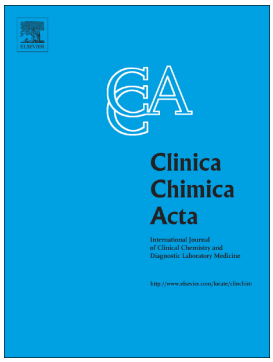

Confirmation practice in pharmacogenetic testing; how good is good enough?

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Confirmation practice in pharmacogenetic testing; How good is good enough?

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Abstract

Pharmacogenetic testing is increasingly implemented in routine diagnostics. However, quality control measures, in particular confirmation practices e.g. the use of two independent genotyping techniques, are subject of debate and there are no clear guidelines. The aim of the current paper is to discuss the current practice in confirmation testing in the field of pharmacogenetics and draw attention to this situation. *DPYD* genotyping is used as a case example to highlight the importance of assigning the correct genotype. Current confirmation practices in laboratories are explored through a survey. Substantial heterogeneity was observed with 54% of the laboratories applying different forms of confirmation practice. Finally, we evaluated over 10 years of genotyping results from two large genotyping facilities, which both use a second, independent genotyping technique. Discrepancies between tests were identified in 9 patients (0.01%), possibly due to allele dropout. We feel that a second, independent technique is useful for genetic tests with a high clinical impact, such as *DPYD* testing. Guidelines can help to align confirmatory laboratory practices for pharmacogenetics, which may need to be specified per gene and test.

Keywords

Pharmacogenetics; genotyping; quality; confirmation

Abbreviations

PGx	Pharmacogenetics
FDA	Food and Drug administration
LDT	laboratory developed tests
PT	proficiency testing
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
RfB	German Reference Institute for Bioanalytics
EQMN	European Molecular Genetics Quality Network
SKML	Dutch Foundation for Quality Assessment in Medical Laboratories
GeT-RM	Genetic Testing Reference Material Coordination Program
LUMC	Leiden University Medical Centre
Erasmus MC	Erasmus Medical Centre
DPYD	Gene encoding dihydropyrimidine dehydrogenase
CPIC	Clinical Pharmacogenetics Implementation Consortium
DPWG	Dutch Pharmacogenetics Working Group
ESMO	European Society for Medical Oncology
SNPs	Single Nucleotide Polymorphisms
GPP	good pharmacogenomics practice
EMA	European Medicines Agency
IVDR	*In Vitro* Diagnostic Regulation
GLIMS	Global Laboratory Information Management System
PCR-RFLP	Polymerase Chain Reaction-Restriction Fragment Length Polymorphism
NGS	next generation sequencing

1. Introduction

Over the past ten years, our knowledge of pharmacogenetics (PGx) has increased significantly. With decreasing assay costs, availability of PGx dosing guidelines and inclusion of PGx information in drug labels PGx testing has become an attractive strategy for routine diagnostics [1]. For some diseases and drugs (pharmaco)genetic testing to predict therapeutic response is already widely accepted in clinical practice (e.g. lung cancer and *EGFR* status) or even mandatory (e.g. abacavir and *HLA-B**5701 allele carriers) [2]. For a limited number of (pharmaco)genetic tests approval of the Food and Drug Administration (FDA) is available, e.g. *CYP2D6* (Luminex) and INFINITI *CYP2C19* assays, possibly increasing its use in clinical care [3-5]. The Roche AmpliChip for cytochrome p450 *CYP2D6* and *CYP2C19* (Roche Molecular Diagnostics, Pleasanton, CA, USA) was the first FDA approved (December 24th, 2004) and commercially available PGx test [6]. If no FDA-approved assay is available, laboratory developed tests (LDTs) can be used [7].

Many laboratories use LDTs. It is important to have quality assurance of the PGx test results from these LDTs, which can be achieved by participating in a proficiency testing program. Proficiency testing programs are regulated by independent organizations, such as the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) [8], the German Reference Institute for Bioanalytics (RfB) [9], the European Molecular Genetics Quality Network (EMQN) in the UK [10], or the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML) [11]. In addition, the Genetic Testing Reference Material Coordination Program (GeT-RM) was set-up to guard quality assurance, assay development, validation and proficiency testing [12]. Another less commonly applied quality control measure used by laboratories to ensure quality of PGx test results is confirmation practice, e.g. the use of two independent genotyping techniques. However, these measures have disadvantages, such as increased costs and labour, and are subject of debate. It is yet unknown if differences in laboratory practices exist as there are no clear guidelines on this particular quality control aspect.

