Resolution of a clinical AmpliChip CYP450 Test™ no call: discovery and characterization of novel *CYP2D6**1 haplotypes

A Han Chinese patient failed *CYP2D6* genotype analysis with the AmpliChip CYP450 Test™. The *CYP2D6* gene locus of the patient and her son were extensively genotyped including copy number variation and gene resequencing. Two SNPs were discovered on the patient's *CYP2D6**1 allele, -498C>A and 1661G>C, while the son's *CYP2D6**1 allele had -498C>A only. AmpliChip failure was attributed to the presence of a *CYP2D6**1 allele carrying the 1661G>C SNP. Functional analyses of -498C>A did not reveal altered activity *in vitro* or *in vivo* suggesting that both novel *CYP2D6**1 subvariants are functional. The implementation of pharmacogenetics-guided drug therapy relies on accurate clinical-grade genotype analysis. Although the AmpliChip is a reliable platform, numerous allelic (sub)variants and gene arrangements are not detected or may trigger no calls. While such cases may be rare, the clinical/genetic testing community must be aware of the challenges of *CYP2D6* testing on the AmpliChip platform and implications regarding accuracy of test results.

Keywords: AmpliChip CYP450 Test™ • cytochrome CYP450 • *CYP2D6* • *CYP2D6**10 • genotyping • pharmacogenetics

Background

CYP2D6 is one of the best characterized polymorphic phase I drug-metabolizing enzymes owing to its role in the metabolism and bioactivation of numerous clinically used drugs. Among those are many antidepressants and antipsychotics, such as risperidone, which is the drug the patient in this case report was taking, opioids, such as codeine and tramadol, and tamoxifen, an estrogen receptor antagonist used to treat breast cancer, as well as antiarrhythmics, adrenergic β -blockers and antiemetics [1,2]. Currently, there are over 100 *CYP2D6* allelic variants defined, not counting numerous variants that are not captured by the Human Cytochrome P450 Allele Nomenclature Database [3]. *CYP2D6*'s genetic diversity is mostly caused by SNPs, single nucleotide variations and insertions or deletions of multiple nucleotides. However, this gene locus is also affected by gene copy number variation (CNV) including gene deletions, duplications and multiplications.

In addition, there are a growing number of variants that contain hybrid genes composed of nonfunctional *CYP2D7* and *CYP2D6* in various constellations. The range of enzymatic activity conveyed by *CYP2D6* allelic variation ranges from nonfunctional to ultrarapid. The complexity of the *CYP2D6* gene locus and challenges to translate genotype information into phenotype have been extensively reviewed by Gaedigk, Hicks *et al.* and Zanger *et al.* [4–6] and references therein; it was also discussed in two recent editorials in this journal [7,8].

Since allele frequencies also substantially vary among populations, the incidences of poor, intermediate, extensive and ultrarapid metabolizer phenotypes differ greatly. Allele and genotype frequencies can be found in the guidelines published by the Clinical Pharmacogenetics Implementation Consortium (CPIC) for the *CYP2D6*/codeine [9] and *CYP2D6*/tricyclic antidepressant gene/drug pairs [10].

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Future
Medicine

Pharmacogenetic testing in the clinical setting is increasingly adopted for CYP2D6 to guide clinicians to individualize drug therapy [11]. To successfully implement pharmacogenetic testing and advance patient treatment, it is of utmost importance to accurately determine a patient's genotype. One of the platforms utilized in clinical testing laboratories is the AmpliChip CYP450 Test™ (referred to as 'AmpliChip' for simplicity); for other test options please see [12,13]. The AmpliChip covers 33 allelic variants by testing for the presence or absence of a series of sequence variations [14]. The AmpliChip and accompanying allele calling algorithm were devised nearly a decade ago using the knowledge available at that time. However, SNPs and single nucleotide variations in novel combinations (haplotypes) not defined by the algorithm are likely to interfere with allele calling and can cause 'no calls' and previously demonstrated [15–17].

