Original Paper

Allele Drop Out Conferred by a Frequent CYP2D6 Genetic Variation For Commonly Used CYP2D6*3 Genotyping Assays

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Key Words

Pharmacogenomics • Pharmacogenetics • Precision medicine • Pyrosequencing

Abstract

Background/Aim: Accurate genotyping of *CYP2D6* is challenging due to its inherent genetic variation, copy number variation (duplications and deletions) and hybrid formation with highly homologous pseudogenes. Because a relatively high percentage (~25%) of clinically prescribed drugs are substrates for this enzyme, accurate determination of its genotype for phenotype prediction is essential. **Methods:** A cohort of 365 patient samples was genotyped for *CYP2D6* using Sanger sequencing (as the gold standard), hydrolysis probe assays or pyrosequencing. **Results:** A discrepant result between the three genotyping methods for the loss of function *CYP2D6**3 (g.2549delA, rs35742686) genetic variant was found in one of the samples. This sample also contained the *CYP2D6* g.2470T>C (rs17002852) variation, which had an allele frequency of 2.47% in our cohort. Redesign of the *CYP2D6**3 pyrosequencing and hydrolysis probe assays to avoid *CYP2D6* g.2470 corrected the anomaly. **Conclusion:** To evidence allele drop out and increase the accuracy of genotyping, intra-patient validation of the same genetic variation with at least two separate methods should be considered.

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Introduction

Most techniques used to detect genetic variations require polymerase chain reaction (PCR), with which comes the risk of allele drop out events. When allele drop out occurs, the genetic variation of interest on one of the alleles is not detected, resulting in an incorrect genotype assignment [1]. Allele drop out events transpire when amplification of one or all alleles does not occur, or the binding of the probe to its target is impeded. Its cause can be due to the presence of separate, usually rare (with a minor allele frequency of < 1% in a given population [2]) genetic variations located within the binding region of primers and/or probes, as well as strong secondary structures of PCR amplicons that block elongation during sequencing [3]. Genetic variations can also generate PCR amplification bias, where one of the alleles is preferentially amplified over the other. The amplification bias created may be quantal or graded, depending on the assay design.

Allele drop out events are especially problematic where a phenotype is predicted from the genotype. Erroneous genotype results can cause phenotype misclassification, which may translate into ill-advised drug administration, removal or dosing adjustment. For example, phenotype misclassification of patients based on the genotype results of phase I metabolizing enzymes, like cytochrome P450 2D6 (*CYP2D6*) can have a substantial impact since prescription and dosing of drugs metabolized by these enzymes may be altered. *CYP2D6* is located on chromosome 22q13.2 and lies downstream of two pseudogenes, *CYP2D7* and *CYP2D8*. *CYP2D6* is an ~4.4kb sequence containing 9 exons and 8 introns followed by a relatively small 0.6kb repeat region and a unique ~2.8kb repetitive element (REP6) (Fig. 1A). *CYP2D6* is especially prone to allele drop out events due to its highly polymorphic nature. In fact, accurate genotyping of this gene is challenging, not only because of its inherent genetic variation, but also due to copy number variation (duplications, referred to as *CYP2D6* × *N*, and deletions, referred to as *CYP2D6**5) and hybrid formation with the highly homologous *CYP2D7* pseudogene lying just upstream of it [4-9]. Because a relatively high percentage (~25%) of clinically prescribed drugs, particularly those used in the field of psychiatry, are substrates for this enzyme, accurate determination of its genotype is essential [10-13].

We identified a discordant genotype result in a sample concerning the *CYP2D6**3 variation (g.2549delA, rs35742686) between three common genotyping methods (Sanger sequencing, end-point genotyping with hydrolysis probes, and pyrosequencing) and determined the cause of the conflicting results.

Materials and Methods

Genomic DNA (gDNA) isolation

The study was performed according to the Declaration of Helsinki of 2013. Following informed written consent, whole blood from 365 healthy, Caucasian of European descent (self-proclaimed) volunteers was collected in EDTA K Monovettes® (Sarstedt; Wr. Neudorf, Austria) via venipuncture. gDNA was isolated from ~350µL blood with the EZ1 DNA Blood 350 µL Kit (Qiagen; Hilden, Germany) on the EZ1 Advanced XL platform (Qiagen) according to the manufacturer's instructions with an elution volume of 50µL. gDNA was quantified with the QIAxpert (Qiagen) spectrophotometer. Only samples with an A_{260}/A_{280} between 1.700 and 1.900 were used for downstream analyses.