The aim of the current paper is to discuss the current practice in confirmation testing in PGx and draw attention to this situation. We first assess current confirmation practices to assure the validity of PGx test results, by means of a questionnaire using *DPYD* genotyping as an impactful case. Secondly, we evaluate genotyping results from Leiden University Medical Center (LUMC) and Erasmus Medical Center Rotterdam (Erasmus MC), where two independent genotyping methods are applied to confirm results.

2. Importance of analytical validity and assigning the correct genotype

The number of executed PGx tests is rapidly increasing, partly due to incorporation of PGx information in drug labels -currently over 260- some of them strongly suggesting or demanding a priori PGx testing (e.g. abacavir, clopidogrel, eliglustat) [13]. For some other diseases or drugs, which have a (pharmaco)genetic application available, the use in clinical practice remains limited and is subject to debate (e.g. bupropion, tamoxifen) [1, 14]. By contrast to many other clinical laboratory tests, a (pharmaco)genetic test is usually performed only once in the lifespan of a patient. As a result, it is of utmost importance that the correct result is reported. Consequences of a false positive or false negative result could be fatal, as is explained in the following example of *DPYD* genotyping for fluoropyrimidines (5-fluorouracil/5-FU, and capecitabine) [15]. There is compelling evidence on the reduction of severe fluoropyrimidine-induced toxicity when using prospective PGx for four *DPYD* variants, and dosing recommendations for these four *DPYD* variants have been published by the Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Dutch Pharmacogenetics Working Group (DPWG) [16-19]. Despite this, clinical implementation is not yet part of routine clinical care in many hospitals [20]. When exposed to standard dosages of fluoropyrimidines, carriers of a *DPYD* variant are at high risk for severe, or even fatal, toxicity. Despite the low frequency of *DPYD* variants, prospective genotyping for *DPYD* variants in all patients prior to initiating fluoropyrimidine treatment was shown to be cost-saving [21]. Thus, it is safer, but not more expensive to genotype patients. Misclassification of the *DPYD* genotype can result in suboptimal therapy (false positive) or even have lethal consequences from fluoropyrimidine treatment in standard dosages (false negative). In addition, therapeutic drug monitoring (TDM) could be used to monitor the 5-FU dose during treatment, but is rarely executed. For capecitabine, the oral pro-drug of 5-FU, TDM protocols need to be developed. This particular example shows the clinical importance and substantial consequences of PGx testing and illustrates why it is of utmost importance to report the correct result.

3. The dilemma

Laboratories apply different genotyping techniques to generate PGx results. Sanger sequencing remains the gold standard for DNA sequencing [22], even though this can be prone to errors [23]. In general, PCR-based assays (including Sanger sequencing) are considered a robust methodology with reliable results. Each assay is subjected to extensive validation by the company or laboratory to reduce the risk of a priori errors. However, after the implementation of a test in clinical practice, it is still possible to have false positive or false negative results, e.g. due to allele dropout [24]. Allele dropout can be caused by a newly acquired variant located at the site of a primer, causing the

binding of this primer to fail. A genetic variant located on that DNA strand will not be genotyped, and the patient is misclassified as homozygous carrier of the variant on the other strand.

