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Characterization of Reference Materials for *CYP3A4* and *CYP3A5*: A GeT-RM Collaborative Project

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Abstract

Pharmacogenetic testing for *CYP3A4* is increasingly provided by clinical and research laboratories; however, only a limited number of quality control and reference materials are currently available for many of the *CYP3A4* variants included in clinical tests. To address this need, the Division of Laboratory Systems, Centers for Disease Control and Prevention (CDC) based Genetic Testing Reference Material Coordination Program (GeT-RM), in collaboration with members of the pharmacogenetic testing and research communities and the Coriell Institute for Medical Research, has characterized 30 DNA samples derived from Coriell cell lines for *CYP3A4*. Samples were distributed to five volunteer laboratories for genotyping using a variety of commercially available and laboratory developed tests. Sanger and next generation sequencing were also utilized by some of the laboratories. Whole genome sequence (WGS) data from the 1000 Genomes Projects was utilized to inform genotype. Twenty *CYP3A4* alleles were identified in the 30 samples characterized for *CYP3A4*: *CYP3A4*4, *5, *6, *7, *8, *9, *10, *11, *12, *15, *16, *18, *19, *20, *21, *22, *23, *24, *35,* and a novel allele, *CYP3A4*38*. Nineteen additional samples with preexisting data for *CYP3A4* or *CYP3A5* were re-analyzed to create comprehensive reference material panels for these genes. These publicly available and well characterized materials can be used to support the quality assurance and quality control programs of clinical laboratories performing clinical pharmacogenetic testing.

Introduction

The *CYP3A4* and *CYP3A5* genes on chromosome 7 encode two important enzymes in the Cytochrome P450 3A subfamily. CYP3A4 is involved in the metabolism of approximately 30–64% of clinically prescribed drugs[1–3] while CYP3A5 contributes to the metabolism of 3% of the top 200 most prescribed drugs and 10% of FDA approved drugs (2005–2016).[2] Among the many pharmaceuticals metabolized by these two enzymes are tacrolimus, cyclosporine and statins which have been thoroughly investigated[4], as well as fentanyl, midazolam, quetiapine and paclitaxel. Of importance, there is considerable substrate overlap meaning that both enzymes contribute to the metabolism of many drugs to various extents. Additional information regarding drugs metabolized by CYP3A4 and

CYP3A5, drug label, clinical annotations, and pathways can be found on PharmGKB (https://www.pharmgkb.org/, last accessed 11/25/2022).

As with all pharmacogenes, genetic polymorphisms in *CYP3A4* and *CYP3A5* account for significant inter-individual variation in enzyme activity that can affect how patients respond to drugs metabolized by these enzymes. Clinical genetic testing laboratories offer tests that can detect specific variants in pharmacogenetic genes, which can be used to predict or explain an individual's response to certain drugs. Physicians can use the results of pharmacogenetic tests to select an appropriate drug and dose for each patient to ensure effective treatment and avoid adverse drug reactions.

To address the lack of standardization of pharmacogenetic test panels, the Association for Molecular Pathology (AMP) Pharmacogenetic Working Group has developed a series of documents that recommend a minimum set of variant alleles to include in clinical pharmacogenetic test panels.[5–9] Most recently, the workgroup has developed recommendations[10] for clinical *CYP3A4* and *CYP3A5* testing. The AMP Pharmacogenetic Workgroup has established four criteria that alleles must meet to be recommended for inclusion in clinical assays. One of these criteria is the availability of reference materials.

To support development of the new *CYP3A4* and C*YP3A5* AMP guidelines, the Division of Laboratory Systems, Centers for Disease Control and Prevention (CDC) based Genetic Testing Reference Material Coordination Program (GeT-RM), the Coriell Institute for Medical Research, and the genetic testing community have collaborated to characterize genomic DNA samples from 30 publicly available cell lines for *CYP3A4* for use as reference materials for clinical testing. In addition, nine samples previously characterized by GeT-RM for *CYP3A5*[11] and ten for *CYP3A4* underwent additional studies to create comprehensive reference material panels for both *CYP3A4* and *CYP3A5* testing.

Materials and Methods

Cell Line DNA and Participating Laboratories

The goal of this GeT-RM study was to create characterized genomic DNA reference materials for as many of the *CYP3A4* alleles that are defined by the Pharmacogene Variation (PharmVar) Consortium and listed on the PharmVar *CYP3A4* gene page (https://www.pharmvar.org/gene/CYP3A4 last accessed 5/15/2023) as possible. DNA from 30 cell lines were selected from the National Institute of General Medical Sciences (NIGMS) Human Genetic Cell Repository and the National Human Genome Research Institute (NHGRI) Sample Repository for Human Genetic Research at the Coriell Institute for Medical Research (Camden, NJ) based on data supplied by the authors or identified by searching the 1000 Genomes Project samples using the Ensemble browser (https://useast.ensembl.org/index.html, last accessed 5/4/2022) for variants in *CYP3A4*. Five laboratories, utilizing a variety of methods and test platforms, participated in this effort: Children's Mercy Research Institute (CMRI, Laboratory 1), RPRD Diagnostics (RPRD, Laboratory 2), Erasmus University Medical Center (Erasmus MC, Laboratory 3), Mayo Clinic (Mayo, Laboratory 4), and Indiana University (IU, Laboratory 5). Eight samples from a previous GeT-RM study[11] (NA12717, NA24008, NA23313, NA07056, NA06993,

HG00276, NA12006, and NA07439) were retested for *CYP3A4* variants, and two samples (NA19160 and HG01269), one having an allele that was not tested in the original panel[11] and one with a rare genotype of interest, were added at a later stage of the project and tested by laboratories 1 and 3. For *CYP3A5*, two laboratories also re-analyzed samples from the previous GeT-RM study to assure methods are accurately identifying their respective genotypes.