In this case report we describe an 81-year-old female patient of Han Chinese ancestry who was not responding to risperidone. To help interpret the lack of a clear effect despite being treated with a relatively high dose for her age with an antipsychotic, CYP2D6 genotype analysis was performed. It was hypothesized that an ultrarapid metabolizer phenotype may contribute to her atypical presentation and guide future therapeutic decision-making. The AmpliChip Test, however, yielded a no call, which was suspected to be caused by a novel haplotype.

The initial aim of our follow-up investigation was to resolve the AmpliChip no call. A secondary goal arose upon the discovery of a yet uncharacterized sequence variation in the patient, that is, to determine whether -498C>A alters CYP2D6 activity by impacting gene expression. This report describes yet another scenario showcasing challenges of CYP2D6 phenotype prediction using the AmpliChip Test.

Methods

Study subjects

The two subjects investigated by the AmpliChip comprised an 81-year-old patient (case) and her healthy son. Both individuals were born in China and reported to be of Fu, Futji-Loye and Han heritage. At the time of presentation to the Hospital del Mar, Barcelona, Spain, the patient and her son lived in Barcelona. Informed written consent was obtained from both individuals to perform genetic testing.

Samples

Genomic DNA was extracted from the patient and her son from whole blood collected by venipuncture using a MagNa Pure Compact Nucleic Acid Isolation Kit and MagNa Pure Compact Instrument (Roche

Molecular Diagnostics, CA, USA) according to the manufacturer's recommendation. DNA concentration was subsequently determined spectrophotometrically using a Nanodrop 2000 instrument.

AmpliChip CYP450 Test

AmpliChip testing was performed as recommended by Roche Molecular Diagnostics [14] at Laboratori de Referencia de Catalunya (Barcelona, Spain).

Follow-up CYP2D6 genotype & CNV analyses

CYP2D6 genotype analysis was performed for both individuals using a combination of a triplex long-range (XL)-PCR and TaqMan® assay technology as described in detail elsewhere [18–20]. The 6.6 kb long template encompassing the entire CYP2D6 gene was subsequently genotyped for the following sequence variations: 100C>T (rs1065852), 1023C>T (rs28371706), 1707Tdel (rs5030655), 1846G>A (rs1800716), 2549A>del (rs35742686), 2613AAGdel (rs5030656), 2850C>T (rs16947), 2935A>C (rs5030867), 2988G>A (rs28371725) and 3183G>A (rs59421388).

Allele-specific XL-PCR was achieved by replacing the forward primer of the 6.6 kb XL-PCR with a primer that only amplifies the CYP2D6*1 allele and not the *10 allele. The resulting product is 5.8 kb long. The sequence of the CYP2D6*1-specific forward primer is as follows: 5'-CAGAAAGCAGTGGAGGAGGACG-3'.

Gene copy number was assessed as previously described in detail [21]. This quantitative assay targets four CYP2D6 gene regions and allows detection of gene copy number and hybrid genes composed of CYP2D6 and 2D7 [20–23].

Allele nomenclature used throughout this report adheres to that recommended by the Human Cytochrome P450 (CYP) Allele Nomenclature database [3] unless indicated otherwise.

CYP2D6 gene resequencing

XL-PCR products for DNA sequence analysis were purified using a GenElute™ PCR Clean-Up Kit (Sigma, MO, USA). Sequencing was performed with BigDye® terminator v3.1 cycle sequencing chemistry and a 3730 capillary DNA analyzer (Life Sciences, CA, USA). The 6.6 kb XL-PCR product was sequenced in its entirety, the CYP2D6*1 XL-PCR product was partially sequenced to cover sequences at positions -498, 100 and 1661, respectively. Sequences were in both directions. Sequencing primers are provided in [Supplementary Table 1](#) (see online at: www.futuremedicine.com/doi/suppl/10.2217/pgs.14.94).

GenBank sequences AY545216, M33388, JX678710 and JX678711 served as references for CYP2D6*1 and *10, respectively.

