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TaqMan real-time PCR quantification strategy of CYP2D6 gene copy number for the LightCycler 2.0

Duc L. Nguyen, Julia Staeker, Barbara Laika, Werner Steimer *

Institut für Klinische Chemie und Pathobiochemie, Klinikum rechts der Isar, Technische Universität München, Germany

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ABSTRACT

Background: The metabolism of many therapeutic drugs depends on the presence and activity of CYP2D6 enzymes. Poor or ultrarapid metabolism may lead to adverse drug effects and lack of therapeutic efficacy. Determining the CYP2D6 gene copy number (GCN) together with SNP genotyping allows predicting the CYP2D6 phenotype and may be beneficial for patients. Efficient TaqMan real-time PCR assays have been developed for this specification but are limited to the Abi Prism system and lack extensive data to demonstrate reliable application for routine purposes.

Materials and methods: We established two TaqMan real-time PCR assays to quantify CYP2D6 GCN on the LightCycler 2.0 platform. With *albumin* as internal control, one assay targets the exon 9 region of the CYP2D6; the other the intron 6.

Results: In 617 samples there is a 99.4% (exon 9 method) and 95.6% (intron 6 method) correlation compared to standard methods. Analyzing deviant results offer indications for polymorphisms such as CYP2D6*16 and exon 9 gene conversions.

Conclusion: Established TaqMan real-time PCR assays to determine CYP2D6 GCN on the LightCycler 2.0 are reliable and may be used in the routine. Comparing deviant results, these assays may even allow the screening for rare polymorphism.

1. Introduction

The cytochrome P450 2D6 (CYP2D6 GenBank M33388) is responsible for the metabolism of many endogenous as well as exogenous compounds [1]. Up to 25% of all therapeutic drugs, particularly antidepressants and antipsychotics are catalyzed and degraded by this enzyme [2–4]. The metabolism for these substrates is highly dependent on the amount of active CYP2D6 enzymes expressed in the liver. At present, more than 80 relevant alleles of the CYP2D6 gene have been identified on the chromosome 22 [5], with varying frequencies between ethnic groups [6]. They produce null, decreased, normal or increased functional CYP2D6 enzymes. The metabolic capacity of these enzymes produces four distinct phenotypes which can be distinguished as: poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM) and ultrarapid metabolizers (UM). Unless pro-drugs are applied PM and IM have the highest risk of adverse drug reactions due to elevated drug concentration in the system. In contrast, UMs have elevated level of drug metabolites and decreased response to drug therapy. Adverse drug reactions and low therapeutic efficacy can be avoided by adjusting drug selection and dosage to each patient's individual need [7,8]. Given the fact that fatal drug reactions are still one of the most common causes of death [9], predicting those phenotypes prior to therapy can result in a significant reduction of avoidable complications in the clinic [10].

Due to the close relationship between CYP2D6 genotype and phenotype [11–13], genotyping CYP2D6 variants is a reliable method to predict individual phenotypes. In Caucasians, CYP2D6*5 (gene deletion) is the second most frequent cause of poor metabolism just after CYP2D6*4. In Asians and Africans it is the most common nonfunctional genotype. For ultrarapid metabolism, gene amplifications (CYP2D6*1×N and CYP2D6*2×N) are responsible for approximately 10–30% of the cases [11,13–15]. Thus, together with SNP genotyping, detecting CYP2D6 GCN is crucial in predicting extreme phenotypes.

Conventional methods to detect CYP2D6 GCN, like Southern blot RFLP and long-range PCR, are well-established but very laborious and inefficient for routine use. Published TaqMan real-time PCR quantification strategies, on the other hand, are efficient and rapid but have little sample sizes, i.e. 64 and 44 samples respectively [16,17], to demonstrate their adequacy for routine application. Both methods were also realized solely for the Abi Prism PCR system and have not yet been adapted on other platforms.

Therefore, we established two TaqMan PCR methods to quantify CYP2D6 gene copy number on the LightCycler 2.0 platform, and by

Abbreviations: CYP, Cytochrome P450; ADR, Adverse drug reaction; GCN, Gene copy number; PPV, Positive predictive value; NPV, Negative predictive value; FAM, Fluorescein; HEX, Hexachlorofluorescein.

* Corresponding author. Ismaninger Straße 22, 81675 München, Germany. Tel.: +49 89 4140 4754; fax: +49 89 4140 4875.
E-mail address: steimer-public@klinchem.med.tum.de (W. Steimer).