To mitigate the risk of allele dropout a laboratory can use a second, independent method that uses different primers to confirm results. However, this results in increased costs, labour and turn-around-time. Should laboratories execute a second method to confirm results, or not? The dilemma of the quality control aspect of PGx testing is based on the probability of a genotyping error to occur, the level of increased effort and costs to detect the error and the consequence of not detecting the error. A genotyping error, e.g. due to allele dropout, can be detected by a second, independent genotyping assay, which is the most adequate, but comprehensive, available method. Abolishing a second method or repetition can thus save both time and costs, possibly increasing the likeliness of use of PGx testing since cost-effectiveness is often reported as a barrier for implementing PGx testing [15]. The consequence of an error in PGx can be substantial, yet it is unrealistic to aim to never have an incorrect result. This dilemma is why differences in confirmation practices in laboratories could exist and why guidelines are required to align laboratory practices. These differences could be overcome by clear guidelines from regulatory authorities, however, notifications from regulatory authorities are also not conclusive about this dilemma. In January 2017, the FDA discussed that regulatory aspects on the quality control of LDTs are still under debate [25]. In Europe, guidelines on good pharmacogenomics practice (GPP) by the European Medicines Agency (EMA) issued in September 2018 include a chapter on quality aspects on PGx analyses. They describe the importance of proper validation prior to using genetic tests in clinical trials or a diagnostic setting and the detection of respective allele-drop-outs, as primer-based technologies are prone for these artefacts. However, no specific standpoint is taken regarding the use of a second, independent technique [26]. Also, the *In Vitro* Diagnostic Regulation (IVDR) of the European Parliament and of the Council on *in vitro* diagnostic medical devices has recently been updated and will come into force in 2022. Yet, these guidelines do not explicitly state what actions to guarantee quality are required in the laboratory.

4.Confirmation practice

4.1 Current confirmation practice in laboratories

In order to investigate the consequences of the lack of clear guidelines we assessed the current confirmation practices of laboratories. A short questionnaire comprising three general questions on *DPYD* genotyping and confirmation practices in the laboratory was sent to laboratories in Europe and the Netherlands participating in the proficiency testing program of the RfB and SKML,

respectively. Details on the set-up of the questionnaire can be found in the **Supplementary Material**. Out of the 475 laboratories, 35 completed the questionnaire. One laboratory participated in both the European (RfB) and Dutch (SKML) questionnaire. 28 laboratories executed genotyping tests. Of all laboratory techniques, the TaqMan assay and melting curve analyses were most frequently used. A large variation between laboratories in confirmation practice was observed. Almost half of the laboratories did not execute a second test (either independent or repetition).

4.2 Two independent genotyping methods as confirmation practice

In addition, we assessed the impact of confirmation methods in PGx. At LUMC and the IFCC PGx reference laboratory at Erasmus MC, the most elaborate confirmation method, executing two independent genotyping tests using two different platforms, are used. We evaluated over 10 years of aggregated genotyping data of these two large genotyping laboratories performing duplicate analyses on two independent platforms. Details of the two laboratories can be found in the **Supplementary Material**. In total, 89.842 duplicate tests were executed for patient care in over 10 years of genotyping. Nine discrepancies (0.01%) between tests were observed. One discrepancy in *CYP3A5**3 was the result of chimerism due to allogeneic hematopoietic stem cell transplantations, which resulted in the determination of the genotype of both patient and donor [27, 28]. Four discrepancies in *CYP3A5**3, one discrepancy in *DPYD**13 and three discrepancies in *CYP2D6**6 were identified, possibly due to allele dropout. The probability of finding a discrepant result when using two independent techniques according to our data was calculated to be 0.01%.

5. Discussion

The topic of confirmatory testing in the rapidly growing field of PGx deserves attention. At this moment, there are no clear guidelines on the required confirmation practice aspects of PGx testing. Should laboratories execute a second method to confirm results, or not? The FDA is in debate on this dilemma and the current guidelines of the EMA are not very precise on the use of confirmation methods. Our supporting data show that there is great heterogeneity between laboratories in confirmation practice. Discrepant results were identified between two tests in about 0.01% of samples.

Our data show a substantial variation of approaches for *DPYD* genotyping used in laboratories across Europe as well as a limited use of second, independent techniques as a confirmation method to assure the correctness of genotyping results. Almost half of the responders do not apply any of the suggested confirmation or replication methods, and implies the need for centrally organized guidelines. We selected *DPYD* as an example for its clinical relevance, as a false negative result or

misclassification can have a fatal outcome. The number of centres which routinely test for *DPYD* is relatively low and it is possible that a questionnaire focussing on a gene that is more commonly tested would have resulted in a higher response rate. However we do not expect major differences in confirmation practice between genes within a laboratory.