Haplotype analysis

Existing *CYP2D6* data previously obtained on a Korean population ($n = 175$) [24] were reanalyzed to further define haplotype structures (or alleles) that contain signature SNPs defining *CYP2D6* alleles. Genotyping of the -498C>A SNP was performed by pyrosequencing on a 6.7 kb long preamplified PCR product as described [24] and detailed in the [Supplementary Material](#).

The haplotypes and pairwise linkages of *CYP2D6* -498C>A and 100C>T were analyzed using the SNPAnalyze software (Version 4.1, Dynacom Co., Yokohama, Japan) and Haploview v4.2 [25].

Functional characterization of -498G>A *in vitro* & *in vivo*

A 1.8 kb fragment of the 5'-flanking region of the *CYP2D6* gene was cloned between the *Xho*I and *Hind*III sites of the pGL2 vector (Promega, WI, USA). *CYP2D6* gene sequences containing -498C and -498A variants were obtained using QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, CA, USA). HepG2 cells were grown at a density of 5×10^5 cells/well in six-well plates and cotransfected with 3 µg of pGL2 *CYP2D6* -498C and pGL2 *CYP2D6* -498A and 0.2 µg β-galactosidase reporter plasmids using the Lipofectamine™ 2000 Reagent (Invitrogen, CA, USA), according to the manufacturer's protocol. After 48 h of transfection, luciferase activity was determined; activity was normalized to the cotransfected β-galactosidase [26].

To assess functional consequences of the -498G>A SNP *in vivo*, dextromethorphan/dextrorphan (DM/DX) metabolic ratios were correlated with genotype in 175 Korean subjects. Subjects received a single oral dose of 30 mg of DM and urine was collected for 8 h after administration [24]. DM and DX levels in the urine were determined by reverse-phase HPLC with fluorescence detection, as described previously [27].

The patient and her son were not available for *in vivo* phenotyping.

Results

Patient history & presentation

A female 81-year-old patient presented to the emergency department (ED) with complaints about persistent visual and auditory hallucinations and agitation. Patient history revealed antecedents of arterial hypertension and one episode of suicidal behavior after voluntary immigration from China 6 years prior. At that time an adaptive disorder was diagnosed and treated with sertraline 100 mg per day with early clinical remission and no known cognitive, visual or auditive impairment. Chronic treatment also included valsartan 150 mg, simvastatin 20 mg, and diazepam 5 mg

per day. The patient started treatment with risperidone 1 mg per day 5 weeks before her ED visit. She acquired a urinary infection (*Klebsiella Pneumoniae*) shortly after risperidone initiation, which was treated for 2 days with fosfomycin (1 g per day). She subsequently also received amoxicillin and clavulanic acid (10 days, 1 g per day) resolving the urinary infection. The patient developed delirium around the same time as the urinary infection and 5 days before starting on fosfomycin; the psychotic symptoms persisted until she presented to the ED 1 month later.

At the ED hallucinations persisted in the absence of delirium. The patient did not have temporal spatial or personal disorientation, but fluctuating delusions of autoreference were observed. There was no indication of a mood disorder or other serotonergic signs or symptoms. Also, environmental and triggering medical factors were discarded including significant socio-cultural isolation. A physical exam including a neurological follow-up was normal. Laboratory tests including hemoglobin, albumin and TSH, as well as maturation factors, tumor markers, an immunology screen and thorax x-ray were normal. X-ray computed tomography and MRI showed mild small-vessel vasculopathy.

The patient was subsequently diagnosed with induced psychotic disorder owing to chronic sertraline intoxication and urinary infection (293.82 Diagnostic and Statistical Manual of Mental Disorders Fourth Edition, text revision [DSM-IV TR]) versus psychotic disorder not otherwise specified (298.9 DSM-IV TR) and sertraline was withdrawn. After 3 weeks of sertraline discontinuation and 12 days of risperidone treatment (4.5–6 mg p.o. per day), only a partial improvement was observed despite excellent tolerability and compliance carefully supervised. Haloperidol up to 4 mg p.o. per day augmentation was then initiated. A slow but consistent remission of symptoms was observed upon which the patient was discharged. No relapse was observed during a 3-month follow-up period. Shortly thereafter, the patient died of unrelated causes.