End-point genotyping and copy number variation experiments

End-point genotyping was performed with hydrolysis probe assays in either OpenArray® or 384-well formats on the QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher Scientific; Waltham, MA). For the OpenArray® format, 50ng/µL gDNA and 2X TaqMan® OpenArray® Genotyping Master Mix were used with the TaqMan® OpenArray® PGx Panel (Thermo) according to the manufacturer's instructions. For 384-well format experiments, 10µL reactions containing 1X TaqMan® Universal PCR Master Mix no AmpErase® UNG, 1X hydrolysis probe assay and 13.5ng gDNA were performed in MicroAmp® Optical 384-well Reaction Plates on the QuantStudio™ 12K Flex Real-Time PCR System (all from Thermo). Cycling parameters in the OpenArray® format were defined in a template file provided by the manufacturer. Cycling parameters for

384-well format experiments were: 60°C for 30 sec., 95°C for 10 min., followed by 50 cycles of 92°C for 15 sec. and 60°C for 90 sec, and a final 30 sec. at 60°C. The two hydrolysis probe assays used in the 384-well format targeted *CYP2D6**3 (g.2549delA, rs35742686); one was from Thermo (Assay ID C_32407232_50) and the other was custom designed and manufactured by Microsynth (Balgach, Switzerland). The sequences of the primers/probes from Thermo are proprietary. *CYP2D6* copy number in each sample was assessed with two hydrolysis probe assays for the target gene (labeled with VIC): one within intron 2 (Hs.04083572_cn) and the other within exon 9 (Hs.00010001_cn), in combination with one assay for the reference gene (labeled with FAM), *RPPH1* (also known as *RNAse P*). 10µL reactions containing 1X Taqman® Universal PCR Master Mix no AmpErase® UNG, 1X target assay, 1X reference assay and 12ng gDNA were performed in MicroAmp® Optical 384-well Reaction Plates on the QuantStudio™ 12K Flex Real-Time PCR System (all from Thermo). Four technical replicates were performed for each sample for each target gene. Cycling parameters for copy number experiments were: 95°C for 10 min. followed by 40 cycles of 95°C for 15 sec. and 60°C for 1 min. *CYP2D6* copy number quantification was performed with CopyCaller software Version 2.1 (Thermo) using default analysis settings.

Extra-long (XL) PCR

50µL XL-PCR reactions contained 1X JumpStart REDAccuTaq Long and Accurate (JS RAT LA) DNA Polymerase buffer (Sigma; St. Louis, MO), 1mM dNTPs (Thermo), 10% DMSO (Sigma), 0.8µM forward and reverse primers and 5 units JS RAT LA DNA Polymerase (Sigma). Primers and cycling parameters to amplify standard duplicated (no hybrid types) and standard non-duplicated *CYP2D6* genes were described in [14]; those used to determine the presence of *CYP2D6/CYP2D7* and *CYP2D7/CYP2D6* hybrids were described in [6]; and those used to amplify *CYP2D7* were described in [15]. *CYP2D6* sequencing primers are shown (for all online suppl. material, see www.karger.com/doi/10.1159/000484380) in Suppl. Table 3. Amplicons were visualized with either agarose gels or the QIAxcel Advanced XL automated gel electrophoresis system with the DNA screening cartridge (method AL420) (Qiagen) to confirm size and specificity. copy DNA (copy DNA) was purified with the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions and quantified by spectrophotometry. copy DNA was commercially sequenced with the Sanger method (Microsynth).

Haplotype determination using allele specific PCR

The primer sequences for allele specific PCRs are shown (see online suppl. material) in Suppl. Table 3. Cycling parameters for fragment 1 were: 94°C for 20 sec., followed by 35 cycles of 94°C for 20 sec, 67.5°C for 30 sec and 68°C for 4 min. These were identical for fragment 2, except the annealing temperature was 61°C. Purification and sequencing were performed as described above.