To assess the usefulness of applying two independent genotyping techniques for confirmatory testing we evaluated genotyping results of almost 90.000 samples tested in two laboratories in over 10 years of genotyping. We identified nine discrepant results (0.01%) between the two independent genotyping techniques. One discrepant result was caused by chimerism following allogeneic hematopoietic stem cell transplantations, and is thus not due to analytical failure. To prevent this particular type of error, a check-box for "transplantation patient" was added to the genotyping request form. Two other stem cell transplantation patients were correctly genotyped after the check-box was added. For the other eight samples, misclassification due to allele dropout was the most probable cause of the discrepancies. In this study, a frequency of 0.01% of misclassification was shown, whereas previous publications show higher frequencies of misclassification (0.27% in 365 patients, Scantamburlo *et al*. [29] and 0.44% in 30.769 genotypes, Blais *et al*. [24]). A difference in discrepant results between the two genotyping centres was identified and might be explained by the different genotyping techniques used in each centre, as the call rate and accuracy of the techniques can be different. Additionally, *CYP2D6* data of one centre was not included, as this centre did not use a second, independent genotyping platform to confirm genotyping results for *CYP2D6*. *CYP2D6* is a highly polymorphic gene and *CYP2D6*-assays could be more prone to allele dropout.

Another important aspect to consider is that allele dropout is test specific: it depends on the positions of variants and the primer positions of the assay. Therefore, caution should be taken in generalizing our results. Specific quality control analyses per assay may be warranted. One could envision for example a minimum amount of samples to be tested to show that allele dropout for that particular assay and primers is low, possibly as a requirement for diagnostic companies to demonstrate. This brings along a second important consideration, which is that the sensitivity of detecting allele dropouts is directly proportional to the amount of heterozygotes present. In other words, discrepancies for *CYP2D6**4 (allele frequency 23% [30]) will be detected much earlier than discrepancies for, e.g. *CYP2D6**7 (allele frequency 0.05% [30]). In this aspect, the determined discrepancy rate of 0.01% might actually be higher for specific variants. In addition, the tests in this study were mainly executed in patients with a Caucasian ethnic background. As frequencies of

genetic variants can vary between different ethnic populations, results could be different in another population.

The large number of genotyping test results is a strength of this study. However, specific allele dropout will depend on the number of samples with a particular variant. The low discrepancy rate shows high concordance and robustness of the methods used. As described before, the consequence of a misclassified genotype can be substantial, resulting in either underdosing or overdosing, sub sequentially leading to inefficacy or, potentially lethal, toxicity (e.g. *DPYD* genotyping). We expect that next generation sequencing (NGS) might replace some of the current assays in the upcoming years. NGS is also subject to allele dropout as it is PCR based, but possibly less compared to current techniques. This is caused by the fact that NGS has multiple coverage depth of the same variants, thus a failed reaction of one primer will not directly results in a misclassification of the variant.

Differences exist between laboratories in which *DPYD* variants are genotyped, or they might not genotype for *DPYD* variants at all. This could have great impact on patient care as DPD phenotypes might be predicted differently between laboratories. The impact could be greater compared to the impact due to differences between laboratories in confirmation practice as quality control of these tests. This also accounts for other variants in other genes, and for the fact that not all associated variants per gene are discovered yet. Besides assay errors, human errors (switch of samples) might also occur. However, this discussion is out of the scope of this paper, were we focus on the dilemma of confirmation practice.

6. Conclusions

We have shown substantial variability between laboratories in the use of a second confirmatory technique for PGx testing. The risk of a discrepancy may differ between assays and the clinical implications will depend on the gene tested. Therefore we feel that a second, independent technique is useful for genetic tests with a high clinical impact, such as *DPYD* testing. Guidelines can help to align confirmatory laboratory practices for PGx, however, they may need to be specified per gene and per test.

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Conflicts of interest

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Highlights:

- The analytical validity of a pharmacogenetic test result is of utmost importance
- A measure to assure analytical validity is the use of a second confirmation method
- Heterogeneity in confirmation practice exists between laboratories
- Guidelines are key to align laboratory practices for pharmacogenetic testing
- Guidelines may need to be specified per gene and test