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Table 1
Primer and probe sequences.

Primer	Sequence	Calc. Tm
CYP2D6	Exon 9	
CYP2D6 ex9 f	5′-CTT CAC CTC CCT GCT GCA G-3′	56.5 °C
CYP2D6 ex9 r	5′-TCA CCA GGA AAG CAA AGA CA-3′	53.6 °C
CYP2D6 ex9 FAM	5′-FAM-CCG GCC CAG CCA TGG-BHQ1-3′	54.4 °C
CYP2D6	Intron 6	
CYP2D6 in6 f	5′-TGA GCC CAT CTG GGA AAC A-3′	56.2 °C
CYP2D6 in6 r	5′-GGT GTC CCA GCA AAG TTC ATG-3′	58.0 °C
CYP2D6 in6 FAM	5′-FAM-GAA GGG TAC AGG CGG GGG-BHQ1-3′	59.0 °C
Albumin		
Alb ex12 f	5′-TGT TGC ATG AGA AAA CGC CA-3′	55.4 °C
Alb ex12 r	5′-GTC GCC TGT TCA ACC AAG GAT-3′	57.5 °C
Alb ex12 HEX	5′-HEX-AAG TGA CAG AGT CAC CAA ATG CTG CAC AG-BHQ1-3′	63.6 °C

analyzing a large number of samples, we tried to assess the reliability of these methods for routine application.

2. Materials and methods

2.1. DNA samples

A total of 617 collected DNA samples were used to test these assays. These samples have been collected from patients with psychiatric disorders since 2003 for previous population studies and extracted by different standard methods from leukocytes (Promega and Qiagen). The study was approved by the Institutional Review Boards (Technische Universät München) and informed consents were given by all donors.

All samples were previously genotyped for CYP2D6*1,*2, *3, *4, *6, *7, *8, *9, *10, *35, *41 with established PCR methods in the lab [18–20]. Gene deletion (*5) and gene amplification were accessed by conventional long-range PCR methods [21,22].

2.2. Primer and probes

The sequences of the primers and probes used in this study are listed in Table 1. CYP2D6 exon 9 primers and probes were applied as previously described by Schaeffeler et al. [16] and CYP2D6 intron 6 primers and probes were adapted from Bodin et al. [17].The intron 6 probe was extended by 5′-GAAG to achieve better melting point alignment. HPLC purified primers were purchased from Invitrogen (Invitrogen GmbH, Karlsruhe, Germany) and probes from Tib Molbiol (Tib Molbiol Syntheselabor GmbH, Berlin, Germany).

2.3. PCR condition

Real-time PCR was performed using the LightCycler 2.0 system (Roche Diagnostics GmbH, Mannheim, Germany). The exon 9 duplex assay consisted of CYP2D6 exon 9 primers and probe. The intron 6 duplex assay contains CYP2D6 intron 6 primers and probe. In both assays albumin was co-amplified as an internal reference gene. For each assay the following components were added to a total of 20 µl/reaction capillary: 1 µl (50–200 ng) of template DNA, 1× LightCycler® Faststart DNA Master HybProbe, 300 nM of each primer, 200 nm of each probe and 2 µM $MgCl_2$. Thermal cycling was initiated with a 2 min incubation at 50 °C and a Faststart polymerase activation for 10 min at 95 °C. After this, 40 cycles of 15 s denaturation at 95 °C and 60 s elongation at 60 °C followed. The assay concluded with a 30 s cooling at 40 °C. In each run, a standard curve was recorded and used as the calibrator. Controls for no-DNA, CYP2D6 wild type, deletion and duplication were included. FAM signals of CYP2D6 amplification are detected by the 530 nm photohybrid and HEX of albumin by the 560 nm one. A color compensation for those two fluorophores was performed.

2.4. Quantification

The human albumin gene is a well-established reference gene for DNA and RNA quantification. Duplex assays using albumin and CYP2D6 crossing point (Cp) data to quantify gene copy numbers (GCN) have also been previously shown to be accurate [16,17]. CYP2D6 gene copy numbers of test samples were obtained by calculating the gene ratios of CYP2D6 vs. albumin with the ΔΔCp method [23] and normalizing them to the gene copy number of albumin, which is 2. In short, this gene ratio (R) is given by the formula: $R = 2^{\Delta\Delta Cp}$, where $\Delta\Delta Cp = \Delta Cp(\text{calibrator}) - \Delta Cp(\text{sample})$ and $\Delta Cp = Cp(\text{CYP2D6}) - Cp(\text{albumin})$.