DNA Preparation

DNA was prepared from each of the selected cell lines by the Coriell Institute for Medical Research using Gentra/Qiagen Autopure (Valencia, CA) per manufacturer's instructions.

Characterization Protocol

Laboratories 1–5 received one 10 μg aliquot of DNA from 30 cell lines and tested all or a subset of the samples using their standard methods and/or additional methods as needed to resolve inconclusive genotype calls. Laboratories 1, 2 and 3 purchased or used previously purchased DNA from the eight previously tested lines. DNA from the other two cell lines, NA19160 and HG01269, was purchased by Laboratories 1 and 3. The testing platforms and genotyping assays used in the study are described below and in Table 1. Results were submitted to LVK and AG for examination of the data for quality, discordances, and determination of consensus genotype. If discordances were noted, the participating laboratories were asked to re-evaluate the data in question and determine the cause of the inconsistency.

Allele Designations and Diplotype Reporting

CYP3A4 allele designations are according to those described by PharmVar (https://www.pharmvar.org/gene/CYP3A4; last accessed 1/25/2023).[12–15] Variant positions are provided throughout this manuscript according to HGVS using NM_017460.6 as a reference sequence. For Human Genome Variation Society nomenclature throughout, see https://www.ncbi.nlm.nih.gov/snp (last accessed 6/2/2023).

Laboratory 1 (CMRI)

Sanger Sequencing—Sanger sequencing was performed on exons WGS data showed to harbor variants of interest. PCR primers were designed to amplify exons 1, 4 through 6, 7 and 10 (including adjacent intronic sequences) and obtained from Integrated DNA Technologies, Coralville, IA. Each 8 μL reaction contained 15 ng of genomic DNA (Coriell Institute, Camden, NJ), 1x KAPA LongRange HotStart ReadyMix™ with dye (Roche Holding AG, Basel, Switzerland), 5% DMSO, forward and reverse primers each at 0.5 μM, and molecular grade water. Reactions were cycled using the following conditions: initial denaturation, 94°C for 3 min followed by 35 cycles at 94°C for 20 sec, annealing for 30 sec at 60°C (amplicons harboring exon 1, exon 7 and exon 10) or 68°C (amplicons harboring exons 4–6), and extended at 68°C for 4 min, and a final hold at 4°C. PCR amplification was verified by agarose gel electrophoresis. PCR fragments for exon 1 (181 bp), exon 7 (395 bp) and exon 10 (415 bp) were purified using a ExoSAP-IT™ or Exo-SAP-IT™ Express PCR Product Cleanup kit (Applied Biosystems, Waltham, MA) per manufacture's protocol

while the 3 kb fragment encompassing exons 4–6 was purified with a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). Regions harboring variants of interest were sequenced using two different primers. PCR templates were Sanger sequenced using BigDye Terminator version 3.1 chemistry and a capillary 3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA). Sequence traces were aligned and analyzed using Sequencher Software 5.4.6 (Gene Codes Corporation, Ann Harbor, MI). NG_008421.1 was used as the reference sequence for alignments. Sequencing primers are provided in Table 2.

TaqMan™ genotyping—Genotyping for NM_017460.6:c.830dup defining *CYP3A4*6* was performed using a pre-designed TaqMan™ genotyping assay (C__32787140_40) in a standard 96-well (0.1mL) reaction format. Each 6.0 μL reaction contained 1.0 μL DNA (15 ng/μL) and 1x TaqMan™ Genotyping Master Mix or TaqPath™ ProAmp™ Master Mix (Applied Biosystems™, Waltham, MA). Cycling was performed per manufacturer's recommendations. Cycling and analysis was performed on a QuantStudio™ 12K Flex Real-Time PCR System with QuantStudio™ 12K Flex Software (v1.3) (Thermo Fisher Scientific, Waltham, MA).

Next Generation Sequencing—Variant data were retrieved from multiple next-generation sequencing data sets including WGS from the 1000 Genomes Project (1K-WGS) (https://www.internationalgenome.org/data-portal/data-collection/30x-grch38, last accessed 7–29-2022).[16] In addition, data were obtained from a targeted gene panel (ADMEseq) which was previously described in detail.[17] Variant lists were created from the aforementioned datasets using a combination of bcftools software version 1.14, the Genome Analysis Toolkit (GATK) software version 3.8 and Variant Effect Predictor (VEP) software version 105.[18–20]

Determination of variant phase—If more than two heterozygous variants were present in a sample's diplotype, variant phase, i.e., whether variants are in *cis* (same allele) or *trans* (opposite allele) was determined via inheritance using 1K-WGS data of trios. For one sample, HG00139, the phase of two variants, NM_017460.6:c.1334T>C and NM_017460.6:c.1088C>T, was experimentally determined by first amplifying a 3.6 kb long *CYP3A4*-specific amplicon, then the template was used for nested allele-specific PCR and Sanger sequencing. Primer sequences used for amplification and Sanger sequencing are provided in Table 2.