Pyrosequencing

Pyrosequencing PCR and sequencing primers for *CYP2D6**3 are shown in Table 2. Reverse PCR primers were HPLC purified and biotinylated at their 5' end. copy DNA was generated with the PyroMark PCR kit (Qiagen). 25µL reactions contained 1X PyroMark PCR mastermix, 1X CoralLoad, 0.2µM forward and reverse primers and either 100ng gDNA or 10ng copy DNA. Cycling conditions were 95°C for 15 min. followed by 45 cycles of 94°C for 30 sec., 60°C for 30 sec., and 72°C for 30 sec., followed by a final extension step at 72°C for 10 min. Amplicons were visualized using the DNA screening cartridge (method AL420) on the QIAxcel Advanced XL automated gel electrophoresis system (Qiagen).

All pyrosequencing reagents, except for the streptavidin sepharose beads (GE Healthcare; Pfaffing, Austria), belonged to the PyroMark Q24 Advanced Reagents Kit (Qiagen). 15µL double-stranded copy DNA was bound to 1µL streptavidin sepharose beads in 40µL binding buffer. Beads carrying the double-stranded, biotinylated amplicons were captured on filter probes with aspiration. While maintaining aspiration, filter probes were immersed in 70% ethanol for 5 sec., denaturation buffer for 5 sec., and finally in 1X wash buffer for 10 sec. Single-stranded, biotinylated amplicons were released into wells containing 1µM sequencing primer in a total of 22µL annealing buffer and annealed for 5 min. at 80°C.

Pyrosequencing reactions were performed on the PyroMark Q24 Instrument (Qiagen) with the following dispensation order: TCAGCAGAT. dATP was provided as dATPαS in order to avoid interference with the luciferase enzyme. Pyrograms were analyzed with the PyroMark Analysis Software version 3.0.0 in allele quantification mode using default settings.

Table 1. CYP2D6*3 and g.2470T>C results from Thermo's hydrolysis probe assay and Sanger sequencing. Only a subset of samples from the 365 sample cohort are shown

SAMPLE #	CYP2D6 CNV ^a	CYP2D6*3 (g.2549delA) GENOTYPE RESULT			CYP2D6 g.2470T>C RESULT (SANGER SEQUENCING)
		Hybrid Detected ^b	HYDROLYSIS PROBE ASSAY	SANGER SEQUENCING	
7	2	none	mutant	heterozygous	heterozygous
14	2	none	heterozygous	heterozygous	wild type
15	2	none	heterozygous	heterozygous	wild type
16	2	none	heterozygous	heterozygous	wild type
17	2	none	heterozygous	heterozygous	wild type
18	2	none	heterozygous	heterozygous	wild type
19	2	none	heterozygous	heterozygous	wild type
20	2	none	heterozygous	heterozygous	wild type
21	2	none	heterozygous	heterozygous	wild type
22	2	none	heterozygous	heterozygous	wild type
23	2	none	mutant	mutant	wild type

^acopy number variation (CNV)
^bCYP2D6/CYP2D7 or CYP2D7/CYP2D6

Statistics

To determine departure from Hardy-Weinberg equilibrium, a chi-squared test with one degree of freedom was used where $p < 0.05$ was considered statistically significant.

Results

While genotyping a 365-sample cohort for *CYP2D6* with both Sanger sequencing (as the gold standard) and a subset of hydrolysis probe assays (see online suppl. material, Suppl. Table 1), a discordant result concerning the *CYP2D6**3 genetic variation was detected in sample #7. Specifically, Sanger sequencing showed that the sample was heterozygous at this position, whereas the hydrolysis probe assay from Thermo Fisher Scientific (Thermo) (assay ID C_32407232_50) showed that the sample was homozygous mutant for *CYP2D6**3 (Table 1). As a first step to reconcile this disagreement and rule out whether an error was introduced by the polymerase used to generate the copy DNA for Sanger sequencing, we re-amplified the *CYP2D6* alleles from sample #7 and re-sequenced the region encompassing *CYP2D6**3. The result matched that of the first Sanger sequencing outcome and showed again that the patient was heterozygous for *CYP2D6**3.

We then compared the immediate 200 nucleotides upstream and downstream of the *CYP2D6**3 position in ten other samples (that were either homozygous mutant or heterozygous for *CYP2D6**3, and in which the results from the Thermo *CYP2D6**3 hydrolysis probe assay and Sanger sequencing matched) with that of sample #7 and found that only sample #7 was heterozygous (T/C) for a genetic variation at g.2470 (rs17002852) (Table 1). While the exact sequences of the primers and probes from Thermo's hydrolysis probe assays are proprietary, *CYP2D6* g.2470 is well within the plausible range for a *CYP2D6**3 assay, and we hypothesized that this variation, if in *trans* with *CYP2D6**3, might interfere with Thermo's hydrolysis probe assay.