AmpliChip CYP450 Test results

The AmpliChip report of the patient returned a result for each sequence variation interrogated, but failed to call a genotype result (Table 1). No technical difficulties/errors were apparent per the test processing information; furthermore, the *CYP2C19* genotype, which is determined simultaneously with the *CYP2D6* genotype by the AmpliChip platform, was reported. Sample quality did not appear to impact test performance because the no call affected *CYP2D6* only. Manual review of the SNP calls identified a homozygous result for 1661G>C that indeed could not be reconciled with the AmpliChip allele definition algorithm. While

Table 1. AmpliChip CYP450 Test™ results for the patient and her son.		
CYP2D6 position (M33388)	AmpliChip SNP calls	
	Patient (case)	Son of case
-1584C>G	WT	WT
31G>A	WT	WT
100C>T	HET [†]	HET [†]
138insT	WT	WT
883G>C	WT	WT
1023C>T	WT	WT
1039C>T	HET [†]	HET [†]
1659G>A	WT	WT
1661G>C	MUT [‡]	HET [†]
1707T>del	WT	WT
1758G>T	WT	WT
1758G>A	WT	WT
1846G>A	WT	WT
1976G>A	WT	WT
*20 cluster	WT	WT
2539-2542delAACT	WT	WT
2549A>del	WT	WT
2613-2615delAGA	WT	WT
2850C>T	WT	WT
2935A>C	WT	WT
3183G>A	WT	WT
3198C>G	WT	WT
3277T>C	WT	WT
4042G>A	WT	WT
*36GC	WT	WT
4180G>C	HET [†]	HET [†]
1863 Repeat Ins	Negative	Negative
Gene deletion (*5)	Negative	Negative
Gene duplication	Negative	Negative
AmpliChip genotype	No call	CYP2D6*1/*10
AmpliChip predicted phenotype	No call	Extensive metabolizer

Sequence variations are listed as shown on the AmpliChip report.
[†]Heterozygous calls.
[‡]Homozygous call for position 1661G>G that triggered the no call.
HET: Heterozygous; MUT: Mutated; WT: Wild-type.

SNPs at positions 100, 1039, 1661 and 4180 can be aligned with *CYP2D6*10B*, this assignment, however, would default 1661 as the sole SNP to the second allele, i.e., *CYP2D6*1* (1661G), a variant that is not defined by the nomenclature committee or the AmpliChip allele calling algorithm. Hence, the discrepancy caused

by homozygosity at position 1661 appeared to be the culprit of the AmpliChip no call.

By contrast, the AmpliChip produced a call for *CYP2D6* for the son of the patient (Table 1). Because the son’s genotype was not conflicted by the SNP at position 1661, it was reported as *CYP2D6*1/*10* by the AmpliChip test.

The *CYP2C19* genotypes of the patient and her son were *CYP2C19*2/*2* and **1/*2*, respectively, predicting poor and intermediate metabolizer phenotypes.

Resolution of AmpliChip no call & characterization of novel haplotypes

Subsequent analyses using a combination of XL-PCR, TaqMan assays and quantitative CNV analysis confirmed the AmpliChip Test results for the patient and her son. Sequence variations detected by genotyping were further confirmed by gene resequencing. As shown in Figure 1, in addition to the expected sequence variations the patient was heterozygous for -498C>A (rs74966855), a SNP located in the upstream region of the *CYP2D6* gene, and homozygous for 1661G>C. The son, in contrast, was heterozygous for these two SNPs. These observations suggested the presence of two novel *CYP2D6*1* subvariants. To confirm this, the *CYP2D6*1* allele was amplified in an allele-specific manner and partially sequenced. The son’s *CYP2D6*1* allele carried the -498C>A, but not the 1661G>C SNP, while both SNPs were present on the case’s *CYP2D6*1* allele. These subvariants are designated as *CYP2D6*1(var1)* and *CYP2D6*1(var2)*.