Laboratory 2 (RPRD)

Genotyping was performed as previously described using the PharmacoScan™ Assay Kit, catalog ID 903010 (Thermo Fisher Scientific, Waltham, MA) following manufacturer's instructions.[21] Arrays were hybridized, stained with a fluorescent antibody, and scanned on the GeneTitan™ Multi-Channel (MC) Instrument (Thermo Fisher Scientific, Waltham, MA). Data were analyzed using the Axiom™ Analysis Suite 5.1.1.1 (Thermo Fisher Scientific, Waltham, MA). Genotype calls were made using the commercially released allele translation table (r9). Variants tested by the PharmacoScan platform are summarized in Table 1. Additionally, variant calls for NM_017460.6:c.1026+12G>A (rs2242480) were retrieved; this variant is interrogated by the PharmacoScan™ Assay but is not used for genotype calling using the current translation table (r9).

Laboratory 3 (Erasmus MC)

AutogenomicsBioFilmChip microarray—DNA samples were analyzed for *CYP3A4* and *CYP3A5* using the AutogenomicsBioFilmChip Microarray CYP450 3A4–3A5 Plus assay (ID 03–9520-00) on an INFINITY HT AutoGenomics platform (Autogenomics, Carlsbad, CA), according to the manufacturer's instructions. Variants tested by this microarray are summarized in Table 1.

Laboratory 4 (Mayo Clinic)

DNA samples were analyzed by Sanger sequencing or by TaqMan™ allele discrimination assays in a custom-designed Open Array™ format (Thermo Fisher Scientific, Waltham, MA) on a QuantStudio™ 12K Flex instrument. Genotyper software, version 1.2.2 (Thermo Fisher Scientific) and a custom-designed proprietary software, GINger version 1.0 (Mayo Clinic, Rochester, MN), were used to analyze TaqMan™ assay results. The TaqMan-based chemistry was designed to detect *CYP3A4*8*, **11*, **12*, **13*, **16*, **17*, **18*, **22*, and **26* alleles (Table 1).

Sanger sequencing was performed for selected exons and c.1026+12G>A in intron 10 using BigDye Terminator chemistry v1.1 and an ABI 3500xl DNA Analyzer (Thermo Fischer, Waltham, MA). Primer sequences are provided in Table 2. Mutation Surveyor (Soft Genetics, State College, PA) was used for analysis. NM_017460.5 was used as the reference sequence for *CYP3A4* for both genotyping and sequence analysis.

Laboratory 5 (IU)

DNA from two samples (NA18603 and HG02029) was sequenced to evaluate all coding regions (exons) of the *CYP3A4* gene. Sanger sequencing was performed using BigDye™ Terminator v3.1 and a 3500xL Analyzer instrument according to the manufacturer's protocols (Thermo Fisher Scientific, Waltham, MA). Primers (Table 2) were designed on Primer3web v4.1.0 (https://primer3.ut.ee/ last accessed 5/15/2023) and provided by Integrated DNA Technologies (Coralville, IA). Data was analyzed using Mutation Surveyor V4.0.7 software (SoftGenetics, State College, PA). NM_017460.6 was used as the reference for sequence analysis.

Results

DNA from the 30 selected cell lines was tested by laboratories for *CYP3A4* using a variety of genotyping and sequencing methods. Previously reported sequence data was also analyzed. Laboratories performed testing and shared data for an additional ten samples if available. The results from all assays/tests used to determine the consensus genotypes are summarized in Supplemental Table 1. Consensus genotypes were determined based on the compiled test results across laboratories and datasets. Each consensus genotype was identified in at least two laboratories. The *CYP3A4* genotype results were consistent among the samples tested and all differences in genotype calls were attributable to laboratories not testing for each star allele. For example, Laboratory 3 did not test for *CYP3A4*10* (NM_017460.6:c.520G>C), which was identified in two samples (HG00122 and HG00734) by Laboratory 2 using the PharmacoScan platform and Laboratories 1 and 4 using Sanger

sequencing. This allele call was also consistent with calls made using WGS data. Similarly, *CYP3A4 *4, *7, *8, *11, *15, *16, *23, *24, *28*, and **35* were not identified by Laboratory 3 because the Autogenomics BioFilmChip microarray used was not designed to detect these variants, and thus were defaulted as *CYP3A4*1*. The identifying variants of these alleles were confirmed by Sanger sequencing and WGS; several samples were also further confirmed by ADMEseq, a targeted NGS panel. *CYP3A4*28* and **35* were also not detected by the PharmacoScan platform and thus resulted in **1* default assignments. Sample NA19160, which is heterozygous for the *CYP3A4*24* allele, was not tested by laboratory 2 but would be expected to also cause a **1* default call as its identifying variant is not interrogated by the PharmacoScan platform. Variant phasing for some samples was performed by Laboratory 1, but not by other laboratories in the study.

