Analytical validation of variants to aid in genotype-guided therapy for oncology

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Abstract

The Clinical Laboratory Improvement Amendments (CLIA) of 1988 requires that pharmacogenetic genotyping methods need to be established according to technical standards and laboratory practice guidelines before testing can be offered to patients. Testing methods for variants in *ABCB1, CBR3, COMT, CYP3A7, C8ORF34, FCGR2A, FCGR3A, HAS3, NT5C2, NUDT15, SBF2, SEMA3C, SLC16A5, SLC28A3, SOD2, TLR4,* and *TPMT* were validated in a CLIA-accredited laboratory. As no known reference materials were available, DNA samples that were from Coriell Cell Repositories (Camden, NJ) were used for the analytical validation studies. Pharmacogenetic testing methods developed here were shown to be accurate and 100% analytically sensitive and specific. Other CLIA-accredited laboratories interested in offering pharmacogenetic testing for these genetic variants, related to genotype-guided therapy for oncology, could use these publicly available samples as reference materials when developing and validating new genetic tests or refining current assays.

Introduction

Genetic variants exist in genes coding for enzymes that are targets of oncology medications or responsible for their metabolism and transport. Pharmacogenetic tests are used to assess whether an individual has the variant allele for these known genetic changes and provide information on risk of toxicity or inefficacy to assist a patient's medical care team in developing therapeutic strategies. For example, the cytotoxic agent 5-fluorouracil undergoes fluoropyrimidine catabolism facilitated by dihydropyrimidine dehydrogenase (DPD). Individuals with reduced- or no-function variants in the *DPYD* gene (codes for DPD) have reduced activity of DPD, reduced 5-fluorouracil clearance, increased half-life and profound dose-related toxicities (eg, mucositis, diarrhea, neutropenia, and neurotoxicity). Treatment outcomes can be improved by testing for the *DPYD* variants and then following recommendations according to the Clinical Pharmacogenetics Implementation Consortium (CPIC, https://cpicpgx.org/, last accessed on 29 October 2018) guidelines which suggest a reduction in the dose by 25% to 50% or avoiding 5-fluorouracil depending on the specific *DPYD* genetic variants present [1]. Pharmacogenetic tests can only be used to improve patient care if the test is analytically and clinically valid.

The U.S. Food and Drug Administration (FDA) includes pharmacogenetic test information in drug labels for several approved oncology medications, including belinostat (*UGT1A1*), irinotecan (*UGT1A1*), nilotinib (*UGT1A1*), pazopanib (*UGT1A1* and *HLA-B*), capecitabine (*DPYD*), cisplatin (*TPMT*), mercaptopurine (*TPMT*), and thioguanine (*TPMT*). The suggestion that patients should be tested for genetic variants in the genes included in drug labels results in a need for more clinical laboratories with the ability to validate and perform pharmacogenetic testing. The Clinical Laboratory Improvement Amendments (CLIA) of 1988 were developed to regulate all facilities or sites in the United States that test human specimens for health assessment or to diagnose, prevent, or treat disease. Pharmacogenetic tests need to be established according to the technical standards and laboratory practice guidelines required by CLIA before testing can be offered to patients.

To achieve regulatory requirements and meet best practice standards, the testing laboratories will often use reference materials for assay development and validation, quality control, and proficiency testing. Genomic DNA samples from cell lines or remaining de-identified patient material are regularly used to develop and validate assays. The Centers for Disease Control and Prevention (CDC) established the Genetic Testing Reference Material Coordination (GeT-RM) Program, in 2010, to address the need for characterized genomic DNA reference materials. Several DNA samples from the Coriell Cell Repositories (Camden, NJ) have been tested for genetic variants in five commonly tested genes: C*YP2D6, CYP2C19, CYP2C9, VKORC1*, and *UGT1A1* [2, 3]. As more genetic variants are established as markers of toxicity or inefficacy to cytotoxic agents, more pharmacogenetic testing methods can be validated to offer testing to patients with cancer. This study provides the rationale for chosen genes and variants as well as the analytical validation of genotyping methods for pharmacogenetic variants. For analytical validation, approximately 200 Coriell DNA samples for the variants of which methods were being validated were screened and Sanger sequencing used as an orthogonal method on a subset of samples both positive and negative for the variants of interest. All of the genes included in the analytical validation are involved in metabolism and transport of medicines (*ABCB1, CBR3, CYP3A7, SLC16A5, SLC28A3, TPMT, NT5C2, NUDT15*), or are targets of medications (*FCGR2A, FCGR3A*), or have an unclear role (*COMT, C8ORF34, HAS3, SBF2, SEMA3C, SOD2, TLR4*), in a CLIA-accredited laboratory, related to genotype-guided therapy for oncology.