Fig. 1. (A) CYP2D6 gene structure. Elements are not drawn to scale. The filled and numbered rectangles indicate exons. The adenine of the starting ATG is +1 and the nucleotide before is -1. (B) Genetic variants detected in the two CYP2D6 alleles from sample #7. CYP2D6 g.2470T>C, *2 (g.2850C>T), *3 (g.2549delA) and *41 (g.2988G>A) are boxed. The conv. SNP refers to a group of seven genetic variations (g.214G>C, g.221C>A, g.223C>G, g.227T>C, g.232G>C, g.233A>C and g.245A>G) collectively called the conversion to CYP2D7 in intron 1 (241-245). (C) Allele-specific amplification of the CYP2D6 allele from sample #7. (+) indicates positive control and (-) indicates the negative control. (D) Haplotype specific genotype of sample #7.

Allele Specific CYP2D6 Sequencing using g.843T>G

Sanger sequencing revealed 26 heterozygous CYP2D6 variations from sample #7 (Fig. 1B), and their rs numbers can be found in (see online suppl. material) Suppl. Table 2. To determine how these 26 variations segregated between the two alleles, we developed allele-specific PCRs that exploited the heterozygous variation at position g.843. Forward and reverse primers were designed to specifically amplify the allele containing the CYP2D6 g.843T>G variation. One set was designed to amplify an ~2.8kb fragment from g.-1909 through g.843, and the other was designed to amplify an ~4kb fragment from g.843 through g.4751. PCR products of the expected size were detected in the positive control (homozygous mutant at g.843, GG) and sample #7 (heterozygous at g.843, TG), but not in the negative control (homozygous wild-type at g.843, TT) or the no template control (Fig. 1C). The full CYP2D6 haplotypes of the positive and negative control are depicted (see online suppl. material) in Suppl. Fig. 1. The allele specific PCRs only amplified CYP2D6, and not the upstream pseudogene CYP2D7, since no amplicon was generated in the negative control (which contains CYP2D7, data not shown), suggesting that the amplicons generated in sample #7 represent the CYP2D6 allele containing g.843T>G. After Sanger sequencing of both allele-specific PCR fragments, the variations found were subtracted from the unsegregated CYP2D6 genotype (Fig. 1B), which revealed an allele-specific genotype of *3/*41 (Fig. 1D). It is important to note that the g.2470T>C and CYP2D6*3 variations were in *trans* (on different alleles).

CYP2D6 g.2470T>C interferes with the CYP2D6*3 hydrolysis probe assay from Thermo

To determine whether the g.2470T>C variation interferes with Thermo's CYP2D6*3 hydrolysis probe assay, we designed a custom hydrolysis assay in which the amplification primers and probe did not overlap CYP2D6 g.2470. We then genotyped a sample set with both

*CYP2D6**3 hydrolysis probe assays. The samples within the set were chosen based on their *CYP2D6* allele haplotypes (Fig. 2). Sample #1 (*1/*1) is homozygous wild type for *CYP2D6**3, sample #2 (*1/*3) is heterozygous for *CYP2D6**3, sample #3 (*3/*3) is homozygous mutant for *CYP2D6**3, and sample #4 (*41/*41) is homozygous wild type for *CYP2D6**3. Note that sample #4 does not contain the g.2470T>C variant, but does harbor all the other variations that are found in sample #7's *CYP2D6**41 allele. Sample #5 (*2/*41) is homozygous wild type for *CYP2D6**3 and heterozygous for g.2470T>C. Note that the *CYP2D6**41 allele from this sample is identical to that from sample #7. Sample #6 (*5/*41) is wild type for *CYP2D6**3 and hemizygous at g.2470T>C. This *CYP2D6**41 allele is also identical to that from sample #7. As already mentioned, sample #7 (*3/*41) is heterozygous for both *CYP2D6**3 and g.2470. Some of these aforementioned samples were combined to generate additional genotypes (Fig. 2). The genotyping results from these samples using the two hydrolysis probe assays are shown in Fig. 3A. Thermo's hydrolysis probe assay result for all samples was as expected, except for samples #6 and #7. Sample #7 (*3/*41, including g.2470T>C on the *CYP2D6**41 allele) was erroneously called homozygous mutant for *CYP2D6**3 and no signal was detected in sample #6, which is hemizygous for g.2470T>C. These data suggest that g.2470T>C prevents Thermo's hydrolysis probe assay from detecting the allele on which it is located. We analyzed the same set of samples with our custom *CYP2D6**3 hydrolysis probe assay, in which the amplification primers or probe did not overlap g.2470. As shown in Fig. 3B, the *CYP2D6**3 results for all samples matched the actual *CYP2D6**3 genotype. The "no call" observed for sample #6, and the homozygous mutant *CYP2D6**3 call observed for sample #7 using Thermo's hydrolysis probe assay were correctly reverted to homozygous wild type and heterozygous, respectively. These data also support the notion that g.2470T>C confers allele drop out with Thermo's *CYP2D6**3 hydrolysis probe assay (Fig. 4).