Sample HG00139 was initially called *CYP3A4*3/*11* due to NM_017460.6:c.1088C>T (defining *CYP3A4*11*) and NM_017460.6:c.1334T>C (defining *CYP3A4*3*) being heterozygous. However, there was no trio information available to confirm that c.1088C>T and c.1334T>C are indeed in *trans* in this sample. Utilizing allele-specific PCR and Sanger sequencing revealed that the two variants were not in *trans* as expected, but in *cis* forming a novel haplotype, *CYP3A4*38*. Therefore, the consensus genotype call for this sample was revised to *CYP3A4*1/*38*.

Another recently discovered allele, *CYP3A4*37*, also has NM_017460.6: c.1334T>C (defining *CYP3A4*3*) in combination with the *CYP3A4*22*-defining variant NM_017460.6:c.522–191C>T.[22] The discovery of *CYP3A4*37* raised concerns regarding samples heterozygous for both c.1334T>C and c.522–191C>T as their genotype could either be **3/*22* or **1/*37* (Supplemental Table 1, results laboratory 3). Another compound heterozygous sample, HG01269, was discovered among the 1000 Genomes WGS data. In this case the phase of c.1334T>C and c.522–191C>T could not be ascertained due to the lack of trio information. Experimental phasing was not attempted because these variants are almost 13 kb apart. Thus, the genotype of HG01269 remains ambiguous: *CYP3A4*1/*37* or **3/*22*.

As a consequence of c.1334T>C not only occurring in *CYP3A4*3* but also **37* and **38*, patients heterozygous for c.1334T>C and either c.522–191C>T or c.1088C>T may require further testing to discriminate *CYP3A4*1/*37* from **3/*22* and **1/*38* from **3/*11*, respectively. It remains unknown whether these respective alternate genotypes convey clinically relevant enzyme activity.

Sample NA19160 was heterozygous for NM_017460.6:c.600A>T (p.Gln200His) and called *CYP3A4*1/*24* while HG00452 was heterozygous for c.600A>G (p.Gln200=). This nucleotide position is triallelic (A>T or A>G, rs113667357). HG00452 was also heterozygous for NM_017460.6:c.878T>C and determined to be *CYP3A4*1/*18* because c.600A>G and c.878T>C are in *trans* (the allele with NM_017460.6:c.600A>G being a novel **1* suballele, **1.009*). This sample may be valuable to ascertain assay specificity, i.e., discriminating c.600A>G from c.600A>T.

Sample NA18941 was the only sample determined to have a *CYP3A4*6* allele by WGS, Sanger sequencing TaqMan™ genotyping, and PharmacoScan. However, the AutogenomicsBioFilmChip microarray used by Laboratory 3 repeatedly produced a no-call for this allele. In retrospect, the reference to variant signal ratio (-/A) was clearly distinct from the ratios observed for all other samples in this study. The *CYP3A4*6* allele was also missed when interrogating this sample on the PharmacoFocus platform (Thermo Fisher Scientific, Waltham, MA), which was not part of the study. Subsequent NGS analysis performed by RESULT laboratory (Dordrecht, The Netherlands) did, however, confirm the presence of the *CYP3A4*6* allele in this sample, thus excluding sample mix up.

This study also generated information for a variant located in intron 10, NM_017460.6:c.1026+12G>A. This common variant defines the *CYP3A4*1G* suballele but is also found on numerous other haplotypes. PharmVar redesignated *CYP3A4*1G* as **36* but recently retired this allele due to the large body of inconsistent findings regarding associations between c.1026+12G>A and CYP3A4 activity. Supplemental Table 2 details each sample's c.1026+12G>A genotype and indicates to which allele the variant was phased. While clinical tests typically do not interrogate or report c.1026+12G>A, this information may be valuable for future investigations that examine the functional impact of the variant. Furthermore, the AMP working group did not recommend this allele for clinical allele testing due to the uncertainty regarding its function.[10] Therefore, the consensus *CYP3A4* genotypes summarized in Table 3 and Supplemental Table 1 do not include c.1026+12G>A and are shown per current PharmVar *CYP3A4* allele definitions (https://www.pharmvar.org/gene/CYP3A4; last accessed 1/25/2023).

Finally, eight samples (NA07439, NA12717, NA24008, NA23313, NA07056, NA06993, HG00276, and NA12006) that were characterized during a previous GeT-RM study[11] were retested with the more comprehensive assays used in this study. All genotypes were consistent with those determined earlier (Table 3 and Supplemental Table 1); information regarding c.1026+12G>A can be retrieved from Supplemental Table 2.