Materials and Methods

Selection of variants

Oncology pharmacogenetic literature was reviewed to select 27 clinically-relevant genetic variants in 17 genes that have been associated with inter-individual variability in efficacy or toxicity of cytotoxic agents. Genetic variants in *ABCB1* were selected because *ABCB1* codes for the drug transporter P-glycoprotein and these variants are associated with variability in achieving complete control of chemotherapy-induced nausea and vomiting when using ondansetron [4-6]. The *CBR3* gene encodes for a carbonyl reductase which

is involved in metabolism of anthracyclines. The selected variant is associated with increased risk of anthracycline-induced cardiomyopathy in pediatric patients [7-9]. Similarly, the *HAS3* and *SLC28A3* variants were included for their role in risk of anthracycline-induced cardiomyopathy [10-16]. Although *TPMT* is involved in metabolism of thiopurines, the particular variant included in this study is associated with increased risk of cisplatin-induced hearing impairment. Similarly, the *COMT* genetic variants were included in this study based on studies reporting the variants play a role in cisplatin-induced hearing impairment in pediatric patients with medulloblastoma, neuroblastoma, or osteosarcoma [17-22]. Like *SLC28A3*, *SLC16A5* codes for a protein that is a member of the solute carrier transporter superfamily. In a recent *in vitro* study, cisplatin induced expression of *SLC16A5* in a dose-dependent manner. The selected *SLC16A5* variant was identified as a marker of hearing loss in a group of testicular cancer patients treated with cisplatin-containing chemotherapy [23]. Many clinically used medications are metabolized by CYP3A enzymes including CYP3A7 which is expressed in a fraction of adult human livers. The *CYP3A7 *1C* allele is associated with lower urinary unconjugated estrogen metabolite levels and increased risk of mortality among individuals with breast cancer treated with medicines that are CYP3A substrates [24, 25]. A genome-wide association study among Korean individuals with advanced non–small-cell lung cancer receiving irinotecan plus cisplatin reported associations between the *SEMA3C* variants and increased risk of grade 4 neutropenia and the *C8orf34* variant and increased risk of grade 3 diarrhea [26]. Genetic variants in *FCGR2A* and *FCGR3A* were included because these genes code for fragment C receptor subtypes that are targets for trastuzumab or rituximab binding. The variants are associated with altered risk of disease progression and progression-free survival [27-35]. The *NT5C2* gene codes for an enzyme involved in dephosphorylation of monophosphorylated gemcitabine and the particular variant included is associated with decreased clearance of intravenous gemcitabine [36, 37]. *NUDT15* was selected as it is important in metabolism of thiopurines and variants in *NUDT15* are associated with increased risk of thiopurine-induced toxicity. The relationship between the function of *SBF2* and taxanes is unknown, yet five variants have been associated with increased risk of taxane-induced peripheral neuropathy in a group of African-Americans [38]. The protein coded for by *SOD2* is a manganese superoxide dismutase that acts as a

mitochondrial antioxidant enzyme by endogenously converting superoxide into oxygen and hydrogen peroxide. The *SOD2* variant selected were reported to affect enzyme function and increase risk of asparaginase-induced hepatotoxicity. *TLR4* is a member of the Toll-like receptor family which plays a role in activation of innate immunity. Individuals with the selected *TLR4* variant were more likely to experience methotrexate-induced gastrointestinal, liver, pneumonitis, and skin and mucosal adverse events [39]. Table 1 summarizes the risk genotypes for each genetic variant related to a specific medication. Currently no clinical guidelines are available to recommend dose adjustment for these selected genetic variants.

Samples

189 existing reference DNA samples in the laboratory obtained from Coriell Cell Repository (Camden, New Jersey) [2, 3] were used for analytical validation (Supplemental Table S1).

Taqman genotyping for selected variants

Commercially available genotyping assays and reagents were used for each variant. DNA was amplified by real time PCR on the LifeTech QuantStudio 12K Flex (software v1.2.2; Grand Island, NY) and subjected to Taqman allelic discrimination using commercially available LifeTech (Grand Island, New York) reagents in a custom designed open array. The assay identification numbers are shown in Table 2.