The presence of the -498C>A variation in the *CYP2D6* gene was scored by pyrosequencing in 175 Korean subjects [24], but to date its haplotype structure has not been reported. We utilized existing sequence and genotype data from that study [24] to determine linkage disequilibrium in 175 Korean subjects. The -498A and 100C SNPs were in 100% linkage disequilibrium (D’ = 1), that is these SNPs are located on opposite chromosomes. Two of the 175 subjects were homozygous for -498A and negative for any other SNPs defining a *CYP2D6*1* (-498A) subvariant (Figure 1), which we observed in the patient’s son and labeled *CYP2D6*1(var1)*. The frequency of *CYP2D6*1(var1)* was 6.6% in the Korean population sample studied.

CNV analysis for the patient and her son revealed three copies for the exon 1, intron 5 and intron 6 regions, but only two copies for exon 9. This CNV pattern is in agreement with the ‘Chinese’ *CYP2D6*10* allele first published by Johansson *et al.* in 1994 [28], but now more commonly referred to the *CYP2D6*36+*10* tandem [29]. The *CYP2D6*36* gene located upstream of the **10* gene copy carries the *CYP2D7*-derived exon 9 conversion and therefore only produces a two-copy sig-

nal for this gene region while all other regions produce a signal corresponding to three gene copies [21].

The final genotype assignment for the patient considering all test results was *CYP2D6**1(*var2*)/**36*+**10* predicting extensive metabolizer status. The final assignment for the son was *CYP2D6**1(*var1*)/**36*+**10* also predicting an extensive metabolizer phenotype. As shown in the pedigree of [Supplementary Figure 1](#), the son inherited the *CYP2D6**36+**10* allele from his mother (case), and an *CYP2D6**1(*var1*) from his father, who was not available for the study.

Functional assessment of -498C>A *in vitro* & *in vivo*

Since the -498C>A SNP is located close to putative HNF4a transcription factor binding sites of the proximal promoter region of the *CYP2D6* gene, the functional effect of this SNP on expression was assessed *in vitro* using a luciferase promoter activity assay. No statistically significant difference of luciferase activity was detected for the expression construct containing -498A compared with that containing the wild-type -498C nucleotide ([Figure 2](#)).

In addition, *in vivo* *CYP2D6* activity previously determined by measuring urinary metabolic ratios of DM/DX in 40 subjects with *CYP2D6**1/*1 and *CYP2D6**1/*10 genotypes [27] did not show a statistically significant association with the -498C>A SNP ([Figure 3](#)).

Discussion

We describe a Chinese Han patient presenting with persistent hallucinations, but lack of antipsychotic efficacy within the context of a psychotic disorder possibly induced by sertraline chronic intoxication. Clinically similar cases of hallucinations have been described in association with the selective serotonin reuptake inhibitors sertraline [30] and citalopram [31]. To our knowledge, this is the first report describing a clinical case with this novel *CYP2D6* haplotype representing a ‘real-life’ example of a complicated clinical case that also highlights challenges of *CYP2D6* genotyping, phenotype prediction and clinical interpretation [4,5].