The *CYP3A5* diplotypes of nine samples determined in a previous Get-RM study[11] were reevaluated by WGS and ADMEseq (n=9) and PharmacoScan Array testing (n=7). Notably, a *CYP3A5*3* allele found in sample HG00436 is a **3.005* suballele which contains the NM_017460.6:c.432+2T>C variant that defined the now retired *CYP3A5*5* allele. PharmVar also retired the *CYP3A5*2* and **4* alleles after finding that their respective defining variants were always in *cis* with NM_017460.6:c.219–237A>G, the variant defining *CYP3A5*3* allele (see the PharmVar CYP3A5 GeneFocus review[23] for additional details). These findings did not change the diplotypes determined during the previous study.[11]

Table 3 lists the *CYP3A4* consensus genotype calls of the 40 samples that were characterized in this study (n=32 newly characterized in this study, eight previously tested[11] for *CYP3A4*). Publicly available reference materials have been created for the following alleles: *CYP3A4*2, *3, *4, *5, *6, *7, *8, *9, *10, *11, *12, *15, *16, *18, *19, *20, *21, *22, *23, *24, *28, *35,* and **38*. In addition, nine samples were reevaluated for *CYP3A5* to provide reference materials for *CYP3A5*3, *6,* and **7* (Table 4).

Discussion

Clinical laboratories often develop pharmacogenetic and other genetic tests as laboratory developed tests or procedures (LDT or LDP). Regulations, accreditation standards, and professional guidance requires clinical laboratories to use reference materials for assay development, validation, quality control, and proficiency testing[24–28] (American College of Medical Genetics and Genomics https://www.acmg.net/PDFLibrary/ACMG%20Technical%20Lab%20Standards%20Section%20G.pdf , last accessed 6/16/2022, Washington State Legislature, http://app.leg.wa.gov/WAC/default.aspx?cite=246-338-090, last accessed 6/16/2022, College of American Pathologists (Northfield, IL), New York State Clinical Laboratory Evaluation Program, https://www.wadsworth.org/regulatory/clep, last accessed 6/16/2022, MMWR https://www.cdc.gov/mmwr/preview/mmwrhtml/rr5806a1.htm, last accessed 6/16/2022). Despite the regulatory and professional guidelines requiring their use, there are few, if any, reference materials available for most clinical genetic tests including those for *CYP3A4* and *CYP3A5*. To address this need, the Centers for Disease Control and Prevention's Genetic Testing Reference Material Program (GeT-RM https://www.cdc.gov/labquality/get-rm/index.html last accessed 6/16/2022) has conducted a number of projects to create characterized and publicly available DNA samples for use as reference materials, including several for pharmacogenetic testing.[11, 21, 29–31]

The *CYP3A4* and *CYP3A5* clinical allele testing recommendations from the Association for Molecular Pathology[10] have created an urgent need for characterized reference materials. The reference materials developed as part of this study will not only provide important resources for quality control, proficiency testing, and research, but also support the development and validation of new pharmacogenetic tests and clinical guidelines.

The need for reference materials to validate assay/platform performance is underscored by the fact that the rare *CYP3A4*6* allele was not called by two platforms despite having signal ratios that were clearly distinct from those observed in all other samples. The difficulties in detecting and calling the *CYP3A4*6* allele may be due to the presence of an additional 'A' base (NM_017460.6:c.830dup), but other sample or assay-specific explanations, such as interference by the presence of another variant, cannot be ruled out. Identification and characterization of NA18941 will allow laboratories to re-evaluate their platforms to ensure that this exceedingly rare variant (global frequency of 0.0001806 per GnomAD http://www.gnomad-sg.org/; last accessed 12/30/2022) is indeed accurately called.

For *CYP3A4,* a recently published guideline by the Royal Dutch Pharmacists Association- Pharmacogenetics Working Group (DPWG) recommends that individuals having two *CYP3A4*22* alleles (poor metabolizers with substantially decreased CYP3A4 activity) should receive 30% of the standard dose of quetiapine (KNMP. CYP3A4: quetiapine; available at https://www.g-standaard.nl/risicoanalyse/B0005991.PDF last accessed 11/18/2022). For *CYP3A5*, CPIC[32] and DPWG guidelines recommend increasing the tacrolimus starting dose for normal and intermediate metabolizers (CYP3A5 expressers) to prevent organ rejection in patients receiving an organ transplant (https://www.pharmgkb.org/gene/PA131/prescribingInfo#guideline-annotations, last accessed 11/7/2022). While there is mounting evidence supporting the clinical utility

of testing *CYP3A4*22* to guide drug therapy, activity and/or clinical utility remain unknown or uncertain for most *CYP3A4* star alleles.[33] Measuring activity in an isoform-specific manner is not trivial as both, CYP3A4 and CYP3A5, often contribute to a drug's metabolism[33] as well as drug interactions (https://drug-interactions.medicine.iu.edu/home.aspx; last accessed 12/30/2022). Activity is also subject to complex multi-layer regulatory mechanisms that impact the expression levels of CYP3A4 and likely also CYP3A5[4, 34–37], making it extremely difficult to assess an allele's contribution to overall activity. In addition, uncertainty regarding the exact haplotype composition (i.e., whether variants are in *cis* or *trans*) further complicates accurate genotyping, which may lead to wrong or indeterminate phenotype assignments (see the PharmVar CYP3A4 https://www.pharmvar.org/gene/CYP3A4; last accessed 1/25/2023). Furthermore, some allelic variants are rare, hampering their characterization *in vivo*.