Table 2. Genotyping results from *CYP2D6**3 pyrosequencing assays. ^aAll samples had 2 *CYP2D6* copies. No *CYP2D6*/*CYP2D7* or *CYP2D7*/*CYP2D6* hybrids were detected in any of the samples. ^bsee reference 17

Actual <i>CYP2D6</i> *3 Genotype	Sample ^a	Pyrosequencing <i>CYP2D6</i> *3 Allelic Ratio (A/-)	
		Langaee et al. ^b	In-house
wild type (A/A)	11	100/0	100/0
	12	100/0	100/0
	13	100/0	100/0
heterozygous (A/-)	7	31/69	60/40
	14	60/40	60/40
	15	59/41	60/40
	16	61/39	61/39
	17	60/40	60/40
	18	59/41	59/41
	19	60/40	60/40
	20	58/42	58/42
	21	57/43	57/43
	22	59/41	59/41
mutant (-/-)	23	6/94	5/95
amplification primers		5' CTGTCCCGTCCTCCTGCA 3'	5' CTGTCCCGTCCTCCTGCA 3'
sequencing primers		5' CCTCATTCCTCCTGGGACGC 3'	5' CCTCATTCCTCCTGGGACGC 3'
		5' AGCTGCTAACTGAG 3'	5' AGCTGCTAACTGAG 3'

*CYP2D6 g.2470T>C interferes with a previously reported CYP2D6*3 pyrosequencing assay*

Depending on the analytical workflow, we use pyrosequencing instead of hydrolysis probe assays to detect *CYP2D6* variants, for example, when design constraints for hydrolysis probe assays occur. Pyrosequencing determines nucleotide identities by detecting pyrophosphate release during strand synthesis, as opposed to chain termination with dideoxynucleotides in Sanger sequencing [16]. One alluring characteristic of pyrosequencing is that it can be used to quantify alleles. For *CYP2D6**3 variation analysis by pyrosequencing, for example, a

Fig. 2: The *CYP2D6* allele specific haplotypes of seven samples used to determine the impact of g.2470T>C on Thermo's hydrolysis probe assay. The *CYP2D6*, g.2470T>C and *3 genotypes of each sample are listed. Some samples were combined to produce a specific genotype.

homozygous wild type sample should theoretically result in an allelic quantification ratio of 100/0, where the numerator is the wild type nucleotide and the denominator is the mutant nucleotide. Accordingly, a heterozygous and homozygous mutant sample should have allelic quantification ratios of 50/50 and 0/100, respectively.

After realizing that the g.2470T>C variation interferes with CYP2D6*3 hydrolysis probe genotyping assay, we scrutinized our current pyrosequencing assay for CYP2D6*3 obtained from that previously described by Langaee et. al [17]. Table 2 shows the pyrosequencing results from this CYP2D6*3 pyrosequencing assay in three CYP2D6*3 homozygous wild type, ten heterozygous and one homozygous mutant samples. In our hands, a small amplification bias towards the wild-type allele was observed, especially in the heterozygous samples. Of particular concern was the value obtained for sample #7, which is heterozygous for g.2470T>C variant. The allelic quantification value for this sample was 33/67 and biased towards the mutant allele, whereas all of the other heterozygous CYP2D6*3 sample values were biased towards the wild-type allele. Interestingly, the last nucleotide in the forward amplification primer of this pyrosequencing assay is complementary to CYP2D6 position