Primer design and Sanger sequencing of samples for accuracy

Primers for each genetic variant were designed specific to the gene of interest (by aligning the gene sequence with that of genes with similar sequences to select a region that is unique to the gene of interest). The following tools were used for primer design: Primer 3 version 2004 which was developed by Rozen and Skaletsky in 2000 [40] (http://bioinfo.ut.ee/primer3-0.4.0/, last accessed on 29 October 2018), NCBI Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/, last accessed on 29 October 2018), and IDT OligoAnalyzer from Integrated DNA Technologies, Inc. (Coralville, IA). Integrated DNA

Technologies, Inc. performed synthesis of the primers. Primer sequences for each genetic variant are provided in Table 3 with PCR amplification conditions.[41,42]

PCR amplification was performed using the following conditions: initial denaturation at 98 ºC for 30 seconds, followed by 35 cycles of denaturation at 98 ºC for 10 seconds, annealing at the specific annealing temperature provided in Table 3 for 10 seconds, primer extension at 72 ºC for 30 seconds, and final extension at 72 ºC for 5 minutes. A "MyCycler Thermal cycler" (Bio-Rad, Hercules) was used and the PCR reaction contained the following reagents: 10ng genomic DNA, 1X Platinum SuperFi PCR Master Mix (Thermo Fisher Scientific, Massachusetts), and 0.112µM of the forward and reverse primers (Integrated DNA Technologies, Inc.).

Purification of the PCR amplicons were performed using the MinElute PCR Purification Kit (QIAGEN, Hilden, Germany). The protocol was adjusted by performing elution twice in 20µL of DNase-free water. Purified samples were mixed with 0.25µM of the primer used for sequencing and submitted to ACGT, Inc. (Wheeling, IL) for Sanger sequencing. Analysis of the sequences was performed using BioEdit biological sequence alignment editor (v7.0.5, Ibis Therapeutics, Carlsbad, CA).

Results

All of the variants were detected using the Taqman reagents. Both the amplification traces and allelic discrimination plots showed good allele separation (http://tools.thermofisher.com/content/sfs/manuals/cms_042798.pdf, last accessed 11/21/2018). The sequencing results were compared to the genotyping results and were 100% concordant (Table 2). The number of variant alleles and non-variant (ie, wild type) alleles detected by sequencing were evaluated to calculate the analytical sensitivity and specificity. The analytical sensitivity was 100% for the detection of variant alleles, with no reported false negatives. The analytical specificity was 100% for detection of non-

variant alleles, with no false positive results reported (confidence intervals varied based on samples tested and allele frequency, Table 2).

DNA samples obtained from Coriell Cell Biorepositories were used to assess intra- and inter-assay variation. Intra- (within) assay variation studies showed that all three replicates of the samples analyzed in the same run, had concordant results. The inter- (between) assay variation study showed that the samples consistently got the same result across three separate runs. All assays were robust and consistent genotyping results were obtained using two different instruments on different days and using input DNA within a concentration range of 15.4 to 50.8ng/µL.

A total of 189 DNA samples from Coriell Cell Repositories were genotyped successfully, with the results provided in Supplemental Table S1. Sanger sequencing was used as an orthogonal method to confirm the accuracy of the array genotyping results for a majority of the 27 variants, except for *SBF2 rs146987383* and *SBF2 rs149501654*. All 189 Coriell reference materials were negative for both the *SBF2 rs146987383* and *rs149501654* variants and known reference materials were obtained from a research laboratory. The number of samples, from the Coriell sample set, carrying a variant allele for each of the 27 genetic variants is summarized in Table 4 to show how many samples in this data set had a variant allele. The variant alleles for *CYP3A7 rs45446698*, *NUDT15 rs116855232*, *NUDT15 rs186364891*, *SBF2 rs141368249*, and *SBF2 rs117957652* are rare and only observed in five, six, one, five, and three samples, respectively (Table 4).

Discussion

Pharmacogenomic testing methods can be complex to create and complicated by gene sequence similarity between members of the same gene family. The benefit of publishing our validated genotyping methods is that other CLIA-accredited laboratories can access this information and confidently establish these methods knowing that the assays were robust, accurate, and had 100% analytical sensitivity and

specificity. Furthermore, identification of samples with the variant allele among genomic DNA samples from the Coriell Cell Biorepositories is useful for other clinical laboratories. These publicly available DNA samples and associated data can be used when developing and validating new genetic tests or refining current assays. The 1000 Genomes Project [43] or ExAC Project [44] may be used as another resource for identifying reference materials. Having validated methods and positive samples will improve standardization of pharmacogenomic testing across clinical laboratories.

The goal of analytical validation in clinical laboratories is to determine how well the test system can detect what it is designed to detect, ie, defined genetic variants from genomic DNA. Analytical validation can be challenging when there is a lack of reference materials for the defined variants, or lack of "truth". We chose the approach of screening approximately 200 Coriell DNA samples for the variants that were being validated and using Sanger sequencing as an orthogonal method on a subset of both positive and negative samples to determine truth. This approach works well when variants are rather common, greater than 0.01 frequency. For rarer alleles, a different approach was chosen. A research laboratory was contacted and DNA samples requested for validation studies.