One example highlighting the challenges of determining allele function is an allele with a single intronic variant, NM_017460.6:c.1026+12G>A (defining the *CYP3A4*1G* allele), which also occurs on many other haplotypes (Supplemental Table 2). Although this variant has been extensively studied, the literature inconsistently associates it with both increased and decreased activity, making it impossible to define function. Since the impact of c.1026+12G>A on CYP3A4 function is controversial, PharmVar retired this allele in January 2023 after it was briefly re-designated as *CYP3A4*36*. Consequently, c.1026+12G>A was also removed from all star allele definitions. For future studies, investigators are encouraged to include c.1026+12G>A in carefully designed studies to determine its functional impact *in vivo* on drug metabolism and its utility as a biomarker.

The Database of Genomic Variants (DGV at http://dgv.tcag.ca/; last accessed 5/16/2023) and references therein indicate that copy number variation (CNV) can occur at the *CYP3A4* and *CYP3A5* gene loci. However, information regarding the nature and frequencies of such events are sparse.[38–40] A search for CNVs using the Progenetixs 1000 Genomes Germline CNVs tool (https://progenetix.org/progenetix-cohorts/oneKgenomes/; last accessed 5/16/2023) that interrogates the same WGS dataset utilized in this project to identify reference materials, did not detect any CNVs for *CYP3A4*. For *CYP3A5*, however, the tool revealed a partial 5245 bp-long gene deletion encompassing exons 11–13 in two related samples (HG02884 and HG02886, family ID GB89). Visualization of read coverage supports the presence of this partial deletion (data not shown). This deletion was not experimentally confirmed in this study, nor were any other study samples tested for CNVs. Given the rarity of CNVs and little published information regarding their nature, PharmVar has not designated star alleles with CNVs. As more is learned about CNVs in these genes and data becomes available PharmVar will consider designating such alleles and GeT-RM will continue to work with the pharmacogenetic testing community to expand the availability of reference materials to include newly defined variants.

In conclusion, the reference materials described in this report (Tables 3, 4 and Supplemental Table 1) will facilitate accurate clinical *CYP3A4* and *CYP3A5* testing and serve as materials for quality control processes. Together, these characterized genomic DNA samples form a comprehensive set of reference materials for testing of the *CYP3A4 and CYP3A5* genes, including alleles with confirmed clinical relevance. GeT-RM will continue to

work to establish cell lines and characterize additional variants in *CYP3A4, CYP3A5,* and other PGx genes that lack reference materials. All reference materials developed by GeT-RM are publicly available from the NIGMS and NHGRI repositories at the Coriell Institute for Medical Research (Camden, NJ). More information on this and other reference material characterization projects is available at the GeT-RM website: https://www.cdc.gov/labquality/get-rm/index.html last accessed 6/24/2022).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry. Use of tradenames and commercial sources is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention, the Public Health Service, or the US Department of Health and Human Services.

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Table 1.

Summary of platforms and genotyping assays used

Star allele	rsID#	Variant defining star allele position per NM_017460.6	Laboratory 2 PharmacoScan yes= allele tested	Laboratory 3 Autogenomics*	Laboratories 1# and 4 TaqMan™† assay ID
*CYP3A4*2*	rs55785340	c.664T>C	yes	yes	n/a
*CYP3A4*3*	rs4986910	c.1334T>C	yes	yes	n/a
*CYP3A4*4*	rs55951658	c.352T>C	yes	n/a	n/a
*CYP3A4*5*	rs55901263	c.653C>G	yes	n/a	n/a
*CYP3A4*6*	rs4646438	c.830dup	yes	yes	C__32787140_40#
*CYP3A4*7*	rs56324128	c.167G>A	yes	n/a	n/a
*CYP3A4*8*	rs72552799	c.389G>A	yes	n/a	Custom Design (AH6R7YP)
*CYP3A4*9*	rs72552798	c.508G>A	yes	n/a	n/a
*CYP3A4*10*	rs4986908	c.520G>C	yes	n/a	n/a
*CYP3A4*11*	rs67784355	c.1088C>T	yes	n/a	C__30634203_10
*CYP3A4*12*	rs12721629	c.1117C>T	yes	yes	C__30634202_10
*CYP3A4*13*	rs4986909	c.1247C>T	yes	n/a	C__29554474_10
*CYP3A4*15*	rs4986907	c.485G>A	yes	n/a	n/a
*CYP3A4*16*	rs12721627	c.554C>G	yes	n/a	C__30634207_10
*CYP3A4*17*	rs4987161	c.566T>C	yes	yes	C__27859822_10
*CYP3A4*18*	rs28371759	c.878T>C	yes	yes	C__27859823_20
CYP3A4*19	rs4986913	c.1399C>T	yes	n/a	n/a
*CYP3A4*20*	rs67666821	c.1461dup	yes	yes	n/a
*CYP3A4*22*	rs35599367	c.522–191C>T	yes	yes	C__59013445_10
*CYP3A4*23*	rs57409622	c.484C>T	yes	n/a	n/a
*CYP3A4*26*	rs138105638	c.802C>T	n/a	n/a	C_172781425_10