The genetic and functional analyses of the patient’s *CYP2D6* genotype-predicted extensive metabolizer status, which contradicted our initial suspicion that an ultrarapid metabolizer phenotype contributed to the patient’s presentation, that is, hallucinations 3 weeks after sertraline withdrawal. At this point in time we can only speculate why this patient did not respond nor showed adverse effects to high doses of risperidone. It could be, however, that long-time treatment with sertraline (prior to the prescription of risperidone) caused *CYP2D6* inhibition and phenocopying to poor metabolizer status [30]. The patient is also predicted to be a

CYP2C19 poor metabolizer (*CYP2C19**2/*2), which may also have contributed to the sertraline toxicity. Variations in genes in the pharmacodynamic pathway may also have played an important role in the patient’s presentation and may explain why haloperidol was successfully used to alleviate her psychotic symptoms. Antipsychotic drugs act on numerous neurotransmitter receptors. Specifically, in addition to the lack of an α -adrenergic blocking activity of risperidone, the binding affinity for risperidone differs from that of haloperidol for antagonist activity at the serotonin HT2A receptor [32]. It has been postulated that hallucinations can be the result of agonism at the level of serotonin receptors, the blockade of dopamine reuptake and interactions with sigma receptors. Therefore, polymorphisms in key genes such as the serotonin (*HT2A*, *HT2C* and *HT6*), but also dopamine receptors (*DRD2* and *DRD3*) as well as the serotonin transporter (*SLC6A4*) could conceivably have contributed to the adverse event [33,34]. A possible role of histamine in the development of hallucinations has also been hypothesized based on histamine’s psychotic enhancement activity. Variation in the histamine receptor genes *HRH3* and *HRH4* have recently been described to be potential predictors of risperidone efficacy in Chinese Han subjects [35,36], which is of particular interest as our patient is Han Chinese. Additional pharmacogenetic studies of polymorphism in these key genes may be informative to substantiate our hypothesis and help explain the hallucinations our patient experienced. Although the AmpliChip Test did not provide actionable information for the patient’s clinical management, it fueled valuable discussions regarding her atypical presentation.

The AmpliChip Test, which is otherwise successfully utilized in our practice to guide complex cases, failed to call the patient’s *CYP2D6* genotype. The test results could also not manually be interpreted, but warranted a full characterization in the research setting. Despite the availability of numerous *CYP2D6* genotyping platforms including the AmpliChip Test, genotype analysis remains a challenge due to the highly polymorphic and complex nature of the *CYP2D6* gene [4,5]. As stated in each test report disclaimer, there is always the possibility that a patient carries a rare variant that is not covered by the test panel, or possesses a novel variant that eludes detection by the test. Novel variants may be characterized by sequence variation(s) that have not been observed before, but could also harbor known sequence variations in novel combinations (or haplotypes). An example of the latter is *CYP2D6**69, which carries key SNPs otherwise defining *CYP2D6**10 and *41 [37]. In other cases, alleles may have been defined based on limited information and will trigger no calls or are other-

Figure 1. Overview of novel CYP2D6 allelic variants in comparison to selected reference sequences. The top sections of the graph provide coordinates for sequence variations, SNP rs IDs and the sequence context for each variation. For the CYP2D6*1 reference sequences AY545216 and M33388, positions are shown with the ATG translation codon being +1 (light gray boxes for M33388 indicate positions that vary due to sequence errors). In the 'checkerboard' panel, sequence variations present in the novel CYP2D6*1(var1) and (var2) variants found in the case and her son are shown in red/hatched and the sequence variations present in their CYP2D6*10 gene (of the *36+*10 tandem) are indicated in black. Yellow/dotted boxes represent a 210 bp region between two A-homopolymer stretches within sequence that could not unequivocally be determined. JX678710 and JX678711 represent variations across entire CYP2D6*10 alleles, the latter was derived from a CYP2D6*36+*10 tandem. CYP2D6*10A, B and C represent CYP2D6 Nomenclature Committee annotations [3]. The bottom panels summarize the location of sequence variations in respect to gene regions and indicate which variations cause amino acid changes. Dark gray bars indicate 5'- and 3'-UTRs; exon and intron numbers are shown as light gray bars. Please see color figure at www.futuremedicine.com/doi/pdf/10.2217/pgs.14.94

