgTC-3′ and 5′-CAggAggAggGgAT-CA-3′, respectively) at a concentration of 0.5 μM and produced a 251-bp PCR product. Three pairs of mutation-specific hybridization probes were added; one probe of each pair was labeled at the 3′ end with fluorescein, which served as the donor fluorophore, and the other probe was labeled at the 5′ end with LightCycler Red 640 (alleles *4* and *6) or LightCycler Red 705 (allele *8*), which served as an acceptor dye. The sequences of sensors and anchors (3 pmol each for one reaction; TIB Molbiol) are shown in Table 1. The following PCR reagents were used: 3 mM MgCl2 (Roche Diagnostics GmbH), 2 μL of LightCycler-DNA Master Hybridization Probes (10X concentration; Roche Diagnostics) and 0.5 μL of the 1:10 diluted preamplicon in a total volume of 20 μL.

After an initial denaturation step (95 °C for 30 s), amplification was performed for 30 cycles (95 °C for 3 s, 65 °C for 10 s, 72 °C for 12 s). Final melting curves were obtained using a denaturation step (95 °C for 20 s), and a final hybridization (40 °C for 20 s) was performed from 40 °C to 95 °C with a temperature transition of 0.1 °C/s. During the melting curve analysis, temperature-dependent hybridization of the sequence-specific hybridization probes to single-stranded DNA was monitored in channels F2 (alleles *4* and *6*) and F3 (*8*). Fluorescence resonance energy transfer occurred from the excited fluorescein dye to the detection dye LC-RED 640 or LC-RED 705.

**ANALYSIS OF MUTATIONS *3* AND *7**

Mutations *3* and *7* were analyzed using forward and reverse primers (5′-TggCTggCAAggTCCTACg-3′ and 5′-TggCTCACgCTgACATT-3′, respectively) at a concentration of 0.5 μM. The resulting PCR product was 478 bp long. The sensor and anchor sequences are given in Table 1. The amounts of PCR reagents used were identical to the protocol described above. The PCR conditions were denaturation at 95 °C for 30 s and amplification for 35 cycles (95 °C for 3 s, 60 °C for 10 s, 72 °C for 20 s). Melting point analysis was performed as described above, using channels F2 (*7*) and F3 (*3*).

**COMPARISON OF BOTH METHODS**

Results of genotyping obtained by both methods were compared. PCR products for the positive controls for each genotype as well as uncertain results obtained with the allele-specific multiplex PCR were sequenced bidirectionally (ABI 377; Perkin-Elmer) to confirm our real-time PCR findings. We also compared the costs for reagents and labor time necessary to perform genotyping using the multiplex PCR or the real-time PCR.

**ANALYSIS OF ALLELE *5***

A homozygous mutation for allele *5*, which represents the null allele, does not produce a PCR product in the preamplification assay. To confirm these findings and to exclude false-negative results, we used a control assay as described previously by Steen et al. (3). The primers *5-F (5′-ACATTCgAgTgATCGCATgAgCA-3′; 10 pmol/μL) and *5-R (5′-gCAgTgCTAAtgCCgCCAaG-3′; 10 pmol/μL) were designed to amplify a 3.5-kb PCR product in the

<table>
<thead>
<tr>
<th>Name</th>
<th>Site of mutation*</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>*6 Sensor</td>
<td>T1795del</td>
<td>5′-CCTGAgTCACACgCCTgCAgCC 3′</td>
</tr>
<tr>
<td>*6 Anchor</td>
<td></td>
<td>5′-LC Red640-CTCCTgCCAggCCCCCTCCAgTTT p-3′</td>
</tr>
<tr>
<td>*8 Sensor</td>
<td>G1846T</td>
<td>5′-LC Red705-CCCgCCCATCCAACgCgCCCTT p-3′</td>
</tr>
<tr>
<td>*8 Anchor</td>
<td></td>
<td>5′-CCCgCTCCTCCgCCTTCCgX-3′</td>
</tr>
<tr>
<td>*4 Sensor</td>
<td>G1934A</td>
<td>5′-LC Red640-CCCAaAggCCCTCCCTT p-3′</td>
</tr>
<tr>
<td>*4 Anchor</td>
<td></td>
<td>5′-CgCCCTCCTCCgCATCTCCC X-3′</td>
</tr>
<tr>
<td>*3 Sensor</td>
<td>A2637del</td>
<td>5′-TCCCAgCGATCgCCTgCA X-3′</td>
</tr>
<tr>
<td>*3 Anchor</td>
<td></td>
<td>5′-LC Red705-TTgAgTCgCTACgCTgCTgAg X-3′</td>
</tr>
<tr>
<td>*7 Sensor</td>
<td>A3023C</td>
<td>5′-ACATTCgAgTgATCGCATgAgCA X-3′</td>
</tr>
<tr>
<td>*7 Anchor</td>
<td></td>
<td>5′-LC Red640CCCAgagCCCTgCgTgAg X-3′</td>
</tr>
</tbody>
</table>