A novel discovery during the validation studies was that several of the variants were in *cis*. Several DNA samples were positive for more than two variants in the same gene (eg, *ABCB1, COMT, SBF2, SEMA3C, SLC28A3*). In clinical testing some examples include *CFTR* (*p.R117H* and *c.1210−12[5][7][9]*) [45], *CYP2D6* [46], and *MTHFR* [47], *cis* variants have been well-documented as both impacting clinical phenotype and as a confounder for clinical interpretation.. If any of these markers are used in risk models for toxicity, these risk models may need to be revised.

Establishing standardized methods is challenging, but once these genotyping methods are validated another difficulty is interpretation and implementation of the test results. Interpretation of pharmacogenomic test results for patients with cancer is particularly complex because both germline and

somatic DNA alterations could inform therapy: somatic mutations can be used to select a targeted therapeutic agent whereas germline genetic variation can highlight possible risk of toxicity or inefficacy of therapy. An approach, for clinical pharmacogenomic testing in oncology is to perform testing on cancer patients as part of precision genomics initiatives/clinics and then discuss test results during molecular tumor boards. Molecular tumor boards are forums through which interprofessional teams discuss and interpret genomic test results and make treatment recommendations. If a clinical genetics testing laboratory with the ability to perform pharmacogenomic tests is aligned with a molecular tumor board, testing can be performed and results can be used to assess a cancer patients' risk of toxicity or treatment inefficacy when decisions are made about which cytotoxic agents will be preferred. If a clinical testing laboratory is in proximity and its services are integrated into a molecular tumor board, there may be added benefits such as shorter turn-around-time and, in some cases, genotyping prior to therapy selection instead of reactive genotyping. This approach along with the provided pharmacogenetic testing methods for genetic variants in *ABCB1, CBR3, COMT, CYP3A7, C8ORF34, FCGR2A, FCGR3A, HAS3, NT5C2, NUDT15, SBF2, SEMA3C, SLC16A5, SLC28A3, SOD2, TLR4,* and *TPMT* have the potential to better understand a patient's risk of toxicity or treatment inefficacy for oncology medications such as taxanes, anthracyclines, platinum agents, trastuzumab, rituximab, and 5-hydroxytriptamine-3 receptor antagonists.

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Table 1: Selected germline genetic variants and genotypes related to oncology therapy.

Gene and rs number	Genotypes	Description and References	Relevant Population	PharmGKB Level of Evidence†	CPIC Level of Evidence†	Medications with suggested testing in FDA label
ABCB1 rs1045642	*T/T*	More likely to experience efficacy and achieve complete control with ondansetron treatment in postoperative or chemotherapy-induced nausea and vomiting [4-6]		2A	C/D	
ABCB1 rs1128503	*T/T*			2A		
ABCB1 rs2032582	*T/T*					
CBR3 rs1056892	*G/G*	Increased risk of anthracycline-induced cardiomyopathy or reduced ejection fraction if cumulative anthracycline exposure is 1 to 250mg/m^2 [7-9]	Pediatric - Caucasian/European	2B	D	
COMT rs4646316 and *rs9332377*	*rs4646316T/T* and *rs9332377C/C*	Decreased risk of cisplatin-induced hearing impairment [17-22]	Pediatric - Caucasian/European	3	C/D	
CYP3A7 rs45446698 (**1C*)	**1/*1* and **1/*1C*	Lower urinary unconjugated estrogen metabolite levels and increased risk of mortality if treated with CYP3A substrates [24, 25]	Caucasian/European breast and lung cancer patients			
C8ORF34 rs1517114	*G/C* and *C/C*	Increased risk of irinotecan-induced grade 3 diarrhea	Asian advanced non–small-cell lung cancer patients	2B	D	
FCGR2A rs1801274	*G/G* and *A/G*	Increased risk of stable or progressive disease and more likely to have shorter progression-free survival following treatment of HER2 + breast cancer with trastuzumab-based therapy [27-29]		2B		
		Increased risk of stable or progressive disease and more likely to have shorter progression-free survival following treatment of lymphoma with rituximab-based therapy [30]				
FCGR3A rs396991	*C/C*	Decreased risk of stable or progressive disease and more likely to have longer progression-free survival following treatment of HER2$^+$ metastatic breast cancer with trastuzumab-based therapy [27-29]		2B	D	
		Decreased risk of stable or progressive disease and more likely to have longer progression-free survival following treatment of lymphoma with rituximab-based therapy [30-35]				
HAS3 rs2232228	*A/A* and *A/G*	Increased risk of anthracycline-induced cardiomyopathy or reduced ejection fraction if cumulative anthracycline exposure is 1 to 450mg/m^2 [10]	Pediatric - Caucasian/European	2B	D	
NT5C2 rs11598702	*A/A*	Decreased clearance of intravenous gemcitabine [36, 37]	Caucasian solid tumor patients	2B	D	