As reference, a standard curve with 100 ng, 20 ng, 8 ng and 0.4 ng DNA was recorded. The average ΔCp of the standard curve points was set as ΔCp(calibrator) with gene ratio of 1. Integrating the ΔCp(calibrator) in this way reduces variation of an important reference value. Thus, a normalized 2 gene copy test sample is expected to have a gene ratio of 1, a deletion sample of 0.5 and a duplication sample of 1.5. In general, this calculation method yields accurate results only when CYP2D6 and albumin PCR amplification efficiencies are equivalent.

2.5. Data interpretation

Crossing point data were automatically collected by the LightCycler software (Idaho Technology Inc.) and further analyzed by Microsoft Office Excel. Statistical calculations were done with SPSS (SPSS Inc.). The Kolmogorov–Smirnov test was performed and normality of data distribution was established for the results of each GCN group followed by a t-test (one-way ANOVA), which demonstrated significant differences between them ($p<0.001$). TaqMan quantification results were used to predict CYP2D6 GCN with the following cut off values. Gene ratios from 0.25 to 0.74 were assigned to the gene deletion group (1 gene copy), 0.75–1.25 to the normal gene copy group (2 gene copies), and >1.25 to the gene amplification group (more than 2 gene copies). For duplicated measurements the mean of the two results was taken.

3. Results

3.1. Assay design and validation

These assays were designed such that the CYP2D6 gene copy number was examined with the reference gene albumin (ALB GenBank: M12523). One primer pair and probe target the exon 9 region of the CYP2D6, the other the intron 6 region. By redesigning the probes, it was possible to use the previously described primers for the LightCycler 2.0 platform. The characteristic lengths of the PCR products for CYP2D6 exon 9 at 89 bp, CYP2D6 intron 6 at 78 bp and albumin at 72 bp were identified with gel electrophoresis. The specificity of those primers was re-evaluated with a CYP2D6 total deletion (CYP2D6*5*5) sample (Fig. 1a and b). Calculated efficiencies via the $10^{-1/\text{slope}}$ formula for target and reference gene tend to vary,

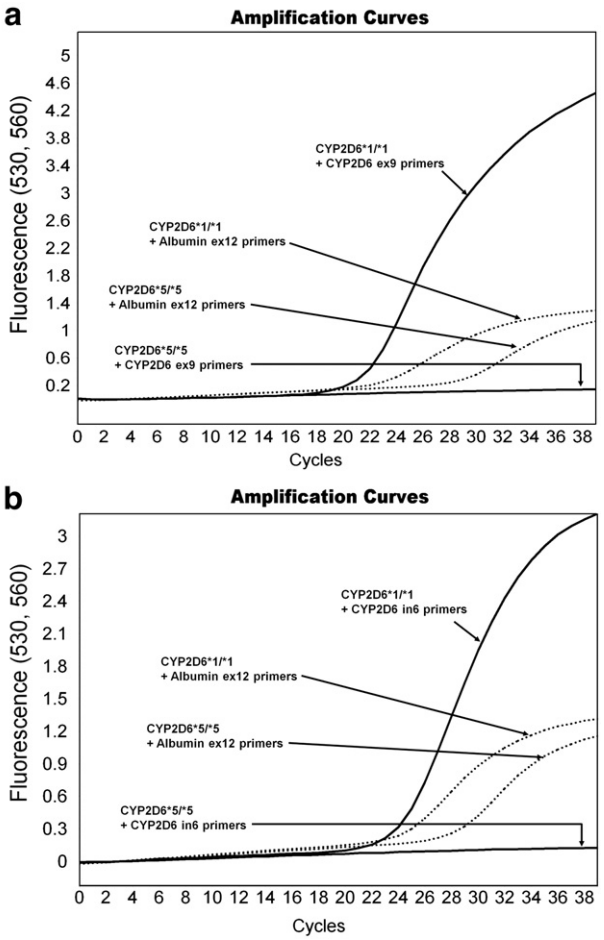

Fig. 1. a. CYP2D6 exon 9 vs. albumin duplex assay. LightCycler output result for exon 9 duplex assay with a CYP2D6*5*5 total deletion sample. Detection of FAM at 530 nm, HEX at 560 nm. b. CYP2D6 intron 6 vs. albumin duplex assay. LightCycler data output for intron 6 duplex assay with a CYP2D6*5*5 total deletion sample. Detection of FAM at 530 nm, HEX at 560 nm.