* The numbering of nucleotides corresponds to Kimura et al. (32).
The presence of allele *5. The CYP2D6 deletion detection assay was improved by adding an internal standard primer, *5-C (5'-CgTCTAgTgggAgACAAAC-3'; 10 pmol/µL), to enable amplification of a 4.2-kb fragment for the wild-type allele. The reaction contained 5 ng of genomic DNA, 2.5 mM deoxynucleotide triphosphates, and 3.5 U/µL polymerase (Boehringer Expand Long). PCR conditions were as follows: denaturation at 94 °C for 2 min, followed by 35 cycles with denaturation at 96 °C for 10 s, annealing at 66 °C for 20 s, and extension at 68 °C for 9 min. Final extension was at 68 °C for 7 min. PCR products were resolved by 0.9% agarose gel electrophoresis and visualized by ethidium bromide staining.

**Results**

The described real-time PCR protocol led to reliable and fast results for all five CYP2D6 mutations investigated. Exact discrimination between the different genotypes was possible because the different genotypes, either wild-type or heterozygous or homozygous for mutations, were associated with hybridization-specific melting temperatures obtained by melting curve analysis. In cases in which a single nucleotide mismatch between sample DNA and fluorescent sensor is present, the sensor is expected to dissociate from the allele at a significantly lower temperature, producing a lower melting peak. This real-time PCR assay revealed peaks with distinctly different melting maxima for the different alleles (Fig. 1). The analysis involving simultaneous detection of alleles *4 and *6 displayed up to four different melting temperature peaks, which were analyzed in one channel. Identification of the genotype was possible without any problems because heterozygous controls were used for each run. Typical results of this analysis are shown in Figs. 2 and 3.

**Allele Frequency**

The results of genotyping using real-time PCR are displayed in Table 2. In total, 10.5% of the individuals were identified as PMs displaying two nonfunctional alleles. Of the individuals investigated, eight (2.5%) showed a more complex genotype of two different PM-associated mutations. Fourteen different genotypes were found in the population studied.

Analysis of one blood sample did not produce a PCR product in the preamplification assay. PCR for detection of the null allele confirmed this individual as homozygous for mutation *5 (null allele). Eleven individuals were heterozygous for allele *5 and were categorized as extensive metabolizers, whereas one other individual was heterozygous for allele *5 and displayed the *6 mutation on the other allele. He was assessed as PM.

Genotyping of two individuals revealed an atypical melting curve in channel 2, which analyzed the alleles *4 and *6. The melting curve of the first individual showed a temperature peak at 66.9 °C located between the mutant allele *4 (63.9 °C) and mutant allele *6 peak (67.8 °C). Sequence analysis confirmed a heterozygous *4 genotype and an additional C→G mutation at position 1792 in exon 3, which encoded a Gln→Glu exchange at amino acid position 150. Sequencing of the second DNA sample confirmed no deletion of T at position 1795, but a silent base exchange, C→T, at position 1776. Two patients displayed another heterozygous mutation in exon 6 (G→A at position 3027), which decreased the temperature peak of the *7 assay wild-type curve by 1.8 °C.

**Comparison of Reagent Costs and Labor Time**

The costs of genotyping using real-time PCR with hybridization probes are higher than the costs for conventional
time PCR method produced 100% reliable results, as confirmed by sequencing. Repeated analyses of the same sample on the LightCycler, performed by three different investigators, confirmed the reproducibility of the results.

**Discussion**

Single-nucleotide polymorphisms and other genomic variations in drug-metabolizing enzymes have been associated with side effects, incompatibility, and variation in therapeutic efficacy of >30 drugs, some with wide therapeutic use. Furthermore, this genetic variability has obvious implications for the development of new drugs and for decisions of drug regulatory agencies. On the other hand, genotyping of CYP2D6 may become a routine part of individually optimized drug treatment in the future because therapeutic efficacy and adverse events in treatment with numerous drugs depend on this enzyme activity (15). Murphy et al. (16) recommended prescreening of individuals to be treated by a compound that is extensively metabolized by CYP2D6. Genotyping for the most relevant alleles associated with a PM can increase patient safety and eliminate the need for continual monitoring of drug plasma concentrations. This approach might be especially useful before enrollment of individuals in phase III clinical trials of new therapeutics known to be metabolized by CYP2D6.

Drugs and carcinogens are excreted from the body after metabolic conversion involving enzymes that mediate oxidative metabolism and conjugation. Many of the corresponding genes exhibit functional polymorphisms that contribute to individual susceptibility to neurotoxins. CYP2D6 is thought to be a candidate gene associated with the incidence of different types of cancer and Parkinson disease (12, 17). However, results of association studies have, to date, been inconsistent (12, 18).