Fig. 3. Allelic discrimination plots from CYP2D6*3 hydrolysis probe assays. (A) The original scatter plots generated from the sample set described in Figure 2 using Thermo's (left) and our custom (right) hydrolysis probe assays. (B) The actual genotypes and genotyping results from the samples are listed for comparison. The custom assay, which avoids CYP2D6 g.2470 in its design, alleviated the "no call" in sample #6 and the incorrect call in sample #7 generated by Thermo's assay.

g.2470. Therefore, we removed this nucleotide from the primer and re-ran the samples. As expected, the allelic quantification ratio for sample #7 changed from 33/67 to 65/35, the latter value of which was now similar with those obtained from the other heterozygous *CYP2D6**3 samples. These data show that overlap of the forward amplification primer with g.2470 results in a strong amplification bias towards alleles that do not contain this variant. It should be noted that both of the *CYP2D6**3 pyrosequencing assays amplify *CYP2D7* (see online suppl. material, Suppl. Fig. 2). Therefore, the pyrosequencing assay data presented in Table 2 was obtained from *CYP2D6* copy DNA.

Frequency of g.2470T>C and loss of function *CYP2D6* mutations

Direct transcription and translation of the *CYP2D6* g.2470T>C variant would result in a synonymous amino acid substitution (p.His232=). The NCBI single nucleotide polymorphism (SNP) database currently lists the allele frequency of *CYP2D6* g.2470T>C as 0.74% in the ExAc_Aggregated_Populations population. However, this value is likely underestimated, since the majority of *CYP2D6* variant testing commonly only include variations with >1% allele frequency and/or functional relevant variants [18]. Indeed, we genotyped the *CYP2D6* g.2470 position in 365 samples using Sanger sequencing and hydrolysis probe assays, and found that the variant allele had a frequency of 2.47%. The minor allele frequencies of the *CYP2D6* loss of function mutations detected in these samples were 3.29% for *CYP2D6**3, 20.00% for *CYP2D6**4, 3.70% for *CYP2D6**5, 1.37% for *CYP2D6**6 and 0.14% for *CYP2D6**7 and *CYP2D6**15. All genotype frequencies fulfilled the Hardy-Weinberg Equilibrium expectation (Table 3). It is noteworthy that *CYP2D6**3 is the third most common loss of function mutation in this cohort.

Fig. 4. Cartoon depicting *CYP2D6**3 allele drop out by g.2470T>C. (A) A *CYP2D6* (*1/*3) genotype. Both alleles would be amplified and the result from Thermo's hydrolysis probe assay would be correctly called as heterozygous for *CYP2D6**3. (B) A *CYP2D6* g.2470T>C and *3 trans heterozygous genotype. Only the *CYP2D6**3 allele is amplified since the g.2470T>C variant does not allow one of the primers to bind. The result from Thermo's hydrolysis probe assay would be incorrectly called as homozygous mutant for *CYP2D6**3.

Discussion

The value of individualized drug therapy is continuously being recognized by regulatory authorities [19, 20]. As such, scientists in the field of pharmacogenomics (PGx), as well as physicians and analytical laboratory staff, are constantly searching for efficient and affordable methodologies to genotype patients. Furthermore, both commercial and private entities are continuously designing and validating these very types of techniques. However, limitations exist with every methodology, despite the cost, robustness or extent of validation. One limitation of genotyping, no matter the method used, includes allele drop out events, due to the presence of variations besides the one of interest. These events have been described for genotyping assays across multiple genes [14, 15, 21-24]. While the majority of these interfering variations are usually rare, they can severely alter accurate prediction of the metabolizing phenotype within a particular individual. As there is no clear governance regarding how many methodologies analytical laboratories are required to perform for a given genetic variation, it is conceivable that there are apparent gaps between genotype and predicted phenotype that consequently hamper PGx guided therapy [8].