NUDT15 rs116855232 and *rs186364861* (**3* and **5*)	**1/*3*, **1/*5*, **3/*3*, **5/*5*, and **3/*5*	Increased risk of thiopurine-induced toxicity, including early leukopenia, neutropenia, alopecia totalis, pancytopenia, and treatment discontinuation		1B	A/B	Mercaptopurine and Thioguanine
SBF2 rs7102464	*T/T* and *C/T*					
SBF2 rs146987383	*C/C* and *G/C*					
SBF2 rs141368249	*A/A* and *G/A*	Increased risk of taxane-induced peripheral neuropathy [38]	African American			
SBF2 rs117957652	*C/C* and *G/C*					
SBF2 rs149501654	*G/G* and *C/G*					
SEMA3C rs7779029	*C/C* and *T/C*	Increased risk of irinotecan-induced severe neutropenia [26]	Advanced non–small-cell lung cancer patients	2B	D	
SEMA3C rs11979430	*T/T* and *C/T*					
SLC16A5 rs4788863	*T/T* and *T/C*	Decreased risk of cisplatin-induced hearing impairment	Caucasian/European testicular cancer patients	3		
SLC28A3 rs885004	*A/A* and *G/A*	Decreased risk of anthracycline-induced cardiomyopathy or reduced ejection fraction [11-16]	Pediatric - Caucasian/European	2B	D	
SLC28A3 rs7853758	*A/A* and *G/A*					
SOD2 rs4880	*C/C*	Increased risk of asparaginase-induced hepatotoxicity	Hispanic or Caucasian/European acute lymphoblastic leukemia patients	3	D	
TLR4 rs4986790	*G/G* and *A/G*	Increased risk of methotrexate-induced gastrointestinal (nausea, vomiting, diarrhea and constipation), liver (elevated liver enzymes), pneumonitis, and skin and mucosal adverse events [39]		3		
TPMT rs12201199	*T/A* and *A/A*	Increased risk of cisplatin-induced hearing impairment	Pediatric – Caucasian/European	3		Cisplatin

†Several of these genetic variants have not been reviewed by PharmGKB or CPIC and have not been assigned a level of evidence.

Table 2: Assay results: intra- and inter-assay concordance, accuracy, precision, sensitivity, and specificity.