Interethnic differences in drug metabolism are explained by environmental as well as genetic factors (5). Whereas the *4 allele is present in high frequency in Caucasians and accounts for >75% of the mutant CYP2D6 alleles, this single-nucleotide polymorphism is almost absent in the Chinese population (5). Bathum et al. (19) reported an allele frequency of 0.04 in a black African population.

Nevertheless, a rapid, reliable, and efficient method of genotyping for diagnosis of functional CYP2D6 metabolizer status seems to be advantageous to identify individuals at risk of developing adverse drug reactions, therapeutic failure, or diseases associated with the CYP2D6 PM genotype.

To demonstrate the reliability and cost-effectiveness of the proposed method, we compared CYP2D6 genotyping by allele-specific multiplex PCR and fluorometric melting point analysis based on real-time PCR.

**Allele Frequencies**

Allele frequencies were comparable to published data (15, 20, 21). In addition, other previously described mutations that decrease enzyme activity exist, e.g., the very
rare mutations *1I to *16, that display allele frequencies \( \approx 0.008 \) could not be detected by our assay. However, it is questionable whether routine assessment of these rare alleles is necessary for a screening procedure aimed at identification of PMs.

In addition to the five mutations investigated, our assay is also able to detect the rare *14 allele (15). This mutation is localized at the same position as allele *8, but it displays a G1846A exchange in contrast to G1846T, which characterizes the *8 allele. Because there was no positive control available, analysis of the *14 mutation was carried out on an artificial oligonucleotide target. The assay showed three different melting temperatures corresponding to mutant allele *8, the wild type, and mutant allele *14, respectively.

The C1792G mutation detected in our population has been described previously (20). The estimated allele frequency of 0.003 is in line with our findings (20). In addition to these previously published mutations, additional mutations probably exist (22). Our assay is able to detect novel mutations by deviation of the melting curves from the predicted melting point if they are located within the sequence of the anchor or sensor, respectively. The C1792T mutation observed in our population was identified by a melting peak that was shifted \(-1.6^\circ C\) from the melting peak for the *6 allele. Sequencing of samples deviating in their melting maxima from the predicted temperature can provide further information on new genomic variations.

**ADVANTAGES OF REAL-TIME PCR METHOD**

Real-time PCR offers a wide range of advantages. The analysis is faster, no time-consuming additional post-PCR processing such as gel electrophoresis is needed, and the risk of contamination is minimized because amplification and genotyping are performed in the same sealed capillary without any additional handling steps. Furthermore, no use of potentially toxic reagents, such as ethidium bromide, is necessary.

As described previously, the allele-specific multiplex PCR did not always show clear results because multiple bands occurred. In these cases, PCR had to be repeated (14).

Temperature-dependent hybridization of the sequence-specific hybridization probes to single-stranded DNA is monitored by the real-time PCR device. Although costs for reagents and consumables are high, the reduced labor time needed and the reliability of the results outweigh these higher costs.

**OTHER METHODS**

Previous studies used phenotyping with a variety of probe drugs, e.g., sparteine and debrisoquine (23). However, this potentially hazardous, time-consuming, and tedious method of ingesting a test drug, collecting urine or blood samples, and calculating the metabolic ratio is rather complicated. Furthermore, phenotyping results may be distorted by some drugs known to be substrates or inhibitors of CYP2D6, e.g., selective serotonin reuptake inhibitors (fluoxetine, fluvoxamine, and paroxetine) (24). Because patients are often treated with a combination of drugs, phenotyping in a clinical setting is not considered a reliable approach because of the anticipated drug interactions (24). To increase the efficiency and to facilitate genotyping, PCR-based methods with subsequent restriction fragment length polymorphism analysis were introduced (25). However, increasing numbers of mutations were identified, and the number of PCR reactions needed to perform genotyping had to increase as well (26). As a result, more convenient methods, such as allele-specific multiplex PCR assays (14, 27–29) and allele-specific amplification by multiplex long PCR, were developed (28). Multiplex PCR with allele-specific oligonucleotide hybridization was another combined approach (30). In contrast, sequencing might be the method of reference, but it is of little value if large sample numbers are to be analyzed.

One thing that all new approaches to genotyping have in common is that they have to be validated by use of well-established standard procedures. In the present study, the real-time PCR procedure was compared with a standard method and was shown to be specific, reliable, and reproducible.

New methods of genotyping should be simple, efficient, and cost-effective, particularly if large numbers of samples are to be screened. Drug selection and dose adjustment to reach therapeutic concentrations might be performed on the basis of genotyping in future therapeutic settings (31). Future methods for assessing the metabolic capacities of enzyme systems should have the potential for automation with microplates and robotic workstations for high throughput.

In conclusion, the proposed real-time PCR method with melting curve analysis is a suitable alternative for screening of PM-associated CYP2D6 mutations. Detection is rapid and highly sensitive, and the savings in labor outweigh the higher costs.

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macology, Georg-August University, Göttingen, Germany).

References