wise flagged when additional SNP(s) are tested. This occurred on the AmpliChip when DNA positive for the *CYP2D6**31 key SNP 4042G>A triggered no calls in a number of patients [16]. The allele definition of *CYP2D6**31 was originally silent on -1584C>G, that is, it was defined without information for this SNP, but was defined by the AmpliChip as -1584C (wild-type). For our patient, homozygosity of 1661G>C triggered an AmpliChip no call, because this result could not be reconciled with the other SNPs that were detected in the patient's sample. 1661G>C is a relatively common SNP and not specific for any particular allelic variant. It is part of numerous *CYP2D6* haplotypes including *CYP2D6**2-like variants (i.e., variants carrying 2850C>T), and variants carrying the 100C>T SNP such as *CYP2D6**4 and *10 according to *CYP2D6* nomenclature [3]. To our knowledge, our case is the first for which an AmpliChip no call was reported in the clinical setting that can be attributed to the 1661G>C SNP and the first report describing *CYP2D6**1(*var1*) and (*var2*) haplotypes in Chinese individuals.

However, a research study that utilized the AmpliChip has suggested that this variant exists in Papua New Guineans. The AmpliChip had unexpected high no call rates for the Kunjingini (45%) and Alexishafen (20%) cohorts [38]. To resolve the high rate of no calls, the authors performed a PHASE analysis using the AmpliChip raw SNP calls and performed limited resequencing. Their data suggested that an allele carrying only the 1661G>C SNP, that is, *CYP2D6**1 (1661C), is frequent in the Papua New Guinean populations of Kunjingini and Alexishafen at 15 and 10%, respectively, and contributed to the AmpliChip no call results. Unfortunately, this allele was not sequenced in its entirety to elucidate its complete sequence and haplotype [38]; hence it remains unknown whether any of the *CYP2D6**1 (1661C) variants found in the Papua New Guinean populations also carried -498A or not. Interestingly, the analysis in the Papua New Guineans also suggested additional novel haplotypes consisting of 100C>T, 1039C>T, 1661C>G and/or 4180G>C. None of the variants described for the Papua New Guineans are defined by the *CYP2D6* Nomenclature Committee.

In our initial attempt at resolving the haplotypes of both alleles in our patient, the entire gene was resequenced including upstream and downstream flanking regions. Unexpectedly, both the patient and her son revealed heterozygosity of -498C>A, a SNP that was previously described in Koreans [24], but not assigned to a haplotype or allele. The *CYP2D6**1/*36+*10 genotype of the patient's son and heterozygosity at position -498C>A did not allow us to draw any conclusions as to whether -498C>A is located on the *CYP2D6**1 or *36+*10

Figure 2. Characterization of the impact of -498C and -498A on *CYP2D6* expression *in vitro*. Activity was measured *in vitro* by a luciferase promoter activity assay in HepG2 cells. Luciferase activity for constructs containing -498C and -498A was not significantly different ($p = 0.11$).

allele. The haplotype analysis of 175 Korean subjects, however, strongly suggested that -498A and 100C are located on opposite alleles (in linkage disequilibrium) and thus -498A is positioned on the *CYP2D6**1 allele. We experimentally confirmed the *CYP2D6**1(*var1*) and (*var2*) suballele variants shown in Figure 1 by partially sequencing the *CYP2D6**1 allele of both individuals.

*CYP2D6**1 and *CYP2D6**10 subvariant assignment according to nomenclature [3] proves difficult as the respective *CYP2D6**1B-E and *CYP2D6**10A, B and D subvariants do not appear to have information listed for the upstream and intronic gene regions. We have previously sequenced two *CYP2D6**10 alleles (Figure 1) to

Figure 3. Characterization of the impact of -498C and -498A on *CYP2D6* activity *in vivo*. The impact of -498C and -498A was assessed in 43 subjects with *CYP2D6**1/*1 and *CYP2D6**1/*10 genotypes. dextromethorphan/dextrorphan was not different among the groups ($p = 0.44$ and $p = 0.46$). MR: Metabolic ratio of dextromethorphan/dextrorphan.

which the *CYP2D6*10* sequence of our patient matches. The closest subvariant per nomenclature appears to be *CYP2D6*10B*, which also matches to the most frequent *CYP2D6*10* haplotype observed in the investigated Korean subjects.