Individuals are currently classified into CYP2D6 metabolizing activity categories based on their genotype. The number of categories and the genotypes that belong to each category vary between PGx expert groups. Recently published data from Caudle et al. regarding the standardization of terms for clinical PGx test results described five CYP2D6 metabolizing phenotype categories: ultrarapid (increased activity compared to rapid metabolizers), rapid (activity between that of ultrarapid and normal metabolizers), normal (fully functional activity), intermediate (activity between that of normal and poor metabolizers) and poor (little to no enzyme activity) [25]. Sample #7 was found to have a *CYP2D6* genotype of *3/*41. The *CYP2D6**3 variant causes a frameshift at p.Arg259 that results in a CYP2D6 protein with little to no function [26, 27]. *CYP2D6**41 alleles are reported to produce decrease of function proteins [28, 29]. This genotype would predict an intermediate (to normal) CYP2D6

Table 3. All loss of function mutations, as well as CYP2D6 g.2470T>C, detected in 365 self-proclaimed Caucasian individuals fulfilled the Hardy Weinberg Equilibrium (HWE) expectation

		Wild type	Heterozygous	Mutant	χ^2 p-value
CYP2D6 g.2470T>C	Observed	347 (95.07%)	18 (4.93%)	0 (0.00%)	
	Expected	347.22 (95.13%)	17.56 (4.81%)	0.22 (0.06%)	0.6291
CYP2D6*3	Observed	342 (93.70%)	22 (6.03%)	1 (0.27%)	
	Expected	341.39 (93.53%)	23.21 (6.36%)	0.39 (0.11%)	0.3189
CYP2D6*4	Observed	237 (64.93%)	110 (30.14%)	18 (4.93%)	
	Expected	233.60 (64.00%)	116.80 (32.00%)	14.6 (4.00%)	0.2660
CYP2D6*5	Observed	339 (92.88%)	25 (6.85%)	1 (0.27%)	
	Expected	338.5 (92.74%)	26 (7.12%)	0.5 (0.14%)	0.4619
CYP2D6*6	Observed	355 (97.26%)	10 (2.74%)	0 (0.00%)	
	Expected	355.07 (97.28%)	9.86 (2.70%)	0.07 (0.02%)	0.7907
CYP2D6*7 & CYP2D6*15	Observed	364 (99.73%)	1 (0.27%)	0 (0.00%)	
	Expected	364 (99.73%)	1 (0.27%)	0 (0.00%)	0.9791

metabolizing phenotype. Based solely on the result given by Thermo's hydrolysis probe assay, this individual's *CYP2D6* genotype would have been *3/*3, which would predict a poor CYP2D6 metabolizing phenotype. The differences between these two phenotype classifications could have dire consequences for the individual in terms of dosing recommendations. For example, a relatively recent dosing guideline update for tricyclic antidepressants (TCAs) based on CYP2D6 phenotype states that i) for CYP2D6 intermediate metabolizers, one should consider a 25% reduction of the recommended starting dose and utilize therapeutic drug monitoring to guide dose adjustments (strength of recommendation: moderate) and ii) for CYP2D6 poor metabolizers, one should avoid TCA use due to the potential for side effects and instead consider an alternative drug not metabolized by CYP2D6; if a TCA is warranted, consider a 50% reduction of the recommended starting dose and utilize therapeutic drug monitoring to guide dose adjustments (strength of recommendation: strong) [30].

We have shown that the *CYP2D6* g.2470T>C variant interferes with two CYP2D6*3 genotyping assays—a hydrolysis probe assay from Thermo and a previously described pyrosequencing assay [17]. The *CYP2D6* g.2470T>C results in complete allele-drop out in the hydrolysis probe assay from a leading provider, and causes a substantial amplification bias in the pyrosequencing assay described by Langaee et al [17]. It is important to underline that this variant, with an occurrence of 2.47%, is not rare. The allele frequency of *CYP2D6**3 is 3.29%. Therefore, the percentage of the population that are expected to harbour the same genotype as sample #7 is ~0.08%. While this percentage seems quite small, its magnitude can be better appreciated in the context of the actual number of individuals in the population. All samples were obtained from self-proclaimed Caucasians of European descent. Assuming a European population of ~500 million, the estimated number of individuals with the same genotype as sample #7 is ~400,000.

Re-design of both assays to avoid g.2470T>C solved the allele drop out. Information regarding allele drop out events in the context of PGx testing is valuable for all those that are involved in developing and/or performing these types of analytics, as well as the patients whose drug therapy may be altered by PGx results. Verification of results with two separate methodologies may help to clarify allele drop out events. In cases where two different methodologies cannot be used, those assays that have been documented to avoid known causes of miscalls will help to increase the quality of PGx analytics as well as its implementation.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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