†TaqMan™ (Thermo Fisher Scientific, Waltham, MA)

*Autogenomics BioFilmChip Microarray CYP450 3A4–3A5 Plus assay (ID 03–9520-00) (Autogenomics, Carlsbad, CA)

#*CYP3A4*6* was genotyped by Laboratory 1 using TaqMan™ (Assay ID: C__32787140_40)

n/a, assay not performed

Star allele defining variants and their respective rsIDs are per the PharmVar *CYP3A4* gene page at https://www.pharmvar.org/gene/CYP3A4 (last accessed 2/1/2023).

Table 2.

Primers used for *CYP3A4* PCR Amplification and Sanger sequencing

Exon	Sequence (5’ to 3’)
	Laboratory 1
1	F: 5’-CACATAGCCCAGCAAAGAGCAACAC-3’#
	R: 5’-AGGAAACAGAGAAGAGGAGC-3’#
	F: 5’-CTCTCATCCCAGACTTGGCCA-3’
	F: 5’-CGGGGTACCTGAAAGGAAGACTCAGAGGAGAGAG-3’
	F: 5’-CGGGGTACCACTCAGAGGAGAGAGATAAGGAAGG-3’
4–6	F: 5’-CTGTGCTGGCTATCACAGATCCT-3’#
	R: 5’-GGTCACTGGAATAACCCAACAGCA-3’#
	R: 5’-GTCCCAGAAGGACATGGCTTTCC-3’
	F: 5’-CTTTCGGGCCAGTGGGATTTATGAAAAAT-3’
	F: 5’-CTTTAGGCCCAGTGGGATTTATG-3’
	F: 5’-AGGATGAAGAATGGAAGAGAATACGG-3’
	F: 5’-CCATGAAGATCACCACAACT-3’
6	F: 5’-ACATCCATGCTGTAGGCCCCAAAG-3’
	R: 5’-CAACTCCCTGTGCTGGCCATC-3’
7	F: 5’-GTTCTGAAAGTCTGTGGCTG-3’#
	R: 5’-CAAATGTACTACAAATCACTGAAC-3’#
	F: 5’-GGATGTGATCACTAGCACATAAT-3’
	F: 5’-TCGACTCTCTCAACAATCCTC-3’
	R: 5’-ACATCCATGCTGTAGGCCCCAAAG-3’
10	F: 5’-ATTAAAATGATTTGCCTTATTCTGGT-3’#
	R: 5’-TGAGGAGGCATTTTTGCTAAGGT-3’#
	F: 5’-TCACCCTGATGTCCAGCAG-3’
	F: 5’-GAAATTGATACAGTTTTACCCAATAAG-3’
	R: 5’-CTTATTGGGTAAAACTGTATCAATTTC-3’
10 – 12	F: 5’-ATGAAACCACCCCCAGTGTAC −3’#
	R: 5’-GAGAACAAATTAGTAAAAGATTAAACAAGCA-3’#
Allele-specific PCR to amplify allele with c.1334C	F: 5’-AGCCTTCCCGAATGCTTCCCACC-3’^
	R: 5’-CAAGTTTCATGTTCATGAGAGCAAACCTCG-3’^
10 and 12	R: 5’-GGAACTTCTCAGGCTCT-3’^^
	F: 5’-CTCATCTCAACAAGACTGAAAGCTCCT-3’^^
	Laboratory 4
1	F: 5’-**GTGCCAGCAAGATCCAATCTAGA**CAACTGCAGGCAGAGCACAG-3’
	R: 5’-**GGGTTCCCTAAGGGTTGGA**GGCAGTCCACTTGCCTTAGC-3’