The *CYP2D6*1(var2)* variant detected in our patient may interfere with call algorithms of genotype platforms that utilize the 1661C>G SNP such as the AmpliChip [14], Affymetrix DMET Plus Array [39] or Luminex X-Tag platform [40] while *CYP2D6*1(var1)* will likely not interfere. The 1661C>G SNP is located within exon 3, but does not confer an amino acid change. Since the -498C>A SNP is not part of any genotype panel, it is not anticipated that this SNP interferes with any genotype platform or genotype calls.

Based on our *in vitro* and *in vivo* data, alleles carrying the -498C>A SNP are not anticipated to impact *CYP2D6* expression levels and activity. Consequently, we propose to classify both *CYP2D6*1(var1)* and *(var2)* as fully functional and assigning an activity score of 1.

Conclusion & future perspective

This report demonstrates that sequence variations that occur in novel haplotypes can interfere with routine *CYP2D6* genotype testing. *CYP2D6* is a highly polymorphic gene locus and novel allelic variants and haplotypes are still being discovered especially in populations that have only been investigated in more detail in recent years [16,41–43] or are identified through discordant observations during testing (such as AmpliChip no calls) [15–17]. In order to accurately interpret such

results, algorithms interpreting genotype data must be periodically updated to reflect current knowledge. Furthermore, more advanced databases, ideally containing complete reference sequences along with functional data for all known allelic variants, need to be available to keep track of the still growing number of *CYP2D6* variations. This will be especially important with the arrival of next-generation sequencing technology-based platforms in the near future. The P450 Nomenclature Committee website is a valuable resource [3,44], but it no longer assigns and posts (sub) variants unless they are associated with a functional consequence. Consequently, many variants may not be readily available or not even be published. Although allelic variants associated with poor, intermediate and ultrarapid metabolizer status are clinically the most important, sequence variations that may functionally be benign, such as the *CYP2D6*1(var2)* alleles we have discovered in this investigation, may interfere with testing. It is therefore of utmost importance to know about any sequence variations that could tentatively interfere with a test, or at least have knowledge of potential interference in certain cases, to allow for proper test interpretation.

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Executive summary

Aim

- The aim of this case report was to resolve an AmpliChip Test™ no call for *CYP2D6*. The genotype test was ordered to aid in the clinical interpretation of a Han Chinese patient presenting with hallucinations and failure to respond to risperidone.

Methods

- The *CYP2D6* gene locus of the patient and her son was extensively characterized. Methods included long-range PCR, TaqMan genotyping assays, quantitative gene copy number determination and gene resequencing.
- The functional impact of -498C>A was assessed *in vitro* using luciferase reporter assays and *in vivo* by phenotyping with dextromethorphan.

Results

- Two novel *CYP2D6* haplotypes are reported, *CYP2D6*1* (-498A, 1661C) and *CYP2D6*1* (-498A).
- The 1661G>C SNP on *CYP2D6*1* (-498A, 1661C) was identified as causing the AmpliChip no call.
- The -498A SNP was not associated with a functional consequence *in vitro* or *in vivo*.
- The frequency of *CYP2D6*1* (-498A) was 6.6% in Koreans.
- Failure to respond to risperidone was not caused by ultrarapid metabolism as initially suspected.

Conclusion

- Rare and novel *CYP2D6* allelic variants may interfere with established *CYP2D6* genotyping platforms such as the AmpliChip Test.
- While such cases may be relatively infrequent or rare, the clinical/genetic testing community must be aware of the complexity of the *CYP2D6* gene locus and challenges of *CYP2D6* testing at large.
- This is not only true for the AmpliChip CYP450 Test, but any test platform as such variants may interfere with test performance, accuracy of test results and ultimately phenotype prediction from genotype data.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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