Gene	rs number	TaqMan Assay ID	Intra assay concordance	Inter assay concordance	Verified by Sanger sequencing	Accuracy	Robustness†	Analytical sensitivity	Analytical specificity
ABCB1	*rs1045642* *rs1128503* *rs2032582*	C___7586657_20 C___7586662_10 C_11711720C_30 and C_11711720D_40	100% (12 samples in triplicate)	100% (18 samples in triplicate)	yes	100% (12 samples)	yes	100% (95% CI; 93-100)	100% (95% CI; 83-100)
CBR3	*rs1056892*	C___9483603_10	100% (14 samples in triplicate)	100% (18 samples in triplicate)	yes	100% (11 samples)	yes	100% (95% CI; 68-100)	100% (95% CI; 78-100)
COMT	*rs4646316* *rs9332377*	C__29193982_10 C__29614343_10	100% (14 samples in triplicate)	100% (18 samples in triplicate)	yes	100% (11 samples)	yes	100% (95% CI; 77-100)	100% (95% CI; 89-100)
CYP3A7	*rs45446698* (**1C*)	C__30634320_10	100% (14 samples in triplicate)	100% (18 samples in triplicate)	no	100% (96 samples)	yes	100% (95% CI; 51-100)	100% (95% CI; 98-100)
C8orf34	*rs1517114*	C___8341581_20	100% (7 samples in triplicate)	100% (7 samples in triplicate)	yes	100% (16 samples)	yes	100% (95% CI; 86-100)	100% (95% CI; 68-100)
FCGR2A	*rs1801274*	C___9077561_20	100% (14 samples in triplicate)	100% (18 samples in triplicate)	yes	100% (9 samples)	yes	100% (95% CI; 70-100)	100% (95% CI; 70-100)
FCGR3A	*rs396991*	C__25815666_10	100% (14 samples in triplicate)	100% (18 samples in triplicate)	yes	100% (10 samples)	yes	100% (95% CI; 72-100)	100% (95% CI; 90-100)
HAS3	*rs2232228*	C___3283947_1_	100% (14 samples in triplicate)	100% (18 samples in triplicate)	yes	100% (12 samples)	yes	100% (95% CI; 68-100)	100% (95% CI; 81-100)
NT5C2	*rs11598702*	C__11196884_20	100% (7 samples in triplicate)	100% (7 samples in triplicate)	yes	100% (16 samples)	yes	100% (95% CI; 61-100)	100% (95% CI; 87-100)
NUDT15	*rs116855232* (**3*) *rs186364861* (**5*)	C_154823200_10 C_181955856_10	100% (7 samples in triplicate)	100% (7 samples in triplicate)	yes	100% (24 samples)	yes	100% (95% CI; 65-100)	100% (95% CI; 96-100)
SBF2	*rs7102464* *rs146987383* *rs141368249* *rs117957652* *rs149501654*	C__29019176_10 C_161447122_10 C_161190467_10 C_152435684_10 C_161562183_10	100% (14 samples in triplicate)	100% (18 samples in triplicate)	yes	100% (12 samples and an additional 9 samples from another laboratory)	yes	100% (95% CI; 81-100)	100% (95% CI; 98-100)
SEMA3C	*rs7779029* *rs11979430*	C____334680_10 C___2621121_10	100% (14 samples in triplicate)	100% (18 samples in triplicate)	yes	100% (12 samples)	yes	100% (95% CI; 78-100)	100% (95% CI; 89-100)
SLC16A5	*rs4788863*	C____156080_10	100% (7 samples in triplicate)	100% (7 samples in triplicate)	yes	100% (16 samples)	yes	100% (95% CI; 85-100)	100% (95% CI; 72-100)
SLC28A3	*rs885004* *rs7853758*	C___2752627_10 C___1820227_30	100% (14 samples in triplicate)	100% (18 samples in triplicate)	yes	100% (11 samples)	yes	100% (95% CI; 70-100)	100% (95% CI; 90-100)
SOD2	*rs4880*	C___8709053_10	100% (7 samples in triplicate)	100% (7 samples in triplicate)	yes	100% (8 samples)	yes	100% (95% CI; 44-100)	100% (95% CI; 77-100)
TLR4	*rs4986790*	C__11722238_20	100% (14	100% (18	yes	100% (12 samples)	yes	100% (95%	100% (95%

			samples in triplicate)	samples in triplicate)				CI; 21-100)	CI; 86-100)
TPMT	*rs12201199*	C__31923406_10	100% (7 samples in triplicate)	100% (7 samples in triplicate)	yes	100% (16 samples)	yes	100% (95% CI; 70-100)	100% (95% CI; 86-100)

†Robustness means obtaining the same genotyping result using two different instruments on different days and using input DNA within a concentration range of 15.4 to 50.8ng/µL

Table 3: Primer sequences and PCR amplification conditions for validated genetic variants before performing verification by Sanger sequencing.