Exon	Sequence (5’ to 3’)
3	F: 5’-**GTGCCAGCAAGATCCAATCTAGA**CCTTTATGACGTCTCCAAATAAGC-3’
	R: 5’-**GGGTTCCCTAAGGGTTGGA**AACTTCTCTCTGTTTGTAGTTAGGT-3’
6	F: 5’-**GTGCCAGCAAGATCCAATCTAGA**AAGATCACAGTCCCTTTCCAAG-3’
	R: 5’-**GGGTTCCCTAAGGGTTGGA**ACCCAACAGCAGGAATATCAG-3’
9	F: 5’-**GGGTTCCCTAAGGGTTGGA**GGAGCCATATTCTCAGAAGGGA-3’
	R: 5’-**GTGCCAGCAAGATCCAATCTAGA**ATGTGGCAGAAATTCTCATCATCCT-3’
10	F: 5’-**GGGTTCCCTAAGGGTTGGA**AGGGATTTGAGGGCTTCACT-3’
	R: 5’-**GTGCCAGCAAGATCCAATCTAGA**TTCTCCTGGGAAGTGGTGAG-3’
12	F: 5’-**GTGCCAGCAAGATCCAATCTAGA**GCATAGCAGGATTTCAATGACC-3’
	R: **5’-GGGTTCCCTAAGGGTTGGA**CAGATGGGCCTAATTGATTCTTTG-3’
13	F: 5’-**GTGCCAGCAAGATCCAATCTAGA**GGAGTGTCTCACTCACTTTGAT-3’
	R: 5’-**GGGTTCCCTAAGGGTTGGA**CCGGTTATTTATGCAGTCCATTG-3’
	Laboratory 5
1	F: 5’-**TGTAAAACGACGGCCAGT**CCCAGTAACATTGATTGAGTTGT-3’
	R: 5’-**CAGGAAACAGCTATGACC**CAGAGTTTCACCATGTTAGCCA-3’
2	F: 5’-**TGTAAAACGACGGCCAGT**GAAGACTTCAGCTGCTTTGAG-3’
	R: 5’-**CAGGAAACAGCTATGACC**AGCCCTTGGGTAAACATTGC-3’
3	F: 5’-**TGTAAAACGACGGCCAGT**TGACGTCTCCAAATAAGCTTCC-3’
	R: 5’-**CAGGAAACAGCTATGACC**ACTGATCTTTGTAGCGAAGGAT-3’
4	F: 5’-**TGTAAAACGACGGCCAGT**CAGACTCTTGCTGTGTGTCA-3’
	R: 5’-**CAGGAAACAGCTATGACC**AGCTCTGTGAACTGTATCAATGT-3’
5–6	F: 5’-**TGTAAAACGACGGCCAGT**GACACTGGGCATCTGGGATA-3’
	R: 5’-**CAGGAAACAGCTATGACC**TGTGCACAGGGGAGAAGATC-3’
7	F: 5’-**TGTAAAACGACGGCCAGT**ACTGGCACCTGATAACACCT-3’
	R: 5’-**CAGGAAACAGCTATGACC**TGGTTGCATATGATGACAGGG-3’
8	F: 5’-**TGTAAAACGACGGCCAGT**TGGCTTCCAGTTGAGAACCT-3’
	R: 5’-**CAGGAAACAGCTATGACC**CAAACCCCACTTTCTGCATT-3’
9	F: 5’-**TGTAAAACGACGGCCAGT**GCATCAGATTTCTGGTCTTCAA-3’
	R: 5’-**CAGGAAACAGCTATGACC**TGCTATGTGGCAGAAATTCTCA-3’
10	F: 5’-**TGTAAAACGACGGCCAGT**ATGAAACCACCCCCAGTGTA-3’
	R: 5’-**CAGGAAACAGCTATGACC**CTGCCAGTAGCAACCATTTG-3’
11	F: 5’-**TGTAAAACGACGGCCAGT**AGCAATGGGCATGACAGTTA-3’
	R: 5’-**CAGGAAACAGCTATGACC**CCAAGCAAATAATTATACAACCACATGA-3’
13	F: 5’-**TGTAAAACGACGGCCAGT**GAATCCAAGATTTATAGTGCTGAAA-3’
	R: 5’-**CAGGAAACAGCTATGACC**CTAACTGGGGGTGGTGGAA-3’

Bold nucleotides indicate the M13 tail

“F” and “R” denote forward and reverse primers, respectively; all primers are shown 5’ to 3’

[#]Primers used to generate PCR amplicon for sequencing or as template for subsequent allele-specific PCR

^Allele specific primer amplifying the allele with c.1334C; allele-specific nucleotide in primer is underlined

^^Sequence primers covering regions c.1088C>T and c.1334T>C

Table 3.

Consensus *CYP3A4* Genotypes

Coriell ID	*CYP3A4*	Coriell ID	*CYP3A4*
HG00122	*1/*10	NA06993^	*1/*22
HG00139	*1/*38	NA07056^	*1/*22
HG00276^	*1/*2	NA07439^	*1/*1
HG00334	*1/*7	NA12006^	*1/*3
HG00368	*1/*8	NA12336	*1/*35
HG00452	*1/*18	NA12717^	*1/*22
HG00525	*1/*4	NA18561	*1/*5
HG00704	*1/*18	NA18603	*1/*21
HG00734	*10/*22	NA18934	*1/*11
HG01269	1/*37 or *3/*22	NA18941	*1/*6
HG01275	*1/*20	NA18966	*1/*16
HG01816	*1/*5	NA18978	*1/*16
HG01865	*1/*4	NA19035	*1/*12
HG02029	*1/*28	NA19109	*1/*15
HG02054	*1/*23	NA19160	*1/*24
HG02134	*1/*18	NA19226	*1/*15
HG02146	*1/*9	NA20813	*1/*7
HG02952	*1/*23	NA21095	*1/*19
HG03159	*1/*12	NA23313^	*1/*22
HG03885	*1/*19	NA24008^	*22/*22

^Samples tested during previous Get-RM study[11]

Table 4.

CYP3A5 Consensus Genotypes

Coriell ID	*CYP3A5*
HG00436	*3/*3
NA07439	*1/*1
NA10856	*1/*3
NA18484	*1/*7
NA18518	*1/*6
NA18564	*1/*1
NA19143	*6/*7
NA19819	*3/*6
NA19920	*7/*7