Gene	rs number	HGVS nomenclature	Sequence accession number	Forward primer sequence	Reverse primer sequence	PCR annealing temperature (°C)	PCR product/amplicon length (bp)
ABCB1	*rs1045642*	*c.3435T>C* or *p.Ile1145=*	NM_000927.4 or NP_000918.2	5'-ACTCTTGTTTTCAGCTGCTTG-3' [41]	5'-AGAGACTTACATTAGGCAGTGACTC-3' [41]	63	231
	rs1128503	*c.1236T>C* or *p.Gly412=*		5'-TGTGTCTGTGAATTGCCTTGAAG-3' [42]	5'-CCTCTGCATCAGCTGGACTGT-3' [42]	63	149
	rs2032582	*c.2677T>G/A* or *p.Ser893Ala/Thr*		5'-ATGGTTGGCAACTAACACTGTTA-3' [42]	5'-AGCAGTAGGGAGTAACAAAATAACA-3' [42]	63	208
CBR3	*rs1056892*	*c.730G>A* or *p.Val244Met*	NM_001236.3 or NP_001227.1	5'-CCAGGACCAGTGAAGACAGA-3'	5'-CCGAAGCAGACGTTTACCAG-3'	63	166
COMT	*rs4646316*	*c.615+310C>T*	NM_000754.3	5'-ACACGCTTCTCTTGGAGGTG-3'	5'-CTGTCTAGCCTCACTCGGG-3'	63	519
	rs9332377	*c.616-367C>A/T*		5'-GCTTGTTGATGGGAGGTCTG-3'	5'-TCCCTTAGAACAGCATGTGG-3'	61	217
C8ORF34	*rs1517114*	*c.736+8162C>G/T/A*	NM_052958.2	5'-CTGTGCTTTCTCGTCTTCAG-3'	5'-CAGCCTGGAACCTACCCTTG-3'	58	238
FCGR2A	*rs1801274*	*c.500A>G* or *p.His167Arg*	NM_001136219.1 or NP_001129691.1	5'-CAAGCCTCTGGTCAAGGTCA-3'	5'-AAGGATTCCCCTTAGCCCCT-3'	58	663
FCGR3A	*rs396991*	*c.841T>C/G* or *p.Phe281Leu/Val*	NM_000569.7: or NP_000560.6	5'-CACATATTTACAGAATGGCAAAGG-3'	5'-GATTCTGGAGGCTGGTGCTACA-3'	58	969
HAS3	*rs2232228*	*c.279A>G* or *p.Ala93=*	NM_001199280.1 or NP_001186209.1	5'-GTGACGGGCTACCAGTTCAT-3'	5'-CACAACCCAAGGGACCTAGA-3'	58	654
NT5C2	*rs11598702*	*c.175+1178A>G/C*	NM_012229.4	5'-GACGGGTTTATAGGTGCAGC-3'	5'-TCAATGACTTCTTGCCCAGT-3'	58	222
NUDT15	*rs116855232* (*3)	*c.415C>T* or *p.Arg139Cys*	NM_018283.3 or NP_060753.1	5'-GCCTTTGTAAACTGGGCTTC-3'	5'-CAAATCTTCTCGGCCACCTA-3'	58	411
	rs186364861 (*5)	*c.52G>A* or *p.Val18Ile*		5'-CATTCCCCAACCTGATAGCC-3'	5'-CAACCGAGCCTTTCCTCTTC-3'	58	296
SBF2	*rs7102464*	*c.2035G>A* or *p.Glu679Lys*	NM_030962.3 or NP_112224.1	5'-ACAGAAACTTGCCCCTGGAG-3'	5'-ACCCAAATACACTGGCAGGA-3'	63	289
	rs146987383	*c.2050C>G* or *p.Leu684Val*		5'-ACAGAAACTTGCCCCTGGAG-3'	5'-ACCCAAATACACTGGCAGGA-3'	63	289
	rs141368249	*c.2081C>T* or *p.Ala694Val*		5'-ACAGAAACTTGCCCCTGGAG-3'	5'-ACCCAAATACACTGGCAGGA-3'	63	289
	rs117957652	*c.3292C>G/T* or *p.Leu1098Val/=*		5'-CCTGTCTTGGTGTAAGAGTCTTCT-3'	5'-ACCTCTTTTTGGAGCCCACT-3'	63	843
	rs149501654	*c.4111G>C* or *p.Val1371Leu*		5'-TCTTCATCCGCAGAACTTCA-3'	5'-AGTGTGCCTTTGGTGGGTAG-3'	63	649
SEMA3C	*rs7779029*	*c.103+13883A>G*	NM_006379.3	5'-GGCTTAGGTCTCTGCCCTTT-3'	5'-GTTCCCATTTCCAGGCTCCA-3'	58	200
	rs11979430	*c.103+36739G>A*		5'-GGAAAGGGCAGACTGTGGTA-3'	5'-ACCAAACCTCTTCAGGGTGA-3'	58	383
SLC16A5	*rs4788863*	*c.121T>C* or *p.Leu81=*	NM_004695.3 or NP_004686.1	5'-AGGTCCCCCTGTTGACTTCT-3'	5'-TGAAATCTGGTGAAACCTTAGGA-3'	58	725
SLC28A3	*rs885004*	*c.862-360C>T*	NM_022127.2	5'-TGTGTCTGCCATCCAGTAGG-3'	5'-CCTGGTGCTAAAAAGACATGG-3'	58	161

	rs7853758	*c.1381C>T p.Leu461=*	or	NM_022127.2 NP_071410.1	or	5'-CCCCTGACAACTCCTTGGTA-3'	5'-CAGGGGCGTGATGTGATTAT-3'	58	239
SOD2	*rs4880*	*c.47T>C p.Val16Ala*	or	NM_000636.3 NP_000627.2	or	5'-CTGTGCTTTCTCGTCTTCAG-3'	5'-CAGCCTGGAACCTACCCTTG-3'	58	238
TLR4	*rs4986790*	*c.776A>G p.Asp299Gly*	or	NM_003266.3 NP_612564.1	or	5'-AGTCCATCGTTTGGTTCTGG-3'	5'-TGCCATTGAAAGCAACTCTG-3'	58	635
TPMT	*rs12201199*	*c.419+94T>A*		NM_000367.3		5'-GTTCTTCGGGGAACATTTCA-3'	5'-AAGTGATTGAGCCACAAGCC-3'	58	975

Accession numbers are available from https://www.ncbi.nlm.nih.gov/snp, last accessed 2/1/2019.

Table 4: Genotype frequencies for validated genetic variants.

Gene and rs number	HGVS nomenclature	Genotype	N	Genotype frequencies
ABCB1 rs1045642	*c.3435T>C or p.Ile1145=*	*T/T*	34	0.18
		T/C	84	0.44
		C/C	71	0.38
ABCB1 rs1128503	*c.1236T>C or p.Gly412=*	*T/T*	28	0.15
		T/C	88	0.47
		C/C	73	0.39
ABCB1 rs2032582	*c.2677T>G/A or p.Ser893Ala/Thr*	*T/T*	33	0.17
		T/G	70	0.37
		G/G	86	0.46
CBR3 rs1056892	*c.730G>A or p.Val244Met*	*G/G*	65	0.34
		G/A	101	0.53
		A/A	23	0.12
COMT rs4646316	*c.615+310C>T*	*C/C*	117	0.62
		C/T	57	0.30
		T/T	15	0.08
COMT rs9332377	*c.616-367C>A/T*	*C/C*	125	0.66
		C/T	59	0.31
		T/T	5	0.03
*CYP3A7 rs45446698 (*1C)*	*c.-232A>C*	*A/A*	184	0.97
		A/C	4	0.02
		C/C	1	0.01
C8orf34 rs1517114	*c.736+8162C>G/T/A*	*C/C*	22	0.12
		C/G	81	0.43
		G/G	86	0.46
FCGR2A rs1801274	*c.500A>G or p.His167Arg*	*A/A*	59	0.31
		A/G	94	0.50
		G/G	36	0.19
FCGR3A rs396991	*c.841T>C/G or p.Phe281Leu/Val*	*T/T*	78	0.41
		T/G	94	0.50
		G/G	17	0.09
HAS3 rs2232228	*c.279A>G or p.Ala93*	*A/A*	81	0.43
		A/G	87	0.46
		G/G	21	0.11
NT5C2 rs11598702	*c.175+1178A>G/C*	*A/A*	102	0.54
		A/G	75	0.40
		G/G	12	0.06
*NUDT15 rs116855232 (*3)*	*c.415C>T or p.Arg139Cys*	*C/C*	183	0.97
		C/T	6	0.03
		T/T	0	0.00
*NUDT15 rs186364861 (*5)*	*c.52G>A or p.Val18Ile*	*G/G*	188	0.99
		G/A	1	0.01
		A/A	0	0.00
SBF2 rs7102464	*c.2035G>A or p.Glu679Lys*	*G/G*	168	0.89
		G/A	18	0.10
		A/A	3	0.01
SBF2 rs146987383	*c.2050C>G or p.Leu684Val*	*C/C*	0	0.00
		C/G	0	0.00
		G/G	189	1.00
SBF2 rs141368249	*c.2081C>T or p.Ala694Val*	*C/C*	4	0.02
		C/T	1	0.01
		T/T	184	0.97
SBF2 rs117957652	*c.3292C>G/T or p.Leu1098Val/=*	*C/C*	0	0.00
		C/G	3	0.02
		G/G	186	0.98
SBF2 rs149501654	*c.4111G>C or p.Val1371Leu*	*G/G*	0	0.00
		G/C	0	0.00
		C/C	189	1.00
SEMA3C rs7779029	*c.103+13883A>G*	*A/A*	134	0.71
		A/G	44	0.23
		G/G	11	0.06
SEMA3C rs11979430	*c.103+36739G>A*	*G/G*	143	0.76
		G/A	39	0.21
		A/A	7	0.03
SLC16A5 rs4788863	*c.121T>C or p.Leu81=*	*T/T*	24	0.13

		T/C	71	0.38
		C/C	94	0.49
SLC28A3 rs885004	*c.862-360C>T*	*C/C*	143	0.76
		C/T	44	0.23
		T/T	2	0.01
SLC28A3 rs7853758	*c.1381C>T or p.Leu461=*	*C/C*	123	0.65
		C/T	56	0.30
		T/T	10	0.05
SOD2 rs4880	*c.47T>C or p.Val16Ala*	*T/T*	86	0.46
		T/C	62	0.33
		C/C	41	0.21
TLR4 rs4986790	*c.776A>G or p.Asp299Gly*	*A/A*	176	0.93
		A/G	13	0.07
		G/G	0	0.00
TPMT rs12201199	*c.419+94T>A*	*T/T*	18	0.10
		T/A	26	0.14
		A/A	